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Hemolytic Activity of Culture Supernatant of *Xenorhabdus nematophilus*, a Symbiotic Bacterium of Entomopathogenic Nematodes

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Abstract Lysis of erythrocytes isolated from human, rabbit, and mouse blood samples was investigated with the culture supernatant of *Xenorhabdus nematophilus* in a primary form. Prior to use, the culture supernatant of the bacteria was concentrated and the concentrate was dialyzed against Tris-HCl buffer (10 mM, pH 8.1) by ultrafiltration using PM-5 membrane with a molecular weight cut-off of 5,000. At 30 °C, the supernatant exhibited no lytic activity towards three types of erythrocytes. However, at 4 °C, the supernatant showed selective lytic activity towards rabbit erythrocytes within 90 min, yet did not lyze human or mouse erythrocytes. Microscopic examination clearly revealed that most of the rabbit erythrocytes had been turned into ghost forms.

Key words: Entomopathogenic nematode, erythrocytes, hemolysis, symbiotic bacteria

Entomopathogenic nematodes, the genera of *Steinernema* and *Heterorhabditis*, are of interest in the development of biological pesticides to replace chemical pesticides, the use of which has raised many environmental and biological concerns [4, 6]. Entomopathogenic nematodes have a wide range of insect hosts, which are killed rapidly upon selective infection, and yet are safe for non-target organisms such as plants, animals, and humans. Nematodes are known to carry *Xenorhabdus* or *Photorhabdus* species in their guts as symbiotic bacteria, and release such bacteria into the hemolymphs of their insect hosts after infection [10]. The bacteria multiply rapidly within the insects and kill the host insects within 24–48 h after infection, thereby providing suitable conditions for nematode reproduction. The bacteria exist in two forms; primary (phase I) and secondary (phase II) forms. The primary form prefers to exist in the infective nematodes, while the secondary form develops in the *in vitro* culture medium of the primary form after a long incubation period [1, 12] or when the bacteria exist in soil as free-living organisms [11].

The symbiotic bacteria of entomopathogenic nematodes are known to produce either endotoxins of the lipopolysaccharide components of the cell wall, or exotoxins of extracellular protein complexes [3]. Similar to other pathogenic bacteria, the symbiotic bacteria also produce an extracellular protease which is considered as one of the major toxic factors contributing to the pathogenicity of the nematode/bacteria complex [5, 9]. Therefore, the toxicity of the symbiotic bacteria is typically examined with the use of a culture supernatant of the bacteria. Currently, the mortality of insects is measured by direct injection of a culture supernatant into the insect hemocoel or by adding a culture supernatant to the diets of the insects. Those two mothods require two days or longer to assay the toxicity of the symbiotic bacteria. In the present study, we investigated the lysis of several animal erythrocytes with the culture supernatant of *Xenorhabdus nematophilus*, and propose a simple alternative method for measuring the toxicity of the symbiotic bacteria, which can be performed within two hours.

Organisms and Culture Conditions

The symbiotic bacterium, *Xenorhabdus nematophilus*, isolated from an insect pathogenic nematode, *Steinernema glaseri*, by Park and Yu [7] was used in the current study. The stock culture of *Xenorhabdus nematophilus* was maintained at 25°C on agar plates of an NBTA medium containing 20 g of a nutrient agar (Difco), 25 mg bromothymol blue, and 40 mg triphenyltetrazolium in 1 l distilled water. On the agar medium, the primary and secondary forms of the symbiotic bacterium can be distinguished by their

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green and dark red colors, respectively, depending on the adsorption of bromothymol blue by the bacterium in the primary form. The primary form of *Xenorhabdus nematophilus* on the agar plate was selectively transferred to a 250-ml flask containing 100 ml of a liquid medium for a seed culture. The flask was incubated at 200 rpm in a rotary shaker for 24 h at 28°C. Subsequently, 40 ml of the seed culture broth was inoculated into a 7-l jar fermentor containing 4 l of the same liquid medium for the main culture. The main culture was performed at 500 rpm with an air flow rate of 0.8 l/min for 24 h at 28°C. The liquid medium for the seed and main cultures contained 50 g yeast extract (Difco), 5 g NaCl, 0.5 g K_2HPO_4, 0.5 g $NH_4H_2PO_4$, and 0.2 g $MgSO_4 \cdot 7H_2O$ per liter.

