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Induction of Hemagglutinating Activity of *Edwardsiella tarda* by Sodium Chloride

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ABSTRACT—The hemagglutinating activity (HA) of *Edwardsiella tarda*, which had been isolated from cultured fish and culture environments, was investigated in relation to NaCl concentration of the growth medium. *E. tarda* cells were cultured in a peptone-yeast extract broth supplemented with 3% NaCl (3%-NaCl culture) and without NaCl (0%-NaCl culture). Hemagglutination assays with guinea pig erythrocytes classified the strains into three HA patterns. Seventeen strains exhibited HA only with the 3%-NaCl culture (type A). A more frequent type (35 strains) displayed HA in both 0%- and 3%- NaCl cultures but the 3%-NaCl culture showed higher HA activity than the 0%-NaCl culture (type B). No HA was detected in both cultures of the other three strains (type C). The NaCl-induced HA was well correlated with the expression of a 19.3 kDa protein, a fimbrial major subunit (FimA). Infection experiments with a selected strain (type A) of *E. tarda* revealed that the 3%-NaCl culture was more virulent to Japanese flounder *Paralichthys olivaceus* than the 0%-NaCl culture, when fish were challenged by an oral route. This induction of the fimbrial protein under higher NaCl concentration may play an important role in the virulence of *E. tarda* in marine environments.

Key words: hemagglutination, *Edwardsiella tarda*, pathogenicity, fimbriae, *Paralichthys olivaceus*

Edwardsiella tarda causes a septicaemia infection (edwardsiellosis) in a variety of cultured freshwater and marine fish species worldwide (Plumb, 1999; Muroga, 2001), and the bacterium is also known as a cause of gastro- and extraintestinal infections in humans (Jordan and Hadley, 1969; Wilson *et al*., 1989). In Japan, fish edwardsiellosis was first reported as the disease in cultured Japanese eel *Anguilla japonica* (Wakabayashi and Egusa, 1973). Subsequently, *E. tarda* was isolated as the causative agent in cultured marine fish species (Kusuda *et al*., 1977; Yasunaga *et al*., 1982) and the disease has been a threat particularly in cultured Japanese flounder *Paralichthys olivaceus* and red sea bream *Pagrus major*. Some therapeutants are effective to reduce mortality tentatively in fish, but usually the disease is chronic rather than acute, causing serious economic damages to the aquaculture industry. In spite of intensive studies on vaccine development against edwardsiellosis of fish (Song and Kou, 1981; Salati *et al*., 1983, 1987; Mekuchi *et al*., 1995a; Tu and Kawai, 1999; Kawai *et al*., 2004), there are still no vaccines commercially available in Japan, mainly due to little efficacy of the vaccine candidates.

Many potential virulence factors of *E. tarda* have been suggested, i.e. production of dermatotoxins against

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rabbit (Ullah and Arai, 1983), cytotoxic hemolysins (Janda *et al*., 1991; Strauss *et al*., 1997), lethal toxins (Hari Suprapto *et al*., 1995), siderophore (Kokubo *et al*., 1990; Igarashi *et al*., 2002), and the ability to resist phagocytic and serum killing (Miyazaki and Kaige, 1985; Iida *et al*., 1993; Iida and Wakabayashi, 1993). However, the role of these factors in the pathogenicity of *E. tarda* has not been fully understood.

In edwardsiellosis of fish, enteric colonization is supposed to be important to develop septicemic condition (Kanai *et al.,* 1988; Ling *et al*., 2001) and the existence of hemagglutinins in *E. tarda* was demonstrated (Nowotarska and Mulczyk, 1977; Wong *et al*., 1989; Janda *et al*., 1991). Recently, a 19.3-kDa protein of *E. tarda* was shown to be a fimbrial major subunit (FimA) possessing hemagglutinating activity, HA (Sakai *et al*., 2003, 2004), and Srinivasa Rao *et al*. (2003) suggested that the *fimA* gene of *E. tarda* was involved in the virulence. In the present study, we describe that HA of *E. tarda* is influenced by NaCl concentrations in culture media and the activity is correlated to both expression of the 19.3-kDa fimbrial protein and the virulence to fish.

