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Bacteriocin ("Vulnificin") Typing of *Vibrio vulnificus*

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Vibrio vulnificus, a halophilic vibrio is an estuarine gram-negative bacteria that is associated with severe and frequently fatal wound infections and life-threatening septicemia. Bacteriocins are defined as antibacterial substance produced by various species of bacteria which are usually active against closely related organisms. Bacteriocins have found widespread application in epidemiological studies as specific markers of bacteria. It was proposed by Ha et al. (1990. J. Korean. Soc. Microbiol. 25: 586.) to give the bacteriocins produced by *V. vulnificus* the name "vulnificins". In the present study, a total of 72 strains of *V. vulnificus* isolated from patients and oysters were subjected to screen potential producers and indicators of vulnificin, applying ultraviolet induction method. Sensitivity of several strains of *Serratia marcesans, Pseudomonas aeruginosa, Shigella flexneri, Salmonella typhi* and *Yersinia enterocolitica* to vulnificins were also examined out. All the tested strains of *V. vulnificus* produced vulnificins active against indicator strains with various different inhibitory patterns. The spectrum of vulnificin activity and sensitive spectrum of indicator strains were considerably broad. Interestingly, almost all strains of *S. marcescens, P. aeruginosa, Salmonella sp., Shigella sp.* and *Y. enterocolitica* tested were sensitive to 1-7 vulnificin(s). Taken together, the present study demonstrated that all of the isolates of *V. vulnificus* produced vulnificins and that 8 good vulnificin producers and 10 good indicators were detected. These strains can be employed efficiently for establishing vulnificin typing scheme of *V. vulnificus* and for the detection of bacteriocinogeny and sensitivity in *V. vulnificus*. Biological role of vulnificin remains to be further elucidated.

Key Words: *V. vulnificus*, Bacteriocin

INTRODUCTION

Vibrio vulnificus, a halophilic vibrio is an estuarine, gram negative bacteria that causes severe and frequently fatal necrotizing wound infections and fulminant life-thretening septicemia in addition to gastroenteritis in humans (2,5,24,28). *V. vulnificus* particularly affects subjects with liver disease, long-term alcohol abuse, diabetes mellitus, hemochromatosis, gastrointestinal disorders, and immunosuppression resulting from corticosteroid therapy, cancer and AIDS (2,24, 28,34). *V. vulnificus* infection is associated with consumption by susceptible hosts of raw shellfish containing the bacteria, especially oyster, or by direct contact to preexisting wounds with sea water (34). Annually, many clinical cases are reported in the United States, Korea, Japan, and Taiwan (5,24,25,30,34). More than 50% of these

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cases succumbed multi-organ failure as a results of a rapidly progressive shock syndrome despite an aggressive antimicrobial therapy and supportive care (24,25).

Gratia, in 1925 (15) was the first to demonstrate isoantagonism among closely related strains of *Escherichia coli,* due to lethal substances produced by them. Later, Fredericq (10) extended this work and found similar antagonistic substances in other *E. coli* strains and called them colicins. Such substances were then demonstrated in *Salmonella* (12), and *Shigella* (11). It was proposed by Jacob et al. (22) to give such isoantagonistic substances, the term "bacteriocins". Bacteriocins were demonstrated in a variety of bacteria and analogue substances were found in other genera of bacteria (19). These are pyocin produced by *Pseudomonas aeruginosa* (21), pesticin produced by *Pasteurella pestis* (1), megacin produced by *Bacilluce megaterium* (32), and vibriocin produced by *Vibrio chorelae* (3,7,16,26).

It was proposed by Ha et al. (16-18) to give the bacteriocin produced by *Vibrio vulnificus* in terms of "vulnificin". Bacteriocins have been widespread application in epidermiological studies as specific markers of bacteria (33,35). The present study were undertaken to investigate further the production and sensitivity of *V. vulnificus* isolated from patients and oysters and vulnificin's inhibitory activity against the growth of *V. vulnificus* and some other bacteria such as *Salmonella sp., Shegella sp., Pseudomonas aeruginosa,* and *Yersinia enterocolitica.*