Measurement of Insect Toxicity by Injection

The insect toxicity of the culture supernatant was measured using 3rd instar *Galleria mellonella* larvae, as previously described by Park and Yu [8]. Into the hemolymph of each larva, 3 µl of the culture supernatant was injected using a microsyringe. Each culture supernatant sample was injected into four larvae. After the injection, each larva was placed on a 9-cm filter paper in an individual petri dish (100 by 15 mm), incubated at 25°C, and then observed for mortality. The controls consisted of larvae injected with 3 µl of a sterile buffer (pH 8.1) containing 10 mM Tris-HCl and 1 M KCl.

Measurement of Lytic Activity Towards Erythrocytes

The erythrocytes were prepared from peripheral blood samples from humans, rabbits, and mice using a Histopaque-1077 following Sigma Procedure No. 1077. Blood samples isolated from the fore arm of humans, the ears of rabbits, and the retro-orbital plexus of mice were diluted two-fold with a phosphate buffer saline solution (PBS) and overlaid on the Histopaque-1077. After centrifugation for 30 min at 500 ×g at room temperature, the precipitated erythrocytes were harvested and washed three times with PBS. The lytic activity of the culture supernatant towards the erythrocytes was measured as described by Bernheimer [2]. The typical method was as follows. A toxin diluent containing 10 mM Tris (pH 7.2), 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.2% gelatin, and 0.15 M NaCl and 0.85% NaCl solution in 10 mM Tris-HCl (pH 7.2) were prepared. In a test tube containing 0.15 ml of the erythrocytes, 0.85% NaCl solution was added to make a final volume of 1.5 ml. After mixing, the mixture was centrifuged at 1,000 rpm for 5 min and the supernatant was then discarded. After repeating this procedure three times, 1.5 ml of the final suspension containing the erythrocytes in 0.85% NaCl was prepared. To 0.3 ml of the final erythrocyte suspension in 0.85% NaCl, the toxin diluent and culture supernatant were added to make a final volume of 1.5 ml. The above mixture of erythrocytes, toxin diluent, and culture supernatant was then shaken gently in a refrigerator for 90 min. After the reaction, the mixture was centrifuged at 1,000 rpm for 7 min, the supernatant was then separated and its optical density at 546 nm was measured using a spectrophotometer (Hewlet-Packard 8402A). The control sample for the total lysis of the erythrocytes was prepared by replacing the culture supernatant with 1 mg saponin powder (Sigma Chemical Co., St. Louis, U.S.A.).

Hemolytic Activity of *Xenorhabdus nematophilus*

At first, the insect toxicity of the culture supernatant of *Xenorhabdus nematophilus* was confirmed by the injection method. The culture supernatant of *Xenorhabdus nematophilus* grown for 24 h was concentrated five-fold, followed by dialysis against Tris-HCl buffer (10 mM, pH 8.1) using an ultrafiltration membrane (PM-5 with a molecular weight cutoff of 5,000). The toxicity of this solution against the insect larvae was measured by injecting the solution directly into the hemolymph of the larvae, as described above. As shown in Table 1, the injection of the initial culture supernatant before concentration caused the death of half of the initial larvae within 24 h and all the initial larvae within 56 h. In contrast, upon injection of the concentrated or concentrated and dialyzed culture supernatants, all initial four larvae were killed within 28 h. Therefore, for the remainder of the study, only the concentrated and dialyzed culture supernatant was used (hereafter referred to as the culture supernatant).

The hemolytic activity of the culture supernatant of *Xenorhabdus nematophilus* was determined using human, rabbit, and mouse erythrocytes. At 30°C, no lytic activity by the culture supernatant was observed towards three types of erythrocytes, therefore, when the mixtures of the culture supernatant and each type of erythrocyte were centrifuged following 90 min of reaction at 30°C, a well separated lower erythrocyte phase and clear upper phase

Table 1. Number of insect larvae killed by injection of culture supernatants of *Xenorhabdus nematophilus* after various elapsed periods.

Injected solution	Incubation period				
	18 h	28 h	36 h	56 h	85 h
Control solution[1]	0	0	0	0	0
Initial culture supernatant	0	2	2	4	4
Concentrated supernatant[2]	0	4	4	4	4
Concentrated and dialyzed supernatant[3]	0	4	4	4	4

For each supernatant, four larvae were initially injected with 3 µl of the supernatant into their hemolymphs.
[1]Control solution was a sterile buffer (pH 8.1) containing 10 mM Tris-HCl and 1 M KCl.
[2]Culture supernatant was concentrated five-fold using a PM-5 membrane.
[3]Concentrated supernatant was dialyzed with a Tris-HCl buffer (10 mM, pH 8.1).

