Relationship between Agglutinative Properties of *Aeromonas salmonicida* Strains Isolated from Fish in Japan and Their Resistance to Mechanisms of Host Defense

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Phenotypic variation in spontaneous agglutination of *Aeromonas salmonicida* was investigated using 114 strains isolated from naturally epizootics of furunculosis in salmonid fish in Japan from 1967 through 1982. These strains were categorized into three different types: an agglutinating (AG) type which produced cell flocks resulting in precipitation and loss of turbidity in cell suspensions; a nonagglutinating (NAG) type, representing no loss in turbidity; and an intermediate (IM), weakly autoagglutinating type. Based on the occurrence of the AG, NAG and IM types determined in 1978 and again 1982, it was shown that a transition occurs progressing from the AG to the NAG via the IM type. The strains involved in the AG and NAG types were virulent and avirulent, respectively, based on LD₅₀ determination in rainbow trout (*Salmo gairdneri*). The NAG type was much less susceptible than the AG to the bactericidal activity of fresh normal rainbow trout serum, to immune bacteriolysis and to phagocytosis by cells from peritoneal exudate. When growth properties of AG and NAG types were compared under mixed culture conditions, the NAG type predominated in the culture broth 2-5 days after inoculation. In contrast, when rainbow trout were inoculated intraperitoneally with cells of both types, the NAG-type cells were selectively eliminated from the kidneys within a period of a few days. The AG-type cells, however, greatly proliferated in the kidneys and brought about clinical furunculosis. These results indicate that the AG type possesses a capacity to escape the defense mechanisms of the host by resisting the bactericidal activity of serum and phagocytosis by leucocytes.

Introduction

Aeromonas salmonicida, the causative agent of furunculosis is an important pathogen of fish from the fry to the adult stage (HERMAN, 1968; KLONTZ *et al.*, 1966; MCCARTHY and ROBERTS 1980). Isolates from naturally occurring furunculosis in salmonids occur in two different forms, those showing spontaneous agglutination (autoagglutination) and those that fail to do so (EVENBERG *et al.*, 1982; ISHIGURO *et al.*, 1981; KAY *et al.*, 1981). The property of agglutination is considered important to the pathogenicity of *A. salmonicida* strains. UDEY and FRYER (1978) reported that the cells of virulent strains are autoagglutinative and possess an additional outer layer (A layer). This A layer is in addition to the normal characteristic of Gram-negative bacteria. In contrast, nonagglutinating strains lack this

layer and are avirulent. A similar correlation was also demonstrated by ISHIGURO *et al.* (1981) and KAY *et al.* (1981). A similar extra surface layer has been found in an atypical, virulent *A. salmonicida* strains from goldfish by HAMILTON *et al.* (1981).

The structure of the A layer in the cell envelopes represents a repeating tetragonal subunit pattern (KAY *et al.*, 1981). The A layer protein has been isolated and characterized as 49 and 54 kilodaltons by KAY *et al.* (1981) and EVENBERG and LUGTENBERG (1982), respectively. They also showed similarity between the amino acid compositions of adhesive fimbriae of enteropathogenic *Escherichia coli* and the microcapsule of *Campylobacter fetus*. A possible role of the A layer producing adhesion to the surface of the host tissues is suggested (UDEY and FRYER, 1978). The immunogenicity of the A layer in the ag-

glutinating strains has been demonstrated by HAHNEL *et al.* (1983).

This extra surface layer may be common to agglutinating or virulent strains for a wide range of diseased fish species. However, the inter-relationships between the A layer, agglutination, adhesion and virulence have not been demonstrated. More work is necessary to define these differences between the agglutinating and nonagglutinating types, in order to understand why the agglutinating type is resistant to the defense mechanisms of the host fish. In this study, the tendency toward phenotypic variation in agglutination is investigated using 114 strains of *A. salmonicida* subsp. *salmonicida*. In addition, the different degrees of susceptibility to the bactericidal action of normal serum, immune bacteriolysis and phagocytosis by leucocytes are tested both bacterial strains using rainbow trout.

Materials and Methods

Bacterial strains and preservation: *Aeromonas salmonicida* subsp. *salmonicida* strains were used. These had been isolated from natural outbreaks of furunculosis in salmonids from 1967 through 1982 in Japan: amago salmon (*Oncorhynchus rhodurus*), chum salmon (*Oncorhynchus keta*), sockeye salmon (*Oncorhynchus nerka*), masu salmon (*Oncorhynchus masou*), pink salmon (*Oncorhynchus gorbuscha*), brook trout (*Salvelinus fontinalis*), Japanese char (*Salvelinus leucomaenis*), and rainbow trout (*Salmo gairdneri*). These isolates, 114 strains, were subcultured and preserved on nutrient agar slants (Eiken Chemical Co., Tokyo) at 15°C, and transferred at six month intervals. In addition, strains ATCC 14174 and NCMB 1102 were maintained and used as reference strains (Table 3).