Materials and Methods

Bacteria

Fifty-five strains of *E. tarda*, which had been isolated from diseased fish (flounder, red sea bream, Japanese eel), intestinal contents of healthy eels and eel-culture ponds from 1989 to 2001 in Japan, were used in this study (Table 1). A flounder strain FK1051 (Pakingking *et al*., 2003) was used to determine the optimal culture conditions for hemagglutinin production. An eel strain KG8401 was used as a positive control for the 19.3-kDa protein (Sakai *et al*., 2003). Bacteria were pre-cultured on Tripto-Soy Agar (TSA, Nissui) containing 0.5% NaCl at 25°C for 48 h.

Table 1. *Edwardsiella tarda* strains used in this study

Source	Location (prefecture)	No. of strains
Diseased Japanese flounder	Hiroshima, Shimane Ehime, Nagasaki	34
Diseased red sea bream	Hiroshima, Ehime	5
Diseased Japanese eel	Tokushima, Shizuoka	7
Eel-culture environments*	Tokushima, Shizuoka	9

Japanese flounder, *Paralichthys olivaceus*; red sea bream, *Pagrus major*; Japanese eel, *Anguilla japonica*
* intestinal contents of healthy eels and eel-pond water

Hemagglutination assays

The pre-culture of *E. tarda* was inoculated in 10 mL of a liquid medium composed of 1% Bacto peptone (Difco, USA) and 0.5% yeast extract (Difco). The bacterial suspension was diluted 100-fold, and 100 μL of the dilution was then inoculated into 10 mL of the liquid medium supplemented with or without NaCl. After shaking culture at 30°C for 15 h, cells were harvested by centrifugation (5,000 $\times$ *g*, 10 min). The cells were washed twice with sterilized phosphate buffered saline (PBS) and the cell concentration was adjusted to A_{530} = 1.0 (ca. 10^9 CFU/mL). Serial 2-fold dilutions of the cell suspension were prepared on 96-well microplates (20 μL/well), and then erythrocytes of guinea pig, horse, or sheep, which had been previously adjusted to 0.5% with PBS, were added in each well (20 μL/well). After mild agitation for 20 sec, the plates were incubated at 25°C for 1 h, and subsequently at 4°C overnight for hemagglutination.

Western blot

Detection of the *E. tarda* 19.3 kDa protein was carried out as described previously (Sakai *et al*., 2003). A rabbit antiserum against the 19.3 kDa protein was provided by Dr. K. Kanai. *E. tarda* cultures in the medium containing 0, 0.5, 1.5, or 3% NaCl was centrifuged (5,000 $\times$ *g*, 10 min) and the resulting pellet was dissolved in SDS-PAGE sample buffer (Laemmli, 1970), then the samples were applied to 15% SDS-polyacrylamide gel. Immunoblotting with the antiserum was performed using ECL Western Blotting Detection System (Amersham Biosciences, USA).

Experimental infection

In the first experiment, ten groups of ten Japanese flounder (average weight, 9.8 g) were put in 25 L tanks with a flow-through system. Water temperature during the experiment was 25.2 $\pm$ 1.8°C. Fish were challenged by oral administration or intraperitoneal inoculation of the strain FK1051. For oral administration, bacteria-loaded food (paste-like pellets) was administered orally to fish (0.05 mL/fish) using a 1 mL syringe without a needle. The amount of inoculated bacteria was 6.0×10^3, 10^5 and 10^7 CFU/fish for the groups administered with the bacteria cultured in broth without NaCl (0%-NaCl culture) and 4.5×10^3, 10^5 and 10^7 CFU/fish for the groups administered with the bacteria cultured in broth supplemented with 3% NaCl (3%-NaCl culture). A control group received bacteria-unloaded food. For intraperitoneal inoculation, fish were injected with the bacterial suspension (0.05 mL/fish) at doses of 3.8×10^2 and 10^4 CFU/fish for the 0%-NaCl culture and 3.6×10^2 and 10^4 CFU/fish for the 3%-NaCl culture. A control group received sterilized PBS.

In the second experiment, Japanese flounder (average weight, 59.3 g) were challenged by the oral administration method. Four groups of 15 fish were put in 90 L tanks with a flow-through system of seawater (water temperature, 24.4−28.7°C). Bacteria-loaded food was administered orally to fish (0.5 mL/fish) as described above. The doses administered were 2.7×10^3 and 10^5 CFU/fish for the 0%-NaCl culture and 2.0×10^3 and 10^5

CFU/fish for the 3%-NaCl culture. A control group received bacteria-unloaded food.

