BIKEN JOURNAL Vol. 12, 145-148, 1969

PRELIMINARY REPORT

A THERMOLABILE DIRECT HEMOLYSIN OF *VIBRIO PARAHAEMOLYTICUS*[1]

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(Received March 1, 1969)

The hemolytic factors of *Vibrio parahaemolyticus* have been studied by several workers. Kato et al. (1966) and Yanagase et al. (1968b) reported the existence of a thermostable hemolysin in the supernatant fluid of a culture of *Vibrio parahaemolyticus* and Kato et al. (1968) proposed that this thermostable hemolysin might be one of the pathogenic factors of this organism. Yanagase et al. (1968a) studied the indirect hemolysin and suggested that it was phospholipase A. This preliminary report presents data indicating that there is a direct hemolysin, which is thermolabile, in *Vibrio parahemolyticus*.

Vibrio parahaemolyticus WP-1, isolated from a patient with gastroenteritis by Dr. M. Hori of Wakayama Prefecture Public Health Research Institute, was used throughout. The medium used for cultivation of the organism had the following constitution; sodium chloride 30 g, disodium phosphate 7 g, monosodium phosphate 3 g, magnesium sulfate 0.1 g, ammonium sulfate 1 g, peptone (Difco) 10 g and glucose 2 g, in 1,000 ml of distilled water. The pH was adjusted to 8.0. One ml of culture in this medium was inoculated into 250 ml of the same medium in a 900 ml Roux bottle and incubated at 37 C for 15 hours without shaking. The supernatant fluid of the culture was collected by centrifugation. Then 35 g of ammonium sulfate per 100 ml of supernatant fluid were added and the resulting precipitate was dissolved in a small amount of phosphate buffer (0.01 M, pH 7.0). This solution was dialyzed overnight against about 300 volumes of phosphate buffer (0.01 M, pH 7.0) and designated as Fraction 1. A further 25 g of ammonium sulfate were added per 100 ml of the resulting supernatant fluid and the precipitate was dissolved in phosphate buffer and dialyzed as before. It was designated as Fraction 2.

Direct hemolysis by Fractions 1 and 2 was tested and the results are shown in Table 1. Fraction 1 caused complete hemolysis at dilutions of 1 : 4 and 1 : 8, and partial hemolysis even at the high dilution of 1 : 128. On the other hand, Fraction 2 caused no hemolysis. This indicates that direct hemolysin in the supernatant fluid is concentrated in Fraction 1 by precipitation with ammonium sulfate.

Experimental data on the heat stability of Fraction 1 are shown in Table 2. Although little hemolysin was inactivated by heating at 45 C for 10 min, heating at either 55 C or 60 C for 10 min inactivated the direct hemolysin significantly, and no direct hemolysis was ob-

1 Supported in part by a grant from the Ministry of Welfare of Japan.

TABLE 1. *Direct hemolysis by ammonium sulfate treated fractions*

	Dilution							
	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512
Fraction 1	3+	3+	2+	2+	+	±	−	−
Fraction 2	−	−	−	−	−	−	−	−

Each sample was serially diluted twofold with phosphate buffer (0.01 M pH 7.0) and adjusted its sodium chloride concentration to 0.9% in 0.5 ml. An equal volume of 2% suspension of human erythrocytes (type O) in phosphate buffer (0.01 M, pH 7.0) containing 0.9% sodium chloride was added to each tube. Mixtures were incubated in a water bath (37 C) for 2 hrs with occasional shaking and then stood at 4 C for 15 hrs. Hemolytic activity was expressed as follows; 3+ : complete hemolysis, 2+, + and ± : degrees of partial hemolysis, and − : no hemolysis. The protein content (measured by the method of Lowry et al. (1956)) of the samples was 3 mg/ml.

TABLE 2. *Heat stability of direct hemolysin of Vibrio parahaemolyticus*

	Dilution							
	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512
Untreated	3+	3+	2+	2+	+	+	−	−
45 C 10 min	3+	3+	2+	2+	+	±	−	−
50 C 10 min	3+	2+	2+	+	±	−	−	−
55 C 10 min	2+	+	−	−	−	−	−	−
60 C 10 min	2+	±	−	−	−	−	−	−

Explanation as in Tabls 1.

served at dilutions of more than 1 : 8. Part of the direct hemolysin was not inactivated by these treatments resulting in partial hemolysis at dilutions of 1 : 4 and 1 : 8. This result indicates that there is a thermolabile direct hemolysin besides a thermostable one in the supernatant of the culture of *Vibrio parahaemolyticus*.

When *Vibrio parahaemolyticus* is cultured on blood agar, a clear zone is seen around the colony, and this kind of hemolysis on blood agar has been known since the first isolation of this organism by Fujino et al. (1953). Kato et al. (1965) found that most strains isolated from patients with gastroenteritis produced clear zones around their colonies on blood agar while most strains isolated from other sources did not. To study this phenomenon further, Wagatsuma (1967) devised a special blood agar (Wagatsuma's Medium) with the following constitution; yeast extract 3 g, peptone (Difco) 10 g, sodium chloride 70 g, dipotassium phosphate 5 g, mannitol 10 g, crystal violet 0.001 g, agar 15 g and washed human erythrocytes at a concentration of 7%, in 1,000 ml of distilled water. Formation of a clear zone around colonies of *Vibrio parahaemolyticus* on Wagatsuma's medium was named Kanagawa phenomenon (Fujino, 1968).