Culture and cell suspensions: All the strains were cultured on agar slants at 20°C for 3 days. Fresh cultures were suspended in sterile physiological saline (0.85% NaCl) and the cell density was adjusted to 0.9 at optical density (OD) 610 nm. Cell suspensions were also prepared by ten-fold serial dilution with saline and used as inocula. Viable cell counts were made on nutrient agar plates incubated at 20°C for 3 days to determine colony forming units (CFU).

Quantitative assay for spontaneous agglutina-

tion: Small portions (2 ml each) of cell suspensions (dispersed cell turbidity was 0.9 at OD 610 nm) were used to determine the capacity for autoagglutination. Each suspension was measured in a spectrophotometer (Hitachi Model 124, Tokyo) equipped with a recorder. After the cell suspension was dispersed with a pipette, the change in turbidity was monitored by the recorder for 100 min at room temperature.

LD₅₀ determination: Juvenile rainbow trout weighing 30–40 g each were used for LD₅₀ determination. These fish were acclimatized for several weeks in laboratory aquaria supplied with fish pathogen free well water at 10°C and fed a commercial dry pellet daily. Five groups consisting of five fish each were injected intraperitoneally (ip) with 0.1 ml of viable *A. salmonicida* cell suspensions which had been prepared by ten-fold serial dilution with sterile saline. Injected fish were held for 2 weeks and mortalities recorded each day.

Acid production from sucrose: Hugh-Leifson medium (peptone 2 g, K₂HPO₄ 0.3 g, agar 3 g, and a trace of bromthymol blue dissolved in 1 liter of distilled water, pH 7.2) was autoclaved at 121°C for 20 min. Filtered sucrose solution was added to the medium at 1% (wt/vol). Subcultures were stabbed in tubes containing 10 ml of the medium and cultured at 20°C for 5 days. Changes in colour were noted. Acid production (yellow) and non-production (greenish blue) were designated suc⁺ and suc⁻, respectively.

Yeast agglutination: Suspensions of glutaraldehyde-fixed yeast cells of *Saccharomyces cerevisiae* (commercial baker's yeast) were prepared by the method of ESHDAT *et al.* (1978). Agglutination of yeast cells by cells of *A. salmonicida* strains was determined in the absence and presence of D-mannose (5–10% in final concentrations of the cell suspensions) by the method of ATKINSON and TRUST (1980).

Mixed culture: Four strains (A-7301, E-7609-3, NCMB 1102 and GN-7512) were used. Equal volume (1 ml each) of viable cell suspensions at 10^{8–9} ml⁻¹ were inoculated into 200 ml of sterile nutrient broth (pH 7.4) in 500-ml Ehrlenmeyer flasks and incubated at 20°C with occasional shakes. After 2 and 5 days, viable cells were enumerated by colony counts. Thirty colonies from cultured nutrient agar plates were chosen at

random and their agglutinative properties and acid production from sucrose determined. Each strain was also prepared in pure culture.

Experimental infection and reisolation: Four strains (A-7301, E-7609-3, NCMB 1102 and GN-7512) were used. Rainbow trout (average wt 60–80 g) received 0.1 ml ip of bacterial inocula which contained 10^8 viable cells ml^{-1} in saline. The fish were returned to laboratory aquaria and held at 10°C. After 2 and 5 days, the kidneys were removed from dissected fish and weighed. Each kidney sample, pooled from three fish, was homogenized in two-fold saline. Viable cell counts were obtained from homogenates and the number of cells g^{-1} kidney calculated. Thirty colonies were then selected on the basis of agglutination and acid production from sucrose determined.

Serum collection: After anaesthetization in *m*-aminobenzoic acid ethylester methanesulphonate solution (Sankyo Co., Tokyo) at $100 \mu\text{g ml}^{-1}$, the blood of the rainbow trout was collected from the caudal vessel. Blood samples were pooled from three or four fish and allowed to stand at room temperature for several min. Clots were removed by centrifuging at 1000 g for 5 min at 4°C and the serum temporarily stored at 5°C until use. Complement from rainbow trout serum was heat-inactivated at 44°C for 20 min (SAKAI 1981a).

Haemolytic activity of serum: Spontaneous (antibody-independent) haemolytic activity (SH_{50}) of normal rainbow trout serum was assayed at 30°C for 60 min with unsensitized red blood cells of goldfish (*Carassius auratus*). Details of the method and units used have been previously reported (SAKAI 1981b). Unless otherwise indicated, GVB²⁺ (isotonic veronal-buffered saline, pH 7.5, containing 0.1% gelatin, 0.15 mM CaCl_2 , and 0.5 mM MgCl_2) was used as a diluent for the haemolytic assay.