During the observation period, i.e. 21 days for the first experiment and 60 days for the second experiment, dead fish were subjected to isolation of bacteria from the kidneys or spleens using TSA or brain heart infusion agar (Nissui, Japan). Isolated bacteria were identified as *E. tarda* with the aid of an anti-*E. tarda* rabbit serum. Bacterial isolation from kidneys was also carried out in the survivors at the termination of the experiments.

Statistical analysis

The experimental infection data were analyzed by Fisher's exact probability test.

Results

Hemagglutination activity (HA) of E. tarda *FK1051*

No hemagglutination was observed in any erythrocytes with the 0%-NaCl culture of FK1051, nor in sheep erythrocytes with the 3%-NaCl culture. The 3%-NaCl culture showed the highest HA against guinea pig erythrocytes (Table 2). The bacteria at a logarithmic phase cultured in the 3%-NaCl medium exhibited higher HA ($1:2^6$) against guinea pig erythrocytes than that at a stationary phase ($1:2^2$) (Table 3), and thus the cells at logarithmic phases were used in the following HA tests using guinea pig erythrocytes. HA was observed in the 1%-NaCl culture and increased as the concentration of NaCl in the medium became higher (Table 4). When seawater was used instead of NaCl, 100% seawater culture exhibited the highest HA ($1:2^3$), though the activity was lower than that of the 3%-NaCl culture. No difference in HA was noted between shaking culture and static culture, and no change in HA was noticed after vigorous washing of cells (3%-NaCl culture) with PBS.

Table 2. Hemagglutination of *E. tarda* FK1051 with mammalian erythrocytes

Erythrocytes	NaCl*1 %	Two-fold serial dilution of *E. tarda* suspension (2^n)							
		0*2	1	2	3	4	5	6	7
Guinea pig	0	–	–	–	–	–	–	–	–
	3	++	++	++	++	++	++	+	–
Horse	0	–	–	–	–	–	–	–	–
	3	++	++	+	+	–	–	–	–
Sheep	0	–	–	–	–	–	–	–	–
	3	–	–	–	–	–	–	–	–

*1 NaCl concentration of the broth for culturing *E. tarda* FK1051
*2 initial bacterial suspension (A530 = 1.0; ca 10^9 CFU/mL)
++, strongly positive; +, positive; –, negative

Table 3. Effect of growth phase of *E. tarda* FK1051 on hemagglutination with guinea pig erythrocytes

Growth phase	Culture time (h)	NaCl*1 %	Two-fold serial dilution of *E. tarda* suspension (2^n)							
			0*2	1	2	3	4	5	6	7
Logarithmic	12	0	–	–	–	–	–	–	–	–
	18	3	++	++	++	++	++	++	+	–
Stationary	24	0	–	–	–	–	–	–	–	–
	24	3	++	++	++	–	–	–	–	–

*1 NaCl concentration of the broth for culturing *E. tarda* FK1051
*2 initial bacterial suspension (A530 = 1.0; ca 10^9 CFU/mL)
++, strongly positive; +, positive; –, negative

Table 4. Effect of salt concentration on hemagglutination of *E. tarda* FK1051 with guinea pig erythrocytes

Salt*1 conc.		Two-fold serial dilution of *E. tarda* suspension (2^n)							
		0*2	1	2	3	4	5	6	7
NaCl	0%	–	–	–	–	–	–	–	–
	1%	++	++	+	–	–	–	–	–
	2%	++	++	++	++	+	–	–	–
	3%	++	++	++	++	++	+	+	–
SW*3	33%	–	–	–	–	–	–	–	–
	66%	+	–	–	–	–	–	–	–
	100%	++	++	+	+	–	–	–	–

*1 salt concentration of the broth for culturing *E. tarda* FK1051
*2 initial bacterial suspension (A530 = 1.0; ca 10^9 CFU/mL)
*3 sea water
++, strongly positive; +, positive; –, negative

Hemagglutination assays of E. tarda *strains*

Hemagglutination assays with guinea pig erythrocytes classified *E. tarda* strains into three different HA patterns (Table 5). Seventeen strains, represented by FK1051, exhibited HA only with the 3%-NaCl culture (type A). Thirty-five strains represented by KG8401 displayed HA in both the 0%- and 3%-NaCl cultures, but the 3%-NaCl culture induced higher HA than the 0%-NaCl culture (type B). No HA was detected in both the cultures of the other three strains, one from diseased Japanese eel and two from eel-culture environments, represented by SU166 (type C).