MATERIALS AND METHODS

Test bacterial strains. A total of 72 strains of *V. vulnificus* isolated from patients and oyster were subjected to this study. Of 72 strains, 25 were isolated from patients (20 strains) and oysters (5 strains) during the period between 1987 and 1990, and 47 were isolated from patients (32 strains) and oysters (15 strains) between 1991 and 1996. These strains were collected from 5 university hospitals in South Korea, including Yonsei University, Chonnam National University, Pusan National University, Wonkang University and Chonbuk National University Hospitals during the periods. In addition, 8 laboratory strains kindly supplied by other investigators were used in selected study. Thirty-three strains which were composed of 25 strains isolated between 1987 and 1990 and 8 laboratory strains were subjected to the first part of this study and 47 strains isolated between 1991 and 1996 were subjected to the second part of this study. Of 8 laboratory strains, 5 strains (*V. vulnificus* D8806, C7184, C2756, H3308, and E2272) were kindly provided by J. D. Oliver, University of North Calorina at Charlotte, NC., two strains (*V. vulnificus* ATCC 29307 and CDC A1402) were kindly supplied by D. H. Hollis of the Center of Disease Control, Atlanta, Ga., and remaining the rest one strain (*V. vulnificus* 80-02-125) was kindly supplied by D. L. Tison, Clinical Laboratory, Multicase Medical Center, Tacoma, WA.

Indicator strains and producer strains. As there is no standard indicator strains for the detection of bacteriocin produced by *V. vulnificus,* we used potential indicator strains for vibriocin typing for *Vibrio chorelae.* The following 6 indicator strains were obtained through the courtesy of Sujata Dastidar, Division of Microbiology, Jadavpur University, Calcutta, India. These strains were used successfully for bacteriocin typing for *V. cholerae.* Other 3 strains of *V. cholerae* were used as indicator strains as shown in Table 1. We called these 9 indicator strain as the "standard" indicator strains. In addition, 47 strains isolated from patients and oysters were also used as the potential indicator and producer strains.

Media and Culture. Stock cultures were maintained on slants of a modified salt water-yeast extract agar (29), composed of 0.1% yeast extract (Baltimore Biological Laboratory), and

Table 1. Sources of standard indicator strains

Indicator strains	Designated as	Sources
Shigella flexneri 3189	A	S. G. Dastida Jadavpur University Calcutta, India
Escherichia coli Row	B	Ditto
Shigella sonnei 56	C	Ditto
Shigella sonnei 17	D	Ditto
Shigella sonnei M56	E	Ditto
S. flexneri 38	F	Ditto
Vibrio cholerae 1969, (ogawa)	G	Authors isolated from stool of a patient in 1969 epidermic in Korea
V. cholerae EL Tor, (ogawa)	H	Ditto
V. cholerae (Inaba) SM 128	I	H. Farkas-Himsley University of Toronto, Canada

1.5% agar in a three-salt solution (0.75 g of KCl, 7.0 g of $MgSO_4 \cdot 7H_2O$, and 23.4 g of NaCl in distilled water to a final volume of 1 liter). Cultures were transferred to fresh medium at intervals of about 10-days. Bacteria used for the production of and sensitivity to "vulnificin" were taken from modified salt water-yeast extract agar slants and grown in brain heart infusion both (BHI broth, Difco Laboratory, Detroit, Mich.), supplemented with an additional 2.5% NaCl.

Bacteriocin typing procedure. The bacteriocin production and detection methods was a combination of stab culture and ultraviolet irradiation induction method as described by others (16-18,26). This method was a modification of double layer technique used by Fredericq (13). Briefly, for detection of bacteriocin production, the cells potential producer strains grown in BHI broth and were transferred on brain heart infusion agar (Difco Laboratories, Detroit, Mich) plates supplemented with 2.5% NaCl and cultivated for 24 hr at 37℃. The bacterial cells were inoculated by a straight stab into 1.5% nutrient agar plates. After incubation for 48 hr at 37℃, the cultures in a petri dish were exposed to ultraviolet radiation from a distance of 15 cm for 15 minutes. An eight watt Germicidal Lamp (Genneral Electric Co.) was used emitting light at 2537Å. A 50 μl amount of an overnight culture of indicator strains (approximately 10^7 bacteria) in the logarithmic growth phase in BHI broth was seeded to 2.5 ml of melted 0.6% semisoled agar (Hershey agar) in a test tube maintained at 45℃ water bath. The indicator culture was layered over the surface of killed cultures by UV irradiation, then solidfied and cultured for 24 hr at 37℃ with extended incubation when necessary. The plates were observed for zone of inhibition. Zones of inhibition indicated bacteriocin (vulnificin) production.