Bactericidal action of serum: Mixtures of equal volume (0.5 ml each) of fresh or heat-inactivated serum and viable cell suspensions of four strains (A-7301, E-7609-3, NCMB 1102 and GN-7512) at 10^{4-8} cell ml^{-1} were incubated in tubes at 20°C for 2 h, and shaken occasionally. As a control, sterile physiological saline (0.5 ml) was applied in place of serum. The antisera (anti-A-7301 and anti-NCMB 1102 serum) were obtained from two groups of rainbow trout (70–120 g), consisting of three fish each, which received ip 5 mg of formalin-killed cells of strains A-7301 and

NCMB 1102. After 3 weeks, agglutinin titers of pooled antisera were determined as 128 for anti-A-7301 serum and 256 for anti-NCMB 1102 serum against cells of NCMB 1102. Following incubation for the assay of bactericidal activity, the number of colonies was counted on nutrient agar plates and the viable cells ml^{-1} calculated (CFU).

Peritoneal exudate cells (PEC): Sterilized liquid paraffin (0.2–0.3 ml/fish) was injected ip into normal rainbow trout (70–120 g) which were maintained in aquaria at 10°C and sacrificed three days after the injections. The fish were bled by caudal peduncle amputation. The body surface of each was disinfected with 70% ethyl alcohol. The abdomen was dissected and the peritoneal cavity carefully washed with filtered Eagle's minimum essential medium (MEM; Gibco, New York) supplemented with 10% foetal bovine serum (Gibco) inactivated by heating at 56°C for 30 min. (MEM was buffered at pH 7.2 with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid [Sigma Chemical Co., Saint Louis, Mo.]-NaOH before filtration). Washings containing MEM and liquid paraffin were collected in silicon-coated tubes and allowed to settle at 20°C for 1 h. The upper layer of liquid paraffin was discarded, and the lower layer of MEM containing the PEC was separated and washed once with MEM by centrifuging at 1000 g for 5 min at 4°C. The live cell density of each PEC sample pooled from two or three fish was adjusted to 10^7 ml^{-1} by the trypan blue exclusion test (SAKAI 1981c).

Phagocytic index of PEC: The phagocytic activity of PEC against viable cells of four *A. salmonicida* strains (A-7301, E-7609-3, NCMB 1102 and GN-7512) was determined. Each viable cell suspension (0.5 ml containing 10^8 bacterial cells in MEM) was added to a mixture composed of 0.5 ml of PEC, 0.5 ml of normal rainbow trout serum as a complement source, and 0.5 ml of the anti-A-7301 serum in which the agglutinin titer was 256 (against NCMB 1102). Tubes containing the mixture were incubated at 20°C for 2 h, with shaking to cause contact between the live bacterial cells and the PEC. Phagocytic activity was determined by applying Wright-Giemsa staining to smears of each mixture, and the numbers of phagocytosed bacteria evaluated by enumerating 500 PEC per sample under an oil-immersion

Fig. 1. Profiles of spontaneous agglutination of *A. salmonicida* strains. Cells of strains A-7301 (A) and E-6709-3 (B) precipitate to the bottoms of the tubes, while cells of strains NCMB 1102 (C) and ATCC 14174 (D) show no precipitation.

microscopy ($\times 1000$). The phagocytic index was calculated as follows:

Phagocytic index

$$= \frac{\text{Number of intracellular bacteria phagocytosed by PEC}}{\text{Number of PEC}}$$

Results

Differences in spontaneous agglutination: When cells of *A. salmonicida* strains were suspended in saline, some strains formed visibly fine bacterial cell flocks and precipitated to the bottoms of the tubes in a short time (1 or 2 h) (A and B in Fig. 1). No precipitation was observed in certain strains (C and D in Fig. 1). A similar difference in precipitation was found in other strains tested. Since cell flocks were easily destroyed with slight shaking, precipitation resulting from agglutination was more variable.

Differences in agglutination were determined by a quantitative turbidimetric method using cell

Fig. 2. Differences in agglutinative properties of *A. salmonicida* strains. Experimental method is detailed in the text.

suspensions of all the strains used. The bacterial strains were categorized into the following three types: AG type, strongly autoagglutinating cells in which changes in turbidity at OD 610 nm followed a reverse sigmoid curve with time; NAG type, a non-agglutinating type, representing no change in turbidity; and IM type, or intermediate cell type, showing a slight loss in turbidity of the suspensions (Fig. 2). Each type reflected differences in the capacity to produce cell flocks. The NAG type had no capacity for agglutination (strains GN-7512 and GH-7501 in Fig. 2, and strain NCMB 1102 shown in Fig. 3). The capacity of the IM type was less than that of the AG type (strain G-7501 and GKB-7301 in Fig. 2). The IM type could be determined by this turbidimetric method but could not be detected by other methods (e.g. precipitation in tubes and cell flock formation on glass slides).

