Comparison of Toxin Composition Between Populations of *Alexandrium* spp. from Geographically Distant Areas

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Axenic clonal isolates of the dinoflagellates *Alexandrium tamarense* and *A. catenella* derived from benthic cysts from Ofunato Bay (Iwate Prefecture, Japan), Tanabe Bay (Wakayama Prefecture, Japan) and motile cells from the Seto Inland Sea were subjected to toxin analysis by HPLC. Toxin contents and compositions of two or four sexually different vegetative cell germinated from each cyst were compared. In *A. tamarense*, the toxin compositions (mole %) of six isolates were relatively constant, but one isolate showed a clear distinction in a lack of *N*-sulfocarbamoyl (Cx) toxins. In *A. catenella*, the toxin composition was rather uniform within a geographical region. Moreover, toxin compositions of *A. catenella* isolates from Tanabe Bay and the Seto Inland Sea were clearly distinguished from those of *A. catenella* from Ofunato Bay. These results indicate the occurrence of inter- and intra-specific indigenous populations from distant localities, and the toxin profiles separate one morphospecies into two regional populations.

Several dinoflagellates of the genus *Alexandrium* produce potent neurotoxins responsible for paralytic shellfish poisoning (PSP). Toxicity of an array of those toxins varies by several orders of magnitude.¹⁾ The northeastern coast of Japan, especially Ofunato Bay, is a representative area for PSP problems; the occurrence of *Alexandrium* spp. and shellfish infestation have been well documented.²⁻⁶⁾ Oshima *et al.*⁷⁾ investigated the toxin profiles of *A. tamarense* from various locations of northern districts. The toxin profile revealed a similar pattern within a geographical region, but this is not the case in different regions. The same trend was observed in the isolates from North America⁸⁻¹⁰⁾ and from widely separated geographical locations (the northeastern Pacific, eastern Canada, Portugal, the United Kingdom, and New Zealand).¹¹⁾ However, toxin composition changes in an isolate occur under different growth conditions.¹¹⁾ Toxin profile varied among the unaxenic isolates of *A. tamarense* or *A. catenella* from different regions, and it was not significantly related to morphological differences.^{11,12)}

In the present study, axenic clonal isolates of *A. tamarense* and *A. catenella* established from benthic

cysts in Ofunato Bay (Iwate Prefecture) and Tanabe Bay (Wakayama Prefecture), and vegetative cells isolated from the Seto Inland Sea were subjected to toxin analysis by HPLC. Toxin compositions among geographically different populations of *Alexandrium* were compared.

Materials and Methods

Organisms and Culture

Cysts of the genus *Alexandrium* were isolated from sediments in Ofunato Bay (Iwate Prefecture, Japan, 1984 and 1988), and Tanabe Bay (Wakayama Prefecture, Japan, 1987). Individual cysts were isolated and germinated in SWIIm medium at 20°C. The germinant cells through the first and second cell divisions were separated individually and grown enough to obtain axenic clones by a micropipette washing method. After preparing axenic culture, mating type was determined by crossing these clones with each other. Three strains (SE6, SE7, SE82) of *A. catenella* were kindly provided by Dr. S. Yoshimatsu (Akashiwo Research Institute of Kagawa Prefecture), which were isolated from a water sample from the Seto

Abbreviation: PSP, paralytic shellfish poisoning; GTX, gonyautoxin; STX, saxitoxin; NEO, neosaxitoxin.

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Table 1. Origin of *Alexandrium tamarense* and *A. catenella* isolates germinated from benthic cysts from Ofunato Bay and Tanabe Bay

Isolates	Origin	Date of sampling/isolation	Mating type	Type of parent cell
<i>A. tamarense</i>				
OF151	Ofunato Bay, Iwate (141°43'E-39°2'N)	Mar 1984/1988	+	T(C)
OF152	"	"	—	T(C)
OF181	"	"	+	T(C)
OF184	"	"	—	T(C)
OF191	"	"	+	T(C)
OF192	"	"	—	T(C)
OF051	"	"	+	T(C)
<i>A. catenella</i>				
OF071	Ofunato Bay, Iwate (141°43'E-39°2'N)	Mar 1988/1988	—	T(C)
OF072	"	"	+	T(C)
OF101	"	"	—	T(C)
OF102	"	"	+	T(C)
TN7	Tanabe Bay, Wakayama (135°22'E-33°41'N)	May 1987/1987	—	T(C)
TN9	"	"	+	T(C)
TN10	"	"	—	T(C)
TN11	"	"	—	T(C)
TN12	"	"	+	T(C)
TN16	"	"	—	T(C)
TN22	"	"	+	T(C)
SE6	Harima Nada, Kagawa	1981	—	M
SE7	"	"	—	M
SE82	"	"	+	M

Motile cells from Harima Nada of the Seto Inland Sea were adopted for comparing.