Reading of Results. The production of vulnificin was evaluated by reading of inhibitory zone over the area of producer growth. The susceptibility of indicator strains to vulnificin was categorized as 3 patterns, very sensitive, sensitive and resistant. The diameter of inhibitory zone (D) was calculated using the following formula. i.e. D = D24 − D0, where D24 is diameter of inhibitory zone 24 hr after incubation and D0 is diameter of bacterial colony. "Very sensitive" signified the value of D more than 10 mm. "Sensitive" signified D from 2 to 10 mm. "Resistant" signified D less than 2 mm or indicating test bacteria did not produce vulnificin.

Table 2. Production of "vulnificin" active against the standard indicator strains

"Standard" indicator strain (Designated as)	No. of producer strains forming vulnificin active on the respective indicator strains			Total	
	Isolates from patients (20 strains)	Isolates from oyster (5 strains)	Standard lab. strains (8 strains)	No.	%
A	19	5	4	28	84.8
B	12	3	4	19	57.6
C	14	4	5	23	69.7
D	8	0	5	13	39.4
E	7	2	3	12	36.4
F	11	1	2	14	42.4
G	13	4	4	21	63.6
H	15	5	5	25	75.8
I	7	1	3	11	33.3

Indicator strains: A, *Shigella flexneri* 3189; B, *Escherichia coli* ROW; C, *Shigella sonnei* 56; D, *Shigella sonnei* 17; E, *Shigella sonnei* M56; F, *Shigella flexneri* 38; G, *Vibrio cholerae* 1969; H, *Vibrio cholerae* EL Tor; I, *Vibrio cholerae* Inaba SM128

Table 3. The sensitivity of 9 "standard" strains to the "vulnificin" produced by *Vibrio vulnificus*

No. of indicator strains sensitive to vulnificin	No. of producer strains inhibiting stated no. of indicator strains			Total (%)
	Isolates from patients	Isolates from oyster	Standard lab. strains	
8	1	0	0	1 (3.0)
7	5	1	3	9 (27.3)
6	4	0	0	4 (12.1)
5	3	3	0	6 (18.2)
4	3	1	1	5 (15.2)
3	3	0	2	5 (15.2)
2	1	0	2	3 (9.1)
1	0	0	0	0 (0)
Total (%)	20 (60)	5 (15)	8 (24)	33 (100)

RESULTS

Production of vulnificin against the standard indicator strains by *V. vulnificus* isolates and laboratory strains. A total of 33 strains of potential producer strains composed of 20 strains of *V. vulnificus* isolated from patients, 5 strains of the isolates from oysters between 1987 and 1990, and 8 strains of laboratory strains of *V. vulnificus* were subjected to investigate for the production of vulnificin, against 9 strains of standard indicator bacteria. The sources of bacteria tested were described in Table 1. As the results, all strains tested produced vulnificin active against one or more strain(s) of the 9 indicator strains (Table 2). Of the 33 strains tested, 28 (84.8%) strains inhi-

Table 4. The spectrum of activity against 9 "standard" indicator strains of the "vulnificins" produced by 33 strains of *Vibrio vulnificus*

No. of indicator strains sensitive to vulnificin	No. of patterns	Sensitivity patterns of indicator strains to vulnificins
8	1	ABCDFGHI
7	1	ABCFGHI
	1	ABCEGHI
	1	ABCEFHI
	1	ABCDFGH
	1	ABCEFGH
	1	ABCDFGH
	1	ACDEGHI
	1	ACDFGHI
	1	BCDEFGH
6	2	ABCDGH
	1	ABCEGH
	1	ABCFGH
	1	AEGHI
	1	ABDFI
	1	ACEHI
	1	ABCGH
	1	ABCDEFGHI
	1	ABCDI
4	1	ACGH
	1	ABGH
	1	ACDH
	1	AFHI
	1	BCEG
3	1	ACH
	1	FGH
	1	AEF
	1	BCH
	1	ADH
2	1	AF
	1	BI
	1	AD
No. of patterns	32	

*ABC GH is the most common sensitive bacteriolytic complex pattern.

bited the growth of indicator strain, *Shigella flexneri* 3189 (designated as A), 25 (75.8%) inhibited *V. cholerae* EL Tor (H), 23 (69.7%) inhibited *S. sonnei* (C), 21 (63.6%) inhibited *V. Cholerae* 1969 (G), 19 (57.6%) inhibited *E. coli* ROW (B) and remaining 11-13 strains (33.3-42.4%) inhibited other indicator strains, respectively. There were no significant difference in the production of vulnificin by specimen source.