Occurrence of AG, NAG types: The occurrence of the AG, IM and NAG types was determined in 1978 using 75 strains isolated prior to that year (Table 1). All the strains isolated from 1967 through 1972 (6–11 years after isolation) were of the NAG type, while those from 1976 through 1978 (0–2 years) were AG. Strains from 1973 through 1975 (3–5 years) included AG, IM and NAG types. The rate of occurrence of the NAG

Table 1. Occurrence of the AG, IM and NAG types in *A. salmonicida* strains isolated from naturally occurring furunculosis in salmonids from 1967 through 1982

Year of isolation	Spontaneous agglutination of isolates			AG (%)
	AG type	IM type	NAG type	AG+IM+NAG
Occurrence determined in 1978				
1967	0	0	1	0
1969	0	0	1	0
1970	0	0	2	0
1971	0	0	4	0
1972	0	0	10	0
1973	2	1	11	14.3
1974	3	1	8	25.0
1975	1	1	12	7.1
1976	7	0	0	100
1977	3	0	0	100
1978	7	0	0	100
(Total)	23	3	49	30.7
Occurrence determined in 1982				
1967	0	0	1	0
1969	0	0	1	0
1970	0	0	2	0
1971	0	0	4	0
1972	0	0	10	0
1973	0	0	14	0
1974	0	0	12	0
1975	0	0	14	0
1976	0	0	7	0
1977	0	0	3	0
1978	1	0	6	14.3
1979	8	1	8	47.1
1980	10	0	1	90.9
1981	5	0	0	100
1982	6	0	0	100
(Total)	30	1	83	26.3

type was higher during the early years of this study. The IM type appeared during the years when both the AG and NAG types were present (Table 1). A similar tendency is more clearly demonstrated for 1982, in which the 114 previously isolated strains were used (Table 1).

In comparison with the results of 1978 and 1982, almost all the strains from 1973 through 1978 (22 out of the 23 strains of the AG type, and 3 strains of the IM type), were determined to be either AG or IM. This is in accordance with the first determination in 1978, indicating a change to the NAG type by the second set of observation made in 1982 (cf. Table 1). This showed a shift from the AG and IM types to the NAG

type in each of the strains, since the same strains which had been isolated from 1973 through 1978 were used for the second set of determination.

A strain (GKB-7301) belonging to IM type in the first determination in 1978 was used to estimate whether the IM type was a possible transitional form from AG to NAG. Agglutinative properties were examined using cell suspensions of thirty colonies isolated from this strains. In 1978, the strain was a mixture of AG and NAG types (Table 2). In 1982, however, thirty colonies isolated from the same strain were NAG (Table 2).

Virulence of AG type and NAG type: Virulence of seven strains was compared by LD₅₀ determination in juvenile rainbow trout. An LD₅₀ of AG

Fig. 3. Differences in flock formation for the AG and NAG types of *A. salmonicida* strains. The capacities for flock formation in the AG-type strain (A-7301) and the NAG-type strain (NCMB 1102) are observed 10 min after setting the cell suspensions on slide glasses under microscopy with low magnification ($\times 80$).

type for three strains was 2.2×10^4 to 4.1×10^5 viable cells determined by ip injection, while four strains of the NAG type was more than 10^8 cells (Table 3). The AG type was virulent and NAG type remained avirulent.

Yeast agglutination: Agglutination of yeast cells by *A. salmonicida* strains was determined in NAG-type cells. Agglutination was not detected in the AG-type cells (Table 3). Mannose inhibit yeast agglutination by the NAG-type cells (Table 3).

Susceptibility of AG and NAG types to serum and antibody: The susceptibility of live bacterial cells of two AG-type strains (A-7301 and E-7609-3) were maintained without serious loss (Table 4). Therefore, these strains were resistant to fresh

Table 2. Occurrence of AG-type and NAG-type colonies isolated from *A. salmonicida* strain GKB-7301 of the IM type*

Year of determination	Spontaneous agglutination of colonies		AG AG+NAG (%)
	AG type	NAG type	
1978	7	23	23.3
1982	0	30	0

* The subculture was smeared on agar plates, and thirty colonies were selected at random. The agglutinative properties (AG/NAG) of the colonies were determined using cell suspensions prepared from the colonies. The strain was determined as IM type in 1978, but was differentiated as NAG type in 1982.

normal rainbow trout serum possessing high SH_{50} activity as in the treatment with heat-inactivated serum showing no SH_{50} activity (Table 4). However, the viable numbers of NAG-type strains (NCMB 1102 and GN-7512) drastically decreased from 10^4 – 10^5 to 10 – 10^2 ml^{-1} when treated with fresh normal serum, although heat-inactivated serum barely reduced the viability (Table 4).

In strain A-7301, the use of anti-A-7301 and anti-NCMB 1102 serums were ineffective to immune bacteriolysis. However, with complement the antiserum caused marked viability in loss with strains NCMB 1102, from approximately 10 to 10^{2-8} ml^{-1} (Table 5).

Responses of AG and NAG types to phagocytic leucocytes: Peritoneal exudate cells (PEC) collected from rainbow trout were composed of large and small leucocytes and the two types occurred in about equal numbers. The large cells were neutrophil- and macrophage-like and measured 10 – 20 μm in diameter when stained by the Wright-Giemsa method. The small cells were lymphocytes measuring 5 – 6 μm and possessing dense nucleus occupying almost the entire cell.