Detection of a 19.3-kDa protein

E. tarda FK1051, KG8401 and SU166, representative strains of each HA type (Table 5), were used in Western blot analysis. In FK1051, the 19.3-kDa protein was detected only in the 3%-NaCl culture, while in KG8401 higher concentration of NaCl in the culture medium increased the expression of the 19.3-kDa

Table 5. Hemagglutination patterns of *E. tarda* strains with guinea pig erythrocytes

Type	Representative strain	NaCl[*1] %	Two-fold serial dilution of *E. tarda* suspension (2^n)								No. of strains isolated from			
			0[*2]	1	2	3	4	5	6	7	flounder[*3]	red sea bream[*3]	eel[*4]	Total
A	FK1051	0	–	–	–	–	–	–	–	–	13	0	4	17
		3	++	++	++	++	++	++	+	–				
B	KG8401	0	++	++	++	++	–	–	–	–	21	5	9	35
		3	++	++	++	++	++	++	++	–				
C	SU166	0	–	–	–	–	–	–	–	–	0	0	3	3
		3	–	–	–	–	–	–	–	–				

[*1] NaCl concentration of the broth for culturing *E. tarda* strains
[*2] initial bacterial suspension (A_{530} = 1.0; 10^9 CFU/mL)
[*3] diseased fish
[*4] diseased eels (kidneys), healthy eels (intestinal contents), or eel-pond water
++, strongly positive; +, positive; –, negative

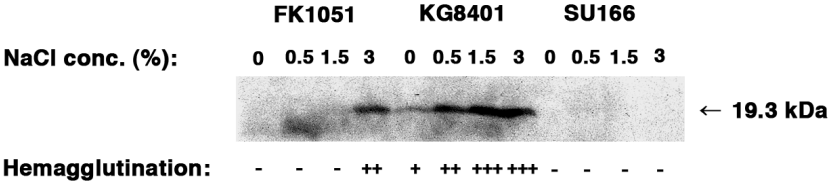

Fig. 1. Detection of the 19.3-kDa protein by Western blot

Table 6. Pathogenicity of *E.tarda* FK1051 in Japanese flounder

Experiment	Challenge method	NaCl[*1] %	Challenge dose (CFU/fish)	No. of dead fish/examined	Mean time to death (days)	No. of fish from which *E. tarda* was reisolated/survived
1	Oral administration	0	6.0×10^7	4/10	11.3	1/6
		0	6.0×10^5	0/10	21 <	1/10
		0	6.0×10^3	0/10	21 <	0/10
		3	4.5×10^7	7/10	8.1	3/3
		3	4.5×10^5	3/10	7.7	0/7
		3	4.5×10^3	2/10	6.5	0/8
	Intraperitoneal injection	0	3.8×10^4	10/10	6.2	–
		0	3.8×10^2	6/10	10.8	4/4
		3	3.6×10^4	8/10	6.8	2/2
		3	3.6×10^2	4/10	9.8	6/6
2	Oral administration	0	2.7×10^5	2/15	32	5/13
		0	2.7×10^3	0/15	60 <	0/15
		3	2.0×10^5	8/15[*2]	36	3/7
		3	2.0×10^3	1/15	35	1/14

[*1] NaCl concentration of the broth for culturing *E. tarda* FK1051
[*2] $< p = 0.05$

protein. No expression of the 19.3-kDa protein was found in any cultures of SU166. These expression profiles of the 19.3-kDa protein were well-correlated with HA of the strains (Fig. 1).