Spectrum of vulnificin activity against 9 standard indicator strains and vulnificin sensitivity patterns of the indicator strains. The sensitivity of 9 standard indicator strains to vulnificins produced by 33 strains of *V. vulnificus* was investigated. As shown in Table 3, 9 strains (27.3%) produced vulnificin inhibiting 7 indicator strains, 6 strains produced vulnificin inhibiting 5 indicator strains, and other strains produced vulnificin inhibiting 2, 3, 4, 6 or 8 indicator strains, respectively. Thirty-two kinds of the sensitivity patterns were observed (Table 4), suggesting that the spectrum of vulnificin activity and the sensitive spectrum of the indicator strains were considerably broad. Among them two strains exhibited ABCDGH vulnificin pattern and other bacterial strains exhibited a single sensitive pattern. Among the patterns observed, 10 strains demonstrated ABCGH complex pattern, indicating that the corresponding indicator strains were inhibited by one or more strain(s) of 33 strains tested, and 6 strains demonstrated the pattern including GH.

Production of vulnificin by 47 strains of *V. vulnificus* isolated from patients and oysters when 47 isolates were used as their indicator strains. Forty-seven strains isolated between 1990 and 1996 were both used as potential indicator and producer strains and were subjected to investigate the production of vulnificin by the isolates. As shown in Table 5, all of the strains tested were found to produce vulnificins active against 6-37 strains of 47 indicators isolates of *V. vulnificus*. Among the vulnificin producers, 15 strains produced vulnificin inhibiting 16-20

Table 5. Production of vulnificin active against 47 indicator strains of *V. vulnificus* isolates with induction by ultraviolet irradiations in 47 strains of *V. vulnificus* isolated from patients and oysters

No. of producers strains forming vulnificin active on indicator strains	No. of producers strains sensitive to stated no. of *V. vulnificus*	No. of producers strains[a] highly sensitive to stated no. of *V. vulnificus*
> 35	2 (4.3%)	0 (0%)
31-35	1 (2.1%)	0 (0%)
26-30	9 (19.1%)	0 (0%)
21-25	11 (23.4%)	0 (0%)
16-20	15 (31.9%)	0 (0%)
11-15	6 (12.8%)	0 (0%)
6-10	3 (6.4%)	0 (0%)
1-5	0 (0%)	37 (78.7%)
0	0 (0%)	10 (21.3%)
Total (%)	47 (100%)	47 (100%)

[a] The diameter of inhibitory zone(D) was more than 15 mm.

Table 6. The sensitivity of 47 indicator strains to vulnificin produced by 47 strains of *Vibrio vulnificus* isolated from patient and oysters[a]

No. of indicator strains sensitive to vulnificins	No. of producer strains inhibiting stated no. of indicator strains	No. of producer strains highly inhibiting stated no. of indicator strains
31-35	5 (10.6%)	0 (0%)
26-30	8 (17.0%)	0 (0%)
21-25	15 (31.9%)	0 (0%)
16-20	12 (25.5%)	0 (0%)
11-15	3 (6.4%)	1 (2.1%)
6-10	4 (8.5%)	4 (8.5%)
1-5	0 (0%)	25 (53.2%)
0	0 (0%)	17 (36.2%)
Total	47 (100%)	47 (100%)

[a] A total of 47 strains isolated from patients and oysters was used both as indicator and producer strains.

indicators with the highest frequency of 31.9% and 11 strains (23.4%) produced vulnificin inhibiting 21-25 indicator strains. Three strains (6.4%) produced vulnificin inhibiting 31 strains of indicator strains. Interestingly, 3 strains (6.4%) produced vulnificin active against more than 31 strains of indicator strains and 37 strains (78.7%) produced vulnificin highly active against the indicator strains, showing more than 16 mm diameter of the inhibitory zone.