Phagocytic activity of PEC against viable bacterial cells was investigated in the presence of anti-*A. salmonicida* (heat-inactivated) and normal rainbow trout serum using AG- and NAG-type cells as particulate antigens. This was followed by a determination of the phagocytic indices. The indices against two AG-type strains (A-7301 and E-7609-3) ranged from 0.15 – 0.20 . Con-

Table 3. Sources and properties of *A. salmonicida* strains, some of which are used in this study

Strain	Source	Spontaneous agglutination	Sucrose marker ^{*1}	Yeast agglutination ^{*2}		LD ₅₀ dose ^{*3} (viable cells/fish)
				Without mannose	With mannose	
A-7301	HFH ^{*4} strain isolated from sockeye salmon	AG type	suc ⁺	—	—	2.2 × 10 ⁴
C-7608-1	HFH strain isolated from rainbow trout	AG type	suc ⁺	—	—	4.1 × 10 ⁵
E-7609-3	HFH strain isolated from pink salmon	AG type	suc ⁺	—	—	1.2 × 10 ⁵
ATCC 14174	American Type Culture Collection (USA)	NAG type	suc ⁻	+	—	> 10 ⁸
NCMB 1102	National Collection of Marine Bacteria (UK)	NAG type	suc ⁻	+	—	> 10 ⁸
GH-7501	Supplied from Inoue (Daiichi Chemical Co.)	NAG type	suc ⁺	+	—	> 10 ⁸
GN-7512	Supplied from Inoue (Daiichi Chemical Co.)	NAG type	suc ⁺	+	—	> 10 ⁸

^{*1} Acid production from sucrose: suc⁺, production; suc⁻, no production.

^{*2} +, Positive; —, negative.

^{*3} By ip injections into rainbow trout.

^{*4} Hokkaido Fish Hatchery, Japan.

Table 4. Bactericidal action of normal rainbow trout serum against AG- and NAG-type cells of *A. salmonicida* strains

Strain	Treatment of cells with serum	Haemolytic ^{*1} activity of serum (SH ₅₀)	Bactericidal action ^{*2} (viable cells/ml)	
			Pre-treatment	Post-treatment
A-7301 (AG type)	Physiological saline		5.6 × 10 ⁴	6.4 × 10 ⁴
	Fresh normal serum	16.5	5.6 × 10 ⁴	1.0 × 10 ⁴
	Heat-inactivated serum ^{*3}	<2	5.6 × 10 ⁴	2.0 × 10 ⁴
E-7609-3 (AG type)	Physiological saline		7.7 × 10 ⁵	5.8 × 10 ⁵
	Fresh normal serum	18.0	7.7 × 10 ⁵	4.3 × 10 ⁵
	Heat-inactivated serum	<2	7.7 × 10 ⁵	8.2 × 10 ⁵
NCMB 1102 (NAG type)	Physiological saline		3.9 × 10 ⁴	2.8 × 10 ⁴
	Fresh normal serum	16.5	3.9 × 10 ⁴	<10
	Heat-inactivated serum	<2	3.9 × 10 ⁴	6.1 × 10 ³
GN-7512 (NAG type)	Physiological saline		2.7 × 10 ⁵	4.0 × 10 ⁵
	Fresh normal serum	18.0	2.7 × 10 ⁵	9.4 × 10 ⁴
	Heat-inactivated serum	<2	2.7 × 10 ⁵	8.5 × 10 ⁴

^{*1} Unsensitized red blood cells of goldfish were used for SH₅₀ assay at 30°C for 60 min.

^{*2} Treated at 20°C for 2 h; numbers of viable cells were determined by colony counts.

^{*3} Heated at 44°C for 20 min.

siderably greater indices (0.82–0.97) with NAG-type strains (NCMB 1102 and GN-7512) were observed (Table 6). This indicated that the NAG types were more susceptible to phagocytosis by leucocytes than those of the AG types. This was demonstrated by large leucocytes containing many

bacterial cells in the PEC with NAG type, but scarcely with the AG type (Fig. 4).

Growth of AG and NAG types in mixed cultures: Differences in the in vitro growth were investigated using AG strains (A-7301 and E-7609-3, both expressing suc⁺) and NAG strains (NCMB 1102,

Table 5. Immune bacteriolysis by rainbow trout antibody and complement against AG- and NAG-type cells of *A. salmonicida* strains

Strain	Treatment of cells with serum	Bactericidal action* ¹ (viable cells/ml)	
		Pre-treatment	Post-treatment
A-7301 (AG type)	Heat-inactivated anti-A-7301 serum* ²	2.1 × 10 ⁶	5.7 × 10 ⁵
	Heat-inactivated anti-A-7301 serum + fresh normal serum	2.1 × 10 ⁶	1.3 × 10 ⁵
	Heat-inactivated anti-NCMB 1102 serum	2.1 × 10 ⁶	8.5 × 10 ⁵
	Heat-inactivated anti-NCMB 1102 serum + fresh normal serum	2.1 × 10 ⁶	3.3 × 10 ⁵
	Heat-inactivated anti-A-7301 serum	8.8 × 10 ⁶	2.4 × 10 ⁶
NCMB 1102 (NAG type)	Heat-inactivated anti-A-7301 serum + fresh normal serum	8.8 × 10 ⁶	7.6 × 10 ²
	Heat-inactivated anti-NCMB 1102 serum	8.8 × 10 ⁶	1.9 × 10 ⁶
	Heat-inactivated anti-NCMB 1102 serum + fresh normal serum	8.8 × 10 ⁶	4.0 × 10 ²

*¹ Treated at 20°C for 2 h; numbers of viable cells were determined by colony counts.