The Kanagawa phenomenon is a result of at least two reactions (Fujino, Miwatani and Takeda; unpublished observation); one is the release of hemoglobin from erythrocytes and the other is a fading of the colour of hemoglobin released (possibly due to conversion of hemoglobin to methemoglobin). The release of hemoglobin from erythrocytes is caused by hemolysin and the fading of the colour is caused by several factors such as the pH of the medium, nitrite and glutathione (Fujino, Miwatani and Takeda; unpublished observa-

tion).

As strain WP-1 used in this experiment showed the Kanagawa phenomenon very clearly (Fig. 1), it was of interest to see whether the direct hemolysin contained in Fraction 1 was responsible for the first reaction of the Kanagawa phenomenon (the release of hemoglobin). To study this, supernatant fluid was dropped into small wells cut in Wagatsuma's medium. If the direct hemolysin is responsible for the release of hemoglobin, a change should be seen in the blood agar around the well containing Fraction 1. As shown in Fig. 2, the Kanagawa phenomenon was seen with Fraction 1, but not with Fraction 2. Thus, the release of hemoglobin was caused by Fraction 1 in the absence of bacterial cells. The reason for the further change of released hemoglobin to give a clear zone around the well is not well understood. However, the pH of Fraction 1 was 7.0 and at this pH value, some hemoglobin changes into methemoglobin within 10 hours at 37 C (Fujino, Miwatani and Takeda; unpublished observation), which may partly explain formation of the clear zone around Fraction 1.

Fraction 1 contains both thermolabile and thermostable direct hemolysin, as indicated in Table 2, so an experiment was carried out on which hemolysin is responsible for the release of hemoglobin from erythrocytes. As shown in Fig. 3, untreated direct hemolysin produced a wide clear zone, but direct hemolysin, heated at either 50 C or 55 C for 10 min, gave a much smaller clear zone. Direct hemolysin heated at 60 C for 10 min did not produce any clear zone. These data suggest that thermolabile direct hemolysin is at least one agent causing the first reaction of the Kanagawa phenomenon of *Vibrio parahaemolyticus*.

The authors are grateful to Miss A. Yoshihara and Mr. T. Ohkawa for their technical assistance.

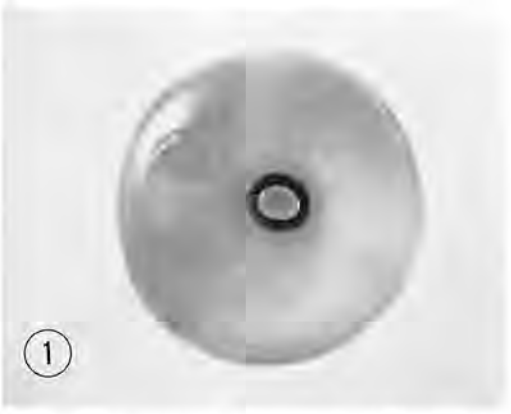

FIGURE 1. *Kanagawa phenomenon of Vibrio parahaemolyticus WP-1.*

A drop of Vibrio parahaemolyticus WP-1 culture was inoculated on Wagatsuma's medium and incubated at 37 C for 15 hrs.

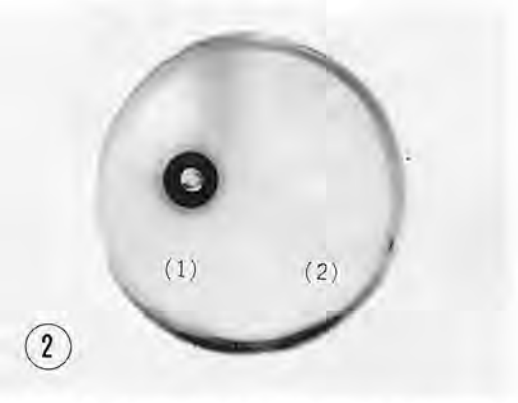

FIGURE 2. *Formation of a clear zone in Wagatsuma's medium by direct hemolysin of Vibrio parahaemolyticus.*

Wagatsuma's medium containing 50 μg/ml of dihydrostreptomycin was prepared and small wells (5 mm in diameter) were cut in it. The bottoms of wells were sealed with agar and 30 μl of samples were dropped into each well and incubated at 37 C for 15 hrs. The samples are the same as those used in the experiment of Table 1; (1): Fraction 1 and (2): Fraction 2.

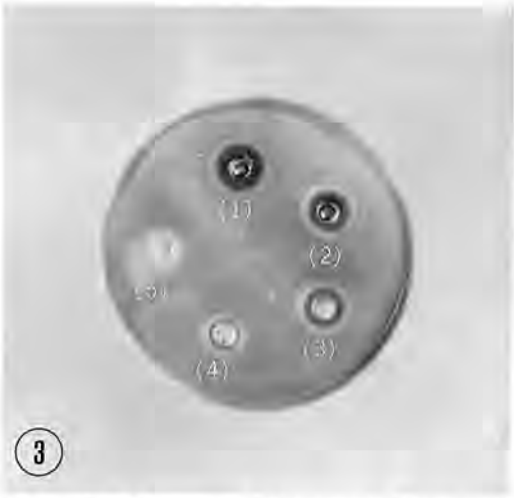

FIGURE 3. *Formation of a clear zone in Wagatsuma's medium by heat treated direct hemolysin of Vibrio parahaemolyticus.*

Explanation is as in Fig. 2. Each well contained 30μl of sample. The samples are the same as those used in the experiment of Table 2; (1) *is untreated direct hemolysin and* (2), (3), (4) *and* (5) *are direct hemolysin heated at 45 C, 50C, 55 C and 60 C, respectively for 10 min.*

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