Experimental infection

The results of infection experiments are summarized in Table 6. In the case of oral challenge in the experiment 1, fish inoculated with the 3%-NaCl culture died earlier and reached higher mortalities compared with fish inoculated with the 0%-NaCl culture, though the difference in the mortality was not significant. In contrast, fish challenged by intraperitoneal infection began to die at 3 to 4 days post-injection and were highly susceptible to both the 0%-NaCl and 3%-NaCl cultures, with the LD_{50} being approximately 10^2 CFU/fish for both cultures. Accumulation of ascites fluid and hypertrophy of spleen and kidney were observed in dead fish of both groups and the inoculated *E. tarda* was re-isolated from all dead fish. *E. tarda* was reisolated from all survivors after intraperitoneal injection, while re-isolation rates from survivors by oral challenge were somewhat low. In the experiment 2, in which larger fish were orally challenged at 10^5 or 10^3 CFU/fish, delayed mortality of fish was observed. Challenge with the 3%-NaCl culture at 10^5 CFU/fish produced significantly higher mortality showing typical clinical signs than that with the 0%-NaCl culture.

Discussion

There have been some studies on hemagglutinins of *E. tarda* using guinea pig erythrocytes, in which both mannose-resistant HA (MRHA) and mannose-sensitive HA were described, as cited before. These hemagglutinins are either fimbriae or afimbrial adhesin protein. Sakai *et al*. (2003) purified a 19.3-kDa protein associated with MRHA in *E. tarda* isolates from fish and identified the protein as a fimbrial major subunit (FimA) of *E. tarda*. As the MRHA was inhibited by fetuin and N-acethylneuraminic acid, they suggested that the carbohydrate binding adhesion might play an important role in colonization of *E. tarda*.

In the present study, the HA of *E. tarda* against guinea pig erythrocytes was enhanced by higher NaCl concentration in the culture media, except that three strains from eel and eel-culture environments had no HA, but no clear-cut relationship was found between the source of strains and HA. In addition, either 0%- or 3%-NaCl culture or both of some strains showed hemolytic activity against guinea pig erythrocytes but no relationship between the hemolytic activity and HA was found. NaCl-induced HA that we demonstrated in this study may explain the reason why Ullah and Arai (1983) failed to detect HA in *E. tarda* isolates from fish and why Sakai *et al*. (2003) showed variations in the HA titers, in which *E. tarda* cells were cultured in or on the media without extra-supplementation of NaCl. Therefore, the cultures using NaCl (3%)-supplemented media should be included in the future HA studies of *E. tarda*. It is known that hyperosmolar conditions decreased fimbrial expression of *Escherichia coli* or adherence to human intestinal cells (Henle 407) of *Salmonella typhi* (Tartera and Metcalf, 1993; Kunin *et al*., 1994). The mechanism of osmoregulation for HA or fimbriae production in *E. tarda* remains to be studied in future.

The present study showed a positive correlation between HA and expression of the 19.3-kDa fimbrial protein, both of which were induced by NaCl. Since intestine is a possible portal for *E. tarda* in Japanese flounder (Mekuchi *et al*., 1995b), we hypothesized that fimbriae of *E. tarda* are associated with adherence to the intestinal epithelial cells and therefore NaCl-induction of HA or the fimbrial protein is important for *E. tarda* to cause the disease in marine fish. In the present oral infection experiments with *E. tarda*, the 3%-NaCl culture of the strain FK1051 with high HA exhibited higher virulence to flounder than the 0%-NaCl culture with no HA. Furthermore, our preliminary experiment with the FK1051 confirmed higher adherence ability of the 3%-NaCl culture to RTG-2 cells, compared with the 0%-NaCl culture (data not shown). These results may support the above-mentioned hypothesis. In contrast, when bacteria were inoculated intraperitoneally to fish, there was no significant difference in the virulence between the 3%-NaCl and 0%-NaCl cultures. This suggests that adherence by fimbriae is not required for further virulence of *E. tarda* once the organism was inside of hosts. Similar relationship between fimbriae or HA and infectivity was reported in *S. typhimurium*; higher virulence of a fimbriate strain with high HA in mice was observed by oral administration but not by intraperitoneal injection (Duguid *et al*. 1976). Considering that *E. tarda* is ubiquitous in flounder culture environments or intestines even when no disease outbreaks are found (Kanai *et al*., 1988; Rashid *et al*., 1994), high expression of hemagglutinins or fimbriae may be important for infection of the organism via non-parenteral routes. A further study on the significance of hemagglutinins or fimbriae in the virulence of *E. tarda* is still needed.

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