*² Heated at 44°C for 20 min.

Table 6. Phagocytic index of rainbow trout PEC against live AG- and NAG-type cells of *A. salmonicida* strains*¹

Strain	Number of fish used for PEC preparation* ²	Phagocytic index of PEC
A-7301 (AG type)	2	0.20
E-7609-3 (AG type)	3	0.15
NCMB 1102 (NAG type)	3	0.97
GN-7512 (NAG type)	2	0.82

*¹ Live bacterial cells were exposed to PEC in the presence of the rainbow trout anti-*A. salmonicida* serum (heat-inactivated) and complement.

*² PEC was pooled.

expressing suc⁻, and GN-7512, expressing suc⁺) in pure and mixed culture conditions. In the pure cultures, the viable cell counts of all strains in the nutrient broth increased equally as shown by colony counts at 2 days and 5 days. The properties of agglutination (AG/NAG) and acid production from sucrose (suc⁺/suc⁻) remained unchanged in the 30 isolated colonies (Table 7). However, when incubated under the mixed culture conditions, the growth of strain NCMB 1102 appeared to be more rapid than that of strain A-7301. This was shown by ratios of colonies exhibiting AG-suc⁺ and NAG-suc⁻ changing from

Fig. 4. Phagocytosis of NAG-type cells (strain NCMB 1102) by rainbow trout leucocytes. Bar represents 20 µm.

21: 9 in the inoculum to 13: 17 after 2 days and then to 6: 24 after 5 days (Table 7). A faster growth rate of the NAG type was also found in the mixed culture of E-7609-3 and GN-7512 (Table 7).

Growth of AG and NAG types in vivo: Differences in the growth of strain A-7301 (AG-suc⁺)

Table 7. Growth of AG- and NAG-type strains of *A. salmonicida* in single and mixed broth cultures*¹

Inoculated strain (viable cells/ml)	Before inoculation				Viable cells (/ml)	After 2 days				Viable cells (/ml)	After 5 days			
	Agglutination		Marker			Agglutination		Marker			Agglutination		Marker	
	AG	NAG	suc ⁺	suc ⁻		AG	NAG	suc ⁺	suc ⁻		AG	NAG	suc ⁺	suc ⁻
A-7301 (AG-suc ⁺ , 2 × 10 ⁷)	30* ²	0	30	0	2.4 × 10 ⁸	30	0	30	0	9.3 × 10 ⁸	30	0	30	0
E-7609-3 (AG-suc ⁺ , 4 × 10 ⁷)	30	0	30	0	3.5 × 10 ⁸	30	0	30	0	6.5 × 10 ⁸	30	0	30	0
NCMB 1102 (NAG-suc ⁻ , 8 × 10 ⁶)	0	30	0	30	5.1 × 10 ⁸	0	30	0	30	2.0 × 10 ⁹	0	30	0	30
GN-7512 (NAG-suc ⁺ , 9 × 10 ⁶)	0	30	30	0	9.7 × 10 ⁷	0	30	30	0	7.8 × 10 ⁸	0	30	30	0
A-7301 (AG-suc ⁺ , 2 × 10 ⁷) + NCMB 1102 (NAG-suc ⁻ , 8 × 10 ⁶)	21	9	21	9	9.8 × 10 ⁸	13	17	13	17	1.1 × 10 ⁹	6	24	6	24
E-7609-3 (AG-suc ⁺ , 4 × 10 ⁷) + GN-7512 (NAG-suc ⁺ , 9 × 10 ⁶)	23	7	30	0	7.8 × 10 ⁸	16	14	30	0	8.5 × 10 ⁸	9	21	30	0

*¹ Numbers of viable cells were determined by colony counts, and agglutination (AG/NAG) and sucrose markers (suc⁺/suc⁻) were determined using thirty colonies isolated from each culture.

*² The number of colonies.