Additionally, forty-seven isolates of *V. vulnificus* were used as indicator strains and examined for their sensitivity to vulnificins produced by 47 isolates. As shown in Table 6, all of the indicator strains were sensitive to vulnificins produced by 1-35 strains, and 21-25 indicator

Table 7. Eight strains of good vulnificin producer among 47 strains tested

Producer	Designated as	Inhibitory pattern active against standard strains (No. of indicators sensitive to stated producer)	No. of indicators sensitive to vulnificins produced by good producer (spectrum of activity against 47 isolates)
1 WK7	P	A B C G H I (6)	28 (59.6%)
2 WK8	Q	A B C E G H I (7)	16 (34.0%)
3 WK12	R	A B C E F H I (7)	10 (21.3%)
11 86-741	S	A B C G H (5)	21 (44.3%)
17 85-HDP	T	A B C E F G H (7)	21 (44.3%)
18 86-PTJ	U	A B C D F G H (7)	18 (38.3%)
22 CU 5	V	A F H I (7)	20 (42.6%)
24 CU 7	W	A B C D F G H I (8)	16 (34.0%)

Table 8. Nine strains of good vulnificin indicator among 47 strains tested

Good indicator strain	Designated as	No. of vulnificin producing strains active against stated indicator (Sensitive spectrum to 47 isolates)
6 YS 4	J1	28 (59.6%)
7 YS 5	J2	31 (66.0%)
8 YS 9	J3	23 (48.9%)
13 YS 86-KST	J5	16 (34.0%)
15 YS 86-7112	K1	22 (46.8%)
17 YS 85-HDP	K2	21 (44.5%)
22 CU 5	K3	27 (57.4%)
27 C 7184	K4	33 (70.2%)
33 ATCC 29307	K5	27 (57.4%)

Table 9. The sensitivity of vulnificin-producing strains of *Vibrio vulnificus* to vulnificin

No. of test strains	No. of strains sensitive to vulnificins	No. of strains highly[a)] sensitive to vulnificin
47	24 (51.1%)	3 (6.9%)

[a)] Inhibitory zone(D) is greater than 15 mm

strains were sensitive to vulnificins produced by 15 producer strains (31.9%). Sixteen to 20 strains were sensitive to vulnificin produced by 12 producer strains (25.5%) and 26-35 strains by 13 strains (27.6%). One to 5 indicator strains were highly sensitive to vulnificin produced by 25 producer strains (53.2%).

Good vulnificin producer strains among the isolates and activity spectrum of vulnificin produced. The vulnificinogeny of 47 strains isolated between 1991 and 1996 of *V. vulnificus* was investigated. Of 47 vulnificinogenic strains, 8 strains (designated as P, Q, R, S, T, U, V, and W) were found to be good producers which exhibited greater inhibitory zone (larger than 15 mm of diameter). Using these strains, the inhibitory patterns active against 9 standard indicator strains and their activity spectrum against 47 isolates were also investigated. As shown in Table 7, good producer strains

Table 10. Sensitive of *Serrata marcescens* to vulnificins produced by 8 good producer strains of *V. vulnificus*

Strain No.	Behavior of strains	Good producer strains[a]							
		P	Q	R	S	T	U	V	W
S. marcescens 10	Sensitive to 4 producers	R[b]	Ⓢ	R	Ⓢ	R	Ⓢ	R	Ⓢ
〃 37	Sensitive to 1 producers	R	R	R	R	Ⓢ	R	R	R
〃 36	Sensitive to 4 producers	R	Ⓢ	Ⓢ	R	R	Ⓢ	R	Ⓢ
〃 30	Sensitive to 2 producers	R	Ⓢ	R	R	R	Ⓢ	R	R
〃 3	Sensitive to 4 producers	R	Ⓢ	Ⓢ	R	R	Ⓢ	R	Ⓢ
No. of strains sensitive to stated producers		0	4	2	1	1	4	0	3

[a] Producer strains;
P, *V. vulnificus* WK 7
Q, *V. vulnificus* WK 8
R, *V. vulnificus* WK 12
S, *V. vulnificus* YS 86-741
T, *V. vulnificus* YS 85-HDP
U, *V. vulnificus* YS 86-PTS
V, *V. vulnificus* CU 3
W, *V. vulnificus* CU 7
[b] Ⓢ, sensitive; R, resistant to vulnificins