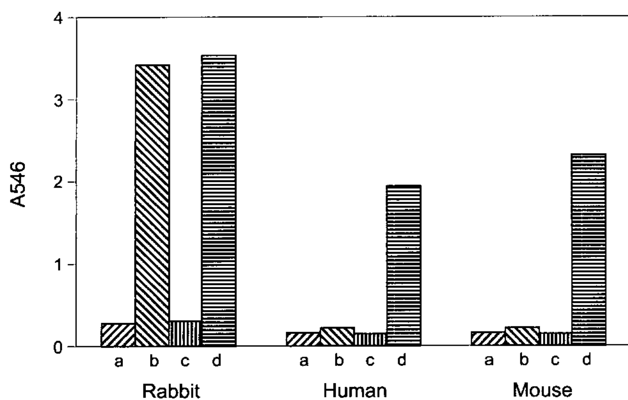

Fig. 1. Lysis of rabbit, human, and mouse erythrocytes incubated at 4°C for 90 min with culture supernatant of *Xenorhabdus nematophilus* (b), heat-treated culture supernatant of *Xenorhabdus nematophilus* at 100°C for 30 min (c), and saponin (d).
Control test (a) was performed by replacing culture supernatant with toxin diluent containing 10 mM Tris (pH 7.2), 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.2% gelatin, and 0.15 M NaCl.

were observed. In contrast, when the erythrocytes were completely disrupted by saponin, the whole mixture of erythrocytes and saponin turned red due to hemoglobin released from the erythrocytes. The totally lyzed erythrocyte suspension also lacked any separated phase of the erythrocytes after centrifugation. However, as the reaction temperature was lowered to 4°C, the culture supernatant lyzed the rabbit erythrocytes, but not the human or mouse erythrocytes. As shown in Fig. 1, the optical density at 546 nm of the supernatant of the reaction mixture of the culture supernatant of *Xenorhabdus nematophilus* and the rabbit erythrocytes was close to that of the rabbit erythrocyte suspension completely lyzed by saponin. Moreover, none of the three types of erythrocytes were

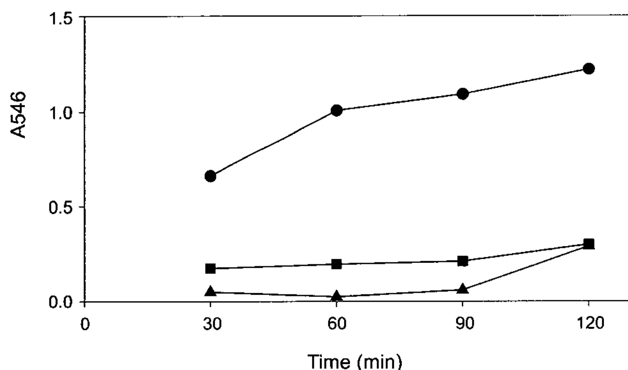

Fig. 2. Course of lysis of rabbit erythrocytes at 4°C with culture supernatant of *Xenorhabdus nematophilus* (●) and heat-treated culture supernatant of *Xenorhabdus nematophilus* at 100°C for 30 min (■).
Control test (▲) was performed by replacing culture supernatant with toxin diluent containing 10 mM Tris (pH 7.2), 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.2% gelatin, and 0.15 M NaCl.

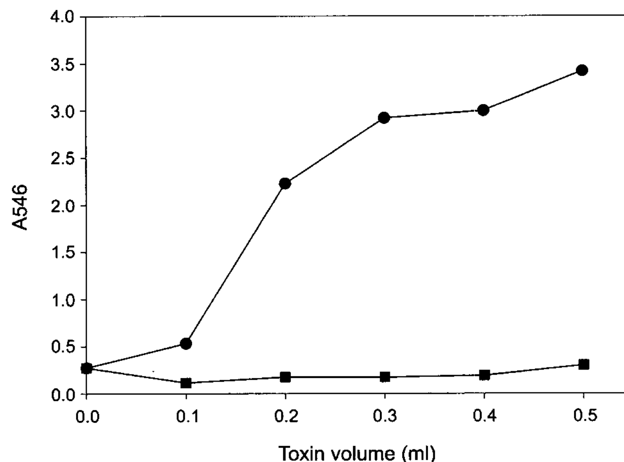

Fig. 3. Dependence of lysis of rabbit erythrocytes on the amount of culture supernatant of *Xenorhabdus nematophilus* (●) and heat-treated culture supernatant of *Xenorhabdus nematophilus* at 100°C for 30 min (■).
Rabbit erythrocytes were incubated with each culture supernatant at 4°C for 90 min. The total volume of rabbit erythrocytes in each culture supernatant was 1.5 ml.