Table 8. Infectivity of AG- and NAG-type strains of *A. salmonicida* in rainbow trout in single and mixed cell injections (ip)*¹

Injected strain (viable cells/fish)	Before injection				Viable cells (/g)	After 2 days				Viable cells (/g)	After 5 days			
	Agglutination		Marker			Agglutination		Marker			Agglutination		Marker	
	AG	NAG	suc ⁺	suc ⁻		AG	NAG	suc ⁺	suc ⁻		AG	NAG	suc ⁺	suc ⁻
A-7301 (AG-suc ⁺ , 3×10 ⁵)	30* ²	0	30	0	4.1×10 ⁴	30	0	30	0	3.6×10 ⁸	30	0	30	0
E-7603-3 (AG-suc ⁺ , 5×10 ⁵)	30	0	30	0	5.1×10 ³	30	0	30	0	1.4×10 ⁸	30	0	30	0
NCMB 1102 (NAG-suc ⁻ , 6×10 ⁵)	0	30	0	30	5.6×10 ²	0	30	0	30	<10	NT* ³	NT	NT	NT
GN-7512 (NAG-suc ⁻ , 2×10 ⁵)	0	30	30	0	<10	NT	NT	NT	NT	<10	NT	NT	NT	NT
A-7301 (AG-suc ⁺ , 3×10 ⁵) +NCMB 1102 (NAG-suc ⁻ , 6×10 ⁵)	12	18	12	18	8.1×10 ⁴	21	9	21	9	2.1×10 ⁷	30	0	30	0
E-7609-3 (AG-suc ⁺ , 5×10 ⁵) +GN-7512 (NAG-suc ⁺ , 2×10 ⁵)	20	10	30	0	7.0×10 ³	30	0	30	0	8.1×10 ⁸	30	0	30	0

*¹ Numbers of viable cells were determined by colony counts using kidney homogenates, and agglutination (AG/NAG) and sucrose markers (suc⁺/suc⁻) were determined using thirty colonies isolated from the homogenates.

*² The number of colonies.

*³ Not tested.

and/or NCMB 1102 (NAG-suc⁻) and strain E-7609-3 (AG-suc⁺) and/or GN-7512 (NAG-suc⁺) were investigated by ip injection into rainbow trout with viable cells. An examination of viable counts and phenotypic properties of colonies isolated from the kidneys showed the infections with AG-type strains alone resulted in progressive increases in the numbers of viable cells in the kidneys at 2 days and 5 days after injection. After 5 days, the fish became moribund and showed inflammations in the vent and haemorrhage at the base of the anal, ventral and pectoral fins, all signs of acute infection in naturally occurring furunculosis. Of the thirty colonies isolated after 2 and 5 days all were of an AG-suc⁺ type. In contrast, the cells of strain NCMB 1102 (NAG type) isolated from the kidneys after 2 days were lower concentration, and completely disappeared 5 days after injection (Table 8). Strain GN-7512 (NAG type) could not be isolated at 2 days. In addition, fish injected with both NAG strains showed no signs of disease. Fish receiving mixed cultures (NAG plus AG) had progressive increases in viable cell counts in the kidneys similar to infections with strain A-7301 and E-7609-3. After 5 days, the fish showed the signs of furunculosis. The ratio of AG-suc⁺: NAG-suc⁻ shifted from 12:18 (in the inoculum) to 21:9 after 2 days and to 30:0 after 5 days. No colony of NAG-suc⁻ was detected among the colonies isolated from trout receiving mixed infections of strains A-7301 and NCMB 1102. Similarly, in the mixed infection of E-7609-3 and GN-7512, only AG-suc⁺ type colonies were isolated after 2 and 5 days (Table 8).

Discussion

It is known that two forms exist among *A. salmonicida* strains with respect to non-immunological, spontaneous agglutination, an autoagglutinating form and a non-agglutinating form (EVENBERG *et al.*, 1892; KAY *et al.*, 1981; UDEY and FRYER, 1978). Japanese strains isolated from 1967 through 1982 were divided into AG, IM and NAG types by a turbidimetric method devised for this study (Fig. 2 and Table 1).

Based on the frequency of the three types in naturally occurring outbreaks of disease, it is apparent that a phenotypic transition develops

from the AG type to the NAG type via an IM type, and that the IM is a transitional form which includes cells of both AG and NAG (Table 2). The phenotypic transition was closely related to the length of preservation after isolation; the transition (AG→IM→NAG) commenced 3–4 years and was complete 5–6 years after isolation (cf. Table 1). This may be explained in terms of the number of subcultures rather than time of preservation in nutrient agar medium. The variation may be reduced by subculture and preservation at temperature of 15°C or less (this study was conducted at 15°C). ISHIGURO *et al.* (1981) have reported that growth at higher than optimal temperature results in the selection of attenuated (non-agglutinating) derivatives in the initial bacterial population of virulent (agglutinating) strains. No evidence for the occurrence of a reverse transition (NAG→AG or NAG→IM→AG) followed by suc⁺/suc⁻ marker was demonstrated, even when the live mixed cells consisting of AG and NAG types were cultured in vitro or injected into fish (Tables 7 and 8) (The majority of isolates in Japan, including the 114 strains used in this study are suc⁺; isolates from the United States, Canada and European countries, including ATCC 14174 and NCMB 1102, are generally suc⁻. It is not known whether suc⁺/suc⁻ markers are associated with different pathogenic effects in fish). These findings are interpreted as showing a lack of one or more genetic elements responsible for agglutinating properties. However, ISHIGURO *et al.* (1981) have suggested that the variation is not due to the loss of a transfer factor or plasmid.