Table 11. Sensitive of *Pseudomonas aeruginosa* to vulnificins produced by 8 good producer strains of *V. vulnificus*

Strain No.	Behavior of strains	Good producer strains[a]							
		P	Q	R	S	T	U	V	W
P. aeruginosa 16	Sensitive to 3 producer	R[b]	R	Ⓢ	R	Ⓢ	R	R	Ⓢ
〃 35	Sensitive to 3 producer	Ⓢ	R	R	R	Ⓢ	Ⓢ	R	R
〃 50	Sensitive to all producer	R	R	R	R	R	R	R	R
〃 67	Sensitive to 5 producer	R	Ⓢ	Ⓢ	R	Ⓢ*	Ⓢ	R	Ⓢ
〃 125	Sensitive to 7 producer	R	Ⓢ	Ⓢ	Ⓢ*	Ⓢ*	Ⓢ*	Ⓢ	Ⓢ*
No. of strains sensitive to stated producers		1	2	3	1	4	3	1	3

[a] See footnote Table 10. [b] Ⓢ, sensitive; R, resistant to vulnificins *Indicates highly sensitive.

showed both broad spectrum of activity inhibiting 6-8 strains of 9 standard indicator strains and different inhibitory patterns. These strains also produced vulnificin inhibiting 10-28 indicator strains among 47 isolates, particularly strain P produced a vulnificin inhibiting 28 strains (59.6%), and strain S and T inhibited 21 strains (44.3%), respectively, and other strains inhibited 10-20 strains (21.3-42.6%), suggesting the activity spectrum of the producer strains are considerably broad.

Good vulnificin indicator strains and their sensitive spectra. Of 47 isolates, as shown in Table 8, 10 strains (J1-J5 and K1-K5) were found to be good indicator strains sensitive to 16-33 vulnificins (34.0-70.2%) produced by the isolates, particularly an indicator strain K4 was sensitive to the vulnificin produced by 33 producer strains (70.2%), suggesting its sensitivity spectrum is also broad.

Sensitivity of *Serratia marcescens*, *Pseudomonas aerugenosa*, *Shigella*, *Salmonella* and *Yersinia enterocoritica* to vulnificins produced by 8 good producer strains of *V. vulnificus*. As shown in Table 9, all 5 strains of *S. marcescens* were sensitive to vulnificins produced

Table 12. Sensitive of *Shigella* strains to vulnificins produced by 8 good producer strains of *V. vulnificus*

Strain No.	Behavior of strains	Good producer strains[a]							
		P	Q	R	S	T	U	V	W
S. flexneri 8	Sensitive to 1 producer	R[b]	R	R	R	R	Ⓢ	R	R
7	Sensitive to 5 producer	R	Ⓢ	R	Ⓢ	Ⓢ	Ⓢ*	R	Ⓢ
9	Sensitive to 5 producer	R	Ⓢ*	Ⓢ	R	Ⓢ	Ⓢ	R	Ⓢ
6	Sensitive to 3 producer	R	Ⓢ	R	R	R	Ⓢ*	R	Ⓢ
11	Sensitive to 4 producer	R	Ⓢ	R	Ⓢ	R	Ⓢ*	R	Ⓢ*
No. of strains sensitive to stated producers		0	4	1	1	2	4	0	4

[a] See footnote Table 10. [b] Ⓢ, sensitive; R, resistant to vulnificins *Indicates highly sensitive.

Table 13. Sensitive of *Salmonella typhi* to vulnificins produced by 8 good producer strains of *V. vulnificus*

Strain No.	Behavior of strains	Good producer strains[a]							
		P	Q	R	S	T	U	V	W
S. typhi 2	Sensitive to 4 producer	R[b]	R	Ⓢ	Ⓢ	R	R	Ⓢ	Ⓢ
〃 LT	Sensitive to 6 producer	Ⓢ	Ⓢ	Ⓢ	R	R	Ⓢ	Ⓢ	Ⓢ*
〃 H901	Sensitive to 2 producer	Ⓢ	R	R	R	R	R	R	Ⓢ
〃 0901	Sensitive to 2 producer	Ⓢ	R	R	R	R	R	R	Ⓢ
〃 3	Sensitive to 6 producer	Ⓢ	Ⓢ	Ⓢ	Ⓢ	Ⓢ	Ⓢ	R	R
No. of strains sensitive to stated producers		4	2	3	2	1	2	2	4

[a] See footnote Table 10. [b] Ⓢ, sensitive; R, resistant to vulnificins *Indicates highly sensitive.

