Note

Identification of a Polymyxin Produced by a Symbiotic Microorganism Isolated from the Brown Planthopper, *Nilaparavata lugens*†

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The existence of intracellular symbiotic microorganisms in several insects has attracted interest.[1～3] It was suggested that the symbionts found in the small brown planthopper, *Laodelphax striatellus*, may supply sterols[4] or amino acids[5] which are not synthesized in the host insect. Another assumed role of the symbionts is that they protect the host insect from invasion by external harmful microorganisms, because the surface of the insect body was infected by fungi when growth of the symbionts was prevented by heat treatment.[5] The results suggested the presence of antimicrobial substances produced by the symbionts. In addition, antimicrobial substances, secreted by the symbionts, may play a role in maintaining the microbial balance in insects due to inhibition of excess growth of certain kinds of symbionts. Recently, intracellular yeast-like symbionts were isolated from the eggs of the brown planthopper, *Nilaparavata lugens*.[6] We attempted to isolate the antimicrobial substances produced by these intracellular symbionts to elucidate the symbiotic relationship between the host insect and the microorganisms. In this paper, we describe the taxonomical results for a selected strain from the symbionts and the identification of the active substance produced by it.

Using the standard agar plate assay method, we screened 120 symbiotic microorganisms, isolated from extracts of eggs of *N. lugens* collected in several Asian countries, for their ability of the production of antimicrobial substances in the culture medium.[6] We selected the strain which showed the strongest and broadest antimicrobial activity against both several gram negative bacteria and the other symbionts isolated from *N. lugens*. The strain, designated as No. 34, was isolated from eggs of *N. lugens* collected in Indonesia, and subjected to further study. The taxonomical studies on the strain were performed according to Bergey's Manual of Determinative Bacteriology, 8th Ed.[7] Strain No. 34 was a gram positive, rod-shaped bacterium showing motility. Nutrient agar colonies were rough and milkywhite. The spores were elliptical and their dominant position was central to terminal. The spores germinated after heating at 80°C for 10 min. From the properties described above and the physiological characteristics of the strain listed in Table I, the strain was determined to belong to the genus *Bacillus*. But further studies will be necessary to confirm this species identification.

To isolate active substances, the strain was cultivated in a glass bottle, equipped with a glass tube as an air inlet and a porous air filter, containing 1 liter of modified LB medium (Bacto-tryptone, 10 g/liter; Bacto-yeast extract, 5 g/liter; glucose, 20 g/liter; NaCl, 0.25 g/liter; the initial pH being adjusted to pH 8.0) at 30°C with bubbling. Five liters of the culture broth was taken at the early stationary phase, and the cells were removed by centrifugation. The supernatant, which showed antibacterial activity against several gram negative bacteria including *E. coli*, was used to isolate an extracellular substance. The antibacterial activity was assayed by monitoring the growth inhibition of *E. coli* NIHJ JC-2 at 650 nm. The substance was still active after incubation at 70°C for 10 min, and adsorbed by the cationic exchanger resin, CM-Sephadex C-25. The active substance was purified 473-fold, on the basis of its

TABLE I. PHYSIOLOGICAL CHARACTERISTICS OF STRAIN NO. 34

Nitrate reduction	Positive
Starch hydrolysis	Negative
Gelatin liquefaction	Negative
H_2S production	Negative
Cell growth at 45°C	Negative
Cell growth at pH 5.7	Positive
Cell growth in 7% NaCl	Negative
Indole formation	Negative
Acetylmethylcarbinol formation	Positive
Catalase test	Positive
Urease test	Negative
Oxidase test	Negative
Carbon utilization: glucose, +; sucrose, +; xylose, +; lactose, +; arabinose, +; trehalose, +; mannitol, +; sodium citrate, +.	

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