The literature is in agreement that agglutinating *A. salmonicida* strains are virulent to fish (EVENBERG *et al.*, 1982; EVENBERG and LUGTENBERG, 1982; HAHNEL *et al.*, 1983; HAMILTON *et al.*, 1982; ISHIGURO *et al.*, 1981; KAY *et al.*, 1981; MCCARTHY and ROBERTS, 1980; UDEY and FRYER, 1978). As the LD₅₀ shows, the results of this study also agree with the previous descriptions (Table 3). The IM type is probably made up of virulent and avirulent cells. ISHIGURO *et al.* (1981) have reported that yeast agglutination is characteristic of nonagglutinating (avirulent) strains, in which the lack of the A layer is common. According to their report, it is possible that the NAG type showing yeast agglutination (which is inhibited by mannose, as de-

scribed by ISHIGURO *et al.*) lacks the A layer, and that the AG type showing no yeast agglutination possesses the A layer (Table 3). In addition, strain ATCC 14174, which was used by ISHIGURO *et al.*, showed the same results with regard to yeast agglutination, agglutinative properties and virulence.

In recent years, the bactericidal action of serum and mucus has been demonstrated in certain species of fish (ELLIS, 1981; FLETCHER and GRANT, 1969; HARRELL *et al.*, 1976; INGRAM, 1980; MACVICAR and FRETCHER, 1970; OURTH and WILSON, 1981 and 1982a, b). SAKAI (1983a, b) demonstrated that normal rainbow trout serum possesses bactericidal action for nonagglutinating *A. salmonicida* and that it is proportional to the level of SH₅₀ activity utilizing an alternative pathway for the activation of complement. The present study showed that, although the NAG strains are markedly susceptible to the serum activity, the AG strains resist it (Table 4). Furthermore, while the NAG types were more susceptible to immune bacteriolysis by a specific antibody and complement than non-specific bactericidal action by the complement alone, the AG strains largely retained their viability even in the presence of the antibody and complement (Table 5). Similar differences were found for phagocytosis by PEC in the presence of the antibody and complement. Phagocytosis by trout leucocytes and opsonization by trout antibody and complement have been reported by GRIFFIN (1983) and SAKAI (1984), implying that phagocytic cells carry Fc and C (complement) receptors on their cell surface. Live AG-type cells resisted phagocytosis by PEC compared to NAG-type cells (Table 6).

Serum and phagocytosis by leucocytes play essential roles in defense of the host in earlier stages of infection by microbes (AVTALION and SHAHRABANI, 1975; ELLIS *et al.*, 1976; ELLIS, 1981; INGRAM, 1980; MCKINNEY *et al.*, 1977; MCVICAR and FLETCHER, 1970; MUNRO, 1982; RIJKERS, 1982; SYPEK and BURRESON, 1983). It is evident that the viability of NAG strains is destroyed by host defense mechanisms such as bactericidal serum activity and phagocytosis (Tables 4, 5 and 6). Therefore, fish never permit the NAG type to persist within hosts (Table 8). Unlike the NAG type, the AG type is capable of avoiding the defense mechanisms by virtue of its resisting

properties, thereby proliferating in and infecting fish, and causing epizootics of furunculosis.

There is a strong likelihood that the AG-type cells have cellular surface component(s) or structure(s) responsible for resistance to killing activities of the serum and to phagocytosis. MUNN *et al.* (1982) and TRUST *et al.* (1982) have claimed an important roles of the A layer in response to bactericidal serum activity in virulent *A. salmonicida*. On the other hand, a few virulence factors (viz. leucocytolysin, protease and haemolysin) and pretection factors against challenges have been characterized as extracellular products of virulent and avirulent strains (CIPRIANO *et al.*, 1981; CIPRIANO 1982a, b; ELLIS *et al.*, 1981; FULLER *et al.*, 1977; KARLSEN, 1962; MELLERGAARD, 1983; MUNRO *et al.*, 1980; MUNRO, 1982; NOMURA and SAITO, 1982; SHIEH and MCLEAN, 1975; TITBALL and MUNN, 1981). The toxic factors in AG (virulent) strains would exert a severe influence on hosts as a result of bacterial propagation within the host tissues in the later stages of infection (ELLIS *et al.*, 1981; FERGUSON and MCCARTHY, 1978). NAG (avirulent) strains, however, may be eliminated from the host before the production of extracellular toxic factors (Table 8). In earlier stages, the virulence factors would be less important than the cellular surface component, because there would not yet be enough toxic products produced in the infected tissues to break down the host defenses. At present, no difference in the cell structures, with the exception of the A layer, has been discovered between the agglutinating and nonagglutinating strains. The A layer, therefore, is more likely seen as a cellular surface component.

Comparing the literature cited and the findings of this study, the following appear to be universal, even though Japanese isolates differ from those from the United States, Canada and European countries in acid production from sucrose: (a) the transition in agglutination proceeds from a positive to a negative type, (b) the reverse does not occur, (c) agglutinating types are virulent, whereas nonagglutinating types are avirulent, (d) the agglutinating type is resistant to fish serum and to phagocytosis by leucocytes but nonagglutinating type is not.

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