Table 14. Sensitive of *Yersinia enterocolitica* to vulnificins produced by 8 good producer strains of *V. vulnificus*

Strain No.	Behavior of strains	Good producer strains[a]							
		P	Q	R	S	T	U	V	W
Y. enterocolitica 551	Sensitive to 2 producer	R[b]	R	R	Ⓢ	R	R	R	Ⓢ
480	Sensitive to 5 producer	Ⓢ	Ⓢ	Ⓢ	R	R	Ⓢ	R	Ⓢ*
841	Sensitive to all producer	Ⓢ	Ⓢ	Ⓢ	Ⓢ	Ⓢ	Ⓢ	Ⓢ	Ⓢ

[a] See footnote Table 10. [b] Ⓢ, sensitive; R, resistant to vulnificins *Indicates highly sensitive.

by 1-4 strain(s) of 8 good producer strains and producer strains Q and U produced vulnificin inhibiting 4 strains of *S. marcescens*, respectively. Of 5 strains of *P. aeruginosa*, 4 strains were sensitive to 3-7 vulnificins and one strain was sensitive to 7 vulnificins, but other one strain (*P. aeruginosa* 50) was resistant to the vulnificins (Table 10). Each five strains of *Shigella* and *Salmonella* tested were sensitive to 1-6 vulnificin(s) and producer strain P produced vulnificin inhibiting none of *Shigella* strains (Table 11, and 12). Interestingly, the sensitivity patterns of *Salmonella typhi* H901 was the same as that of *S. typhi* 0901 (Table 13). Three stra-

ins of *Y. enterocolitica* tested were sensitive to two or more vulnificins, and interestingly one of the strains were sensitive to all individual vulnificin produced by 8 good producers (Table 14). This particular strain may be used as a good indicator strain for the screening of vulnificin production.

DISCUSSION

In our previous study, we observed that *V. vulnificus* produced an antibacterial substance that inhibited the growth of *V. vulnificus, E. coli, Salmonella* and *Shigella*, and we attempted to isolate phage particle. However, serial passages were not successful to detect infectious particles or to increase in potency in the presence of sensitive "indicator" strain. Therefore, we classed the growth inhibitory substance produced by *V. vulnificus* as a bacteriocin, "vulnificin" (18). In the present study, a total of 72 strains of *V. vulnificus* isolated from patients and oysters were subjected to screen for the production of vulnificin, using "standard" indicator strains that were used for vibriocin typing of *Vibrio cholerae* (27) in the early part of this experiment and in the all the test strains produced vulnificin inhibiting the growth of one or more indicator strain(s), and the spectrum of vulnificin activity was considerably broad with 32 different inhibitory patterns. Among the sensitive patterns, patterns including ABCGH or CH were most common, namely *V. vulnificus* strains that produced vulnificins inhibiting *S. flexneri* 3189 (A), *E. coli* Row (B), *S. sonnei* 56 (C), *V. cholerae* 1969 (G) and *V. cholerae* EL Tor (H) were relatively predominant. These results strongly suggest that the spectrum of vulnificin activity and the sensitive spectrum of indicator strains we used are considerably broad and the indicator strains employed in the present study can used efficiently as the "standard" indicator strains to screen for the production of vulnificin.