T(C), one of the tetrad cells divided from a germinated cyst; M, motile cell isolated from water sample.

Inland Sea in 1980. Axenic cultures were established by us. Details of origin are presented in Table 1. All cultures were maintained in SWIIm medium at 15°C for *A. tamarense* and at 20°C for *A. catenella*.¹³⁾

Cultures for toxin analyses used in this experiment were grown at 15 or 20°C with 14L: 10D cycle at 100 $\mu\text{Em}^{-2}\text{s}^{-1}$ with cool white bulbs.¹³⁾ Approximately 10^6 cells at the mid-exponential growth phase were inoculated in 2 l of SWIIm medium contained in a 3 l flask. Cells ($\text{ca. } 2 \times 10^6$) were harvested in the mid-exponential growth stage at the mid-point of the light phase in the photocycle, in which toxin content and toxin composition were stable, as established in the previous study.^{13,14)}

Toxin analysis

The sampled cells were concentrated by centrifugation (2,000 $\text{g} \times 5$ min) at 20°C. The cells were rinsed once with cold distilled water and

re-centrifuged. The pelleted cells were resuspended in 0.05 M acetic acid and were stored at -20°C until toxins were extracted. The frozen samples were thawed and disrupted by mild sonication at 0°C . After the sonication the cell debris was removed by centrifugation (10,000 $\text{g} \times 10$ min). The supernatant was filtered through a membrane filter with 10,000 MW cut-off (Ultrafree C3GC, Millipore) by centrifugation (8,000 $\text{g} \times 5$ min), and stored at -20°C , until analysis.¹⁴⁾ Toxin analyses were carried out by means of an HPLC-fluorometric PSP toxin analyser (Waters 600E system, 470 fluoromonitor) on Wakosil 5C8 column (4.6 $\times$ 250 mm; Wako Pure Chemical) as described by Oshima *et al.*¹⁴⁾

Results and Discussion

Table 1 shows a list of the isolates examined. All isolates were identified as *A. tamarense* or *A. catenella* on the basis of morphological taxonomy

Table 2. Individual toxin content per cell and percent composition of total toxin from *Alexandrium tamarense* and *A. catenella* analysed by HPLC