destroyed by the culture supernatant of *Xenorhabdus nematophilus* heat-treated for 30 min at 100°C. This indicates that the lysis of the rabbit erythrocytes was caused by a heat-labile toxin component contained in the culture supernatant of *Xenorhabdus nematophilus*. The lysis of the rabbit erythrocytes by the culture supernatant of *Xenorhabdus nematophilus* was almost completed within a 60-min incubation period. As shown in Fig. 2, with a heat-treated culture supernatant or a blank solution without the added culture supernatant, the rabbit erythrocytes were not destroyed within 120 min of incubation at 4°C.

As shown in Fig. 3, with a fixed incubation period of 90 min at 4°C, the extent of the lysis of the rabbit erythrocytes increased with the amount of the culture supernatant of *Xenorhabdus nematophilus* added into the incubation solution. The lysis of the erythrocytes was initiated upon the addition of 0.1 ml of the culture supernatant, then attenuated when more than 0.3 ml of the culture supernatant was added into 1.5 ml of the incubation solution. As shown in Fig. 4, microscopic examination confirmed that most of the rabbit erythrocytes incubated with the culture supernatant of *Xenorhabdus nematophilus* were turned into ghost forms, while the rabbit erythrocytes incubated with the heat-treated culture supernatant of *Xenorhabdus nematophilus* remained intact.

Future investigations to elucidate the mechanism of the lysis of rabbit erythrocytes by the culture supernatant of *Xenorhabdus nematophilus* will be focused on two aspects. First, the structural difference between the rabbit erythrocyte membrane and the human and mouse erythrocyte membranes will be examined. Second, the enzymatic activity and the properties of the toxin component present in the

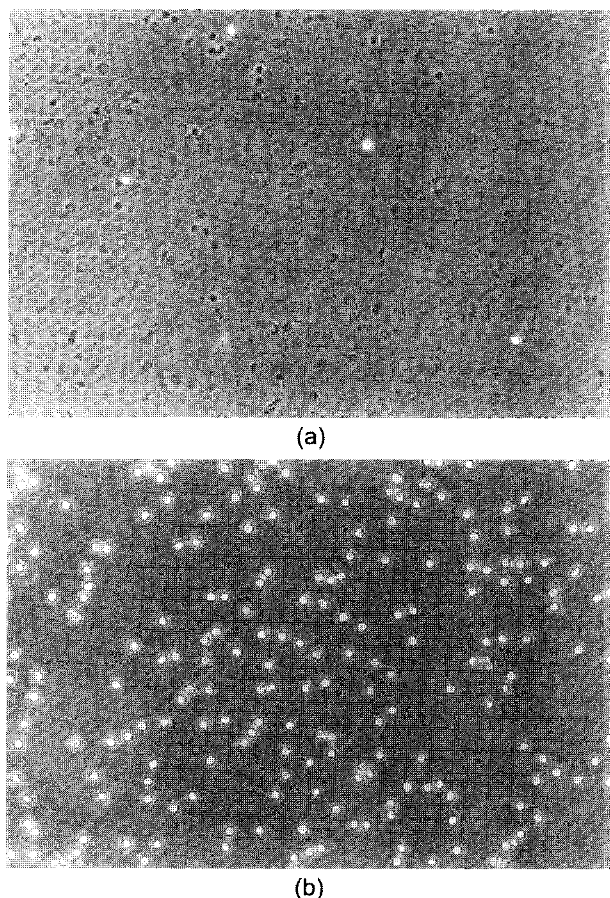

Fig. 4. Photographs of rabbit erythrocytes lyzed by culture supernatant of *Xenorhabdus nematophilus* (a) and intact rabbit erythrocytes incubated with heat-treated culture supernatant of *Xenorhabdus nematophilus* (b).

The erythrocytes/culture supernatant mixtures were incubated at 4°C for 90 min. Magnification: ×200. The size of the intact rabbit erythrocytes is about 5 μm.

culture supernatant of *Xenorhabdus nematophilus* will be characterized.

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