In order to establish successfully the reliable vulnificin typing scheme for *V. vulnificus* as an epidemiological tool, it may be necessary to find good producer and indicator strains among the isolates from a particular country or area. To investigate this, therefore, production of and sensitivity to vulnificin was attempted, employing 47 strains of *V. vulnificus* isolated from patients and oyster as potential producer and indicator strains. We observed that all the test strains produced vulnificin inhibiting 6-35 of 47 indicator strains and 8 strains (P, Q, R, S, T, U, V and W strains) were good producer strains that produced vulnificin inhibiting 10-28 indicator strains among 47 isolates, particularly producer strain P produced a vulnificin active against 28 strains (59.6%) (Table 7). In addition, we also observed that out of 47 indicator stains, 10 strains (J1-J5 and K1-K5) were good indicator strains that were sensitive to 16-33 vulnificus, particularly an indicator strain K4 was sensitive to vulnificins produced by 33 producer strains (70.2%), suggesting that sensitive spectrum is also broad. At the present, there is no report on the vulnificin production by *V. vulnificus* by other investigator(s) and we are not able to compare our present results with other data concerning vulnificin production and sensitivity.

The vibriocin typing of *V. cholerae*, by Farkas-Himsley et al. (1962) were the first report on the lethal biosynthesis of an antibacterial substance by *V. cholerae*. Thereafter, Farkas-Himsley et al. (8), Chakrabarty et al. (4) and Ha et al. (16,18) found that some strains of *E. coli* were surprisingly sensitive to vibriocin. Datta et al. (6), Lee (26) and Ha (16) demonstrated that antibacterial spectrum of vibriocin was broad. Mitra et al. (27) established the single vibriocin typing scheme for *V. cholerae*. They reported that 13 types were common and the typing scheme appeared to be simple and adequate for vibrio group of organisms, and the producer as well as the indicator bacteria behaved remarkably stably in the typing scheme over many

years (27).

In our present study, of interest were the observation that vulnificin was not strictly species-specific and inhibited some strains of *P. aeruginosa, Serratia marcescens, S. typhi, S. flexneri, V. cholerae* and *Y. enterocolitica*. Interestingly, one of 3 strains of *Y. enterocolitica* was sensitive to all individual vulnificins produced by all the 8 strains of good vulnificin producer and we think this particular strain can be used as a good indicator strain for screening of vulnificin production. Although we do not know if the correlation between vulnificinogency and establishment of *V. vulnificus* exists, the vulnificinogeny may be ecologically important and be one of many factors which contributes to the relative competitiveness of *V. vulnificus* strains in the intestine. Fredericq et al. (14) reported that colicin B and V were more frequently produced by the dominant intestinal flora in case of infection by *S. paratyphi* B and *S. typhi* than the flora of healthy individuals. Tatarinova et al. (31) reported that colicinogenic enterobacteria accompanying *Shigella* infections were more common when pathogen itself was Col^+, and that out of 193 cultures of *Shigella sonnei* examined, colicinogenic strains were more commonly associated with protracted and more severe cases of bacillary dysentery than Col^- strains. Friedman et al. (9) reported that a strain of *Shigella* was rapidly eliminated by a colicinogenic strain of *E. coli* after two strains had been injected into the peritoneal cavity of mice and that the elimination of the *Shigella* strain coincided with an increase in the colicin titer in the peritoneal cavity. The considerable advances made in the genetics and molecular biology of bacteriocinogeny may help not only to increase our understanding of the ecological significance of vulnificins, but also to establish the relationship between vulnificinogenic bacteria and Col plasmid as unit of selection. Anyhow, more work is needed before we can assess precisely the ecology of vulnificin in different environments, and the significance of vulnificin in the establishment of pathogens and in the prevention of infection deserves further study.

In conclusion, vulnificin typing of *V. vulnificus* as an epidemiological tool is a specific procedure and one that can be performed in laboratories that have a minimum of space, time, reagents, equipment, and personnel. The procedure is usually much simpler than phage typing or serotyping, which are harder to establish and control. In addition, as shown in our present study, the high percentage of vulnificin production and sensitivity to vulnificin make this technique more reliable than the other typing systems. The method should be internationally standardized as has been done with phage typing of *Staphylococcus* and *Salmonella,* serological typing of *Salmonella* and bacteriocin typing of *P. aeruginosa* (35) and *E. coli* (33). Only when a typing system is internationally established, no matter what the principle, will valid and reliable epidemiologic control of infection due to this ubiquitous pathogen be established.

Taken together, our results showed that all the strains tested produced vulnificin with broad antibacterial spectrum and that out of the isolates, 8 and 10 strains were good producer and good indicator bacteria, respectively. These results strongly suggest that the strains can be used efficiently for establishing applicable vulnificin typing scheme and for the detection of vulnificinogeny.

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