Isolates	Sexuality	Toxins, fmol/cell (% composition of total toxin)													Total
		GTX1	GTX2	GTX3	GTX4	dcGTX3	GTX5	GTX6	epiGTX8	GTX8	C4	neoSTX	dcSTX	STX	
OF151	+	1.0 (2.0)	—	0.6 (1.1)	13.8 (26.5)	—	—	—	0.7 (1.4)	35.4 (67.8)	0.6 (1.1)	Tr	—	—	52.2 (100)
OF152	—	4.2 (13.6)	0.1 (0.3)	0.9 (2.9)	25.2 (80.5)	—	—	—	—	—	—	0.9 (2.7)	Tr	Tr	31.3 (100)
OF181	+	1.0 (2.7)	0.2 (0.4)	1.5 (4.1)	7.9 (21.0)	—	—	—	0.7 (1.9)	25.9 (69.1)	—	Tr	0.3 (0.7)	—	37.4 (100)
OF184	—	1.8 (7.8)	—	0.5 (2.1)	5.6 (23.9)	—	—	—	0.7 (3.2)	11.9 (50.4)	0.2 (0.7)	2.1 (8.8)	0.7 (3.1)	—	23.5 (100)
OF191	+	0.6 (0.9)	Tr	0.2 (0.3)	6.7 (11.0)	Tr	—	—	0.8 (1.4)	33.1 (54.2)	0.9 (1.5)	16.9 (27.6)	—	2.0 (3.2)	61.1 (100)
OF192	—	0.6 (1.3)	0.1 (0.1)	0.2 (0.5)	5.8 (12.1)	—	—	—	0.7 (1.5)	29.5 (62.1)	0.4 (0.8)	10.3 (21.6)	—	Tr	47.6 (100)
OF051	+	1.0 (1.8)	—	0.6 (1.2)	16.2 (30.1)	—	—	—	0.6 (1.2)	26.4 (49.0)	1.0 (1.8)	8.2 (15.1)	—	Tr	53.9 (100)
OF071	—	—	—	0.0 (0.1)	—	—	0.7 (1.4)	—	0.4 (0.8)	13.3 (28.1)	0.1 (0.2)	31.9 (67.5)	—	0.9 (2.0)	47.3 (100)
OF072	+	—	—	0.1 (0.4)	—	—	0.3 (1.4)	—	0.3 (1.4)	6.9 (33.2)	—	10.4 (50.0)	—	2.8 (13.5)	20.7 (100)
OF101	—	—	—	0.1 (0.4)	Tr	—	Tr	—	1.3 (5.3)	14.1 (57.6)	—	7.5 (30.6)	—	1.5 (6.1)	24.5 (100)
OF102	+	—	—	0.6 (0.9)	—	—	0.2 (0.4)	—	0.3 (0.6)	9.3 (15.9)	—	46.5 (79.4)	—	1.6 (2.8)	58.5 (100)
TN7	—	0.2 (0.5)	—	0.0 (0.1)	2.2 (5.9)	—	13.5 (35.7)	0.2 (0.5)	0.5 (1.4)	19.0 (50.2)	1.1 (2.9)	Tr	Tr	1.0 (2.8)	37.8 (100)
TN9	+	0.3 (1.5)	—	0.1 (0.3)	0.9 (5.2)	—	3.1 (17.8)	3.5 (20.0)	0.5 (3.1)	8.6 (48.9)	0.6 (3.1)	—	—	—	17.6 (100)
TN10	—	Tr	—	0.0 (0.1)	1.7 (5.1)	—	6.1 (18.4)	0.2 (0.6)	0.4 (1.1)	23.7 (70.9)	1.3 (3.9)	—	—	Tr	33.5 (100)
TN11	—	0.2 (1.7)	—	0.0 (0.3)	1.6 (13.0)	—	2.4 (19.0)	Tr	0.2 (1.7)	6.5 (51.7)	1.3 (10.3)	Tr	—	0.3 (2.3)	12.6 (100)
TN12	+	Tr	—	Tr	1.7 (5.5)	—	3.1 (9.9)	14.8 (48.1)	0.2 (0.6)	9.3 (30.4)	1.1 (3.5)	—	—	0.6 (2.1)	30.7 (100)
TN16	—	—	—	0.0 (0.1)	1.5 (6.7)	0.0 (0.1)	1.7 (7.6)	9.2 (40.6)	0.3 (1.4)	8.9 (39.2)	1.0 (4.5)	—	—	—	22.6 (100)
TN22	+	Tr	—	Tr	2.4 (13.6)	—	6.4 (35.5)	0.5 (2.9)	0.5 (2.9)	7.3 (40.5)	0.8 (4.5)	—	—	Tr	17.9 (100)
SE6	+	—	—	0.0 (0.1)	1.0 (3.5)	—	5.6 (20.3)	3.9 (14.2)	0.5 (2.0)	15.3 (55.9)	1.1 (4.0)	—	—	—	27.4 (100)
SE7	—	—	—	0.0 (0.2)	1.2 (4.8)	—	5.1 (20.9)	0.7 (2.9)	0.4 (1.5)	10.8 (44.7)	0.5 (2.2)	5.5 (22.8)	—	—	24.2 (100)
SE82	+	Tr	—	Tr	0.5 (3.0)	—	0.8 (5.0)	5.8 (35.3)	0.1 (0.8)	7.1 (43.4)	0.6 (3.4)	1.5 (9.1)	—	—	16.4 (100)

All isolates were established from benthic cysts from Ofunato and Tanabe Bays except for the isolates from the Seto Inland Sea established from motile cells.
Tr, trace; —, not detected; 0.0, <0.05.

described by the criteria of Fukuyo¹⁶⁾ and isozyme analysis,¹⁷⁾ and reactivity of monoclonal antibody.¹⁸⁾ Each isolate showed either (+) or (-) mating type in sexuality (Table 1), according to heterothallic characteristics.¹⁹⁻²¹⁾ When toxin content and toxin composition of sexually different isolates germinated from each cyst were compared, distinct similarities of toxin composition were observed in each pair except for one pair of *A. tamarens* (OF151 and OF152) (Table 2). In *A. catenella*, about twice the difference in toxin content was observed in four isolates from Ofunato Bay (Table 2). Toxin content of *A. tamarens* from Ofunato Bay was nearly twice as high than that of *A. catenella* from Tanabe Bay (Table 2). The differences in toxin content (f mol/cell) between isolates from geographically distant regions were also reported by Cembella *et al.*¹¹⁾ As de-

scribed by Anderson,²²⁾ northern isolates from Japan largely contain highly potent carbamate toxins (*e.g.* NEO and GTX1-4), whereas southern isolates largely contain weaker *N*-sulfocarbamoyl toxins (*e.g.* GTX5, GTX6, GTX8, epiGTX8, and C4) (Table 2).

The previous papers^{13,14)} and several investigators,^{11,23,24)} concluded that toxin composition is a stable property under identical growth conditions, and that composition differences have a genetic basis in *A. tamarens* and *A. catenella*. The toxin compositions of seven isolates of *A. tamarens* from Ofunato Bay and four isolates, seven isolates, and three isolates of *A. catenella* from Ofunato Bay, Tanabe Bay, and the Seto Inland Sea, respectively, were compared (Fig. 1). In *A. tamarens*, the toxin composition of six isolates from Ofunato Bay, except for OF152

Fig. 1. Comparison of the average toxin composition (as % total toxin) of *A. tamarens* (A) and *A. catenella* (B, C, D) collected from different regions.

A, 7 isolates from benthic cysts (Ofunato Bay); B, 4 isolates from benthic cysts (Ofunato Bay); C, 7 isolates from benthic cysts (Tanabe Bay); D, 3 isolates from motile cells (Seto Inland Sea). Error bars denote one standard deviation. Abbreviations: C1, epiGTX 8; C2, GTX 8; NEO, neoSTX; (dc)STX, dcSTX + STX.

which were deficient in Cx toxins, included GTX8 (49–69 mol%) and GTX4 (11–30 mol%) as major components, and GTX1, GTX2, GTX3, and NEO (Trace–27 mol%) as minor components, with the sporadic appearance of dcSTX and STX (Table 2).

Sekiguchi *et al.*⁶⁾ reported that the seasonal blooms (summer in 1980, spring in 1981, and both in 1982) of *A. tamarense* were closely related to scallop toxicity. Kodama and Ogata⁴⁾ indicated that *A. tamarense* was responsible for the high toxicity of scallop in spring blooms but not in autumn blooms, while the association of *A. catenella* to shellfish toxicity was not clear in this region. Seven isolates of *A. catenella* from Tanabe Bay showed a higher proportion of *N*-sulfofocarbamoyl toxins (79–95%), including GTX5 and GTX6, and the toxin compositions were very similar to those of the isolates from the Seto Inland Sea except for containing NEO (Table 2). As shown in Fig. 1, toxin profiles of the isolates of *A. catenella* from Ofunato Bay clearly differed from those of the isolates from Tanabe Bay and the Seto Inland Sea, not only in lacking GTX1 + GTX4 and GTX6, but also in containing NEO to a higher proportion, although *A. catenella* from Ofunato Bay is completely identical to that from Tanabe Bay in the criteria of morphology,¹⁶⁾ isozyme analysis,¹⁷⁾ and monoclonal antibody.¹⁸⁾ The same trend in toxin composition variation related to regional populations was documented on the isolates of northern Japan,^{7,25)} North America,^{8–10)} and widely separated geographical regions.¹¹⁾ Ofunato Bay and Tanabe Bay are geographically distant from each other (more than 1,000 km), and Ofunato Bay belongs to the Oyashio, while Tanabe and the Seto Inland Sea belong to the Kuroshio. Considering these facts, it is probable that intraspecific indigenous populations have developed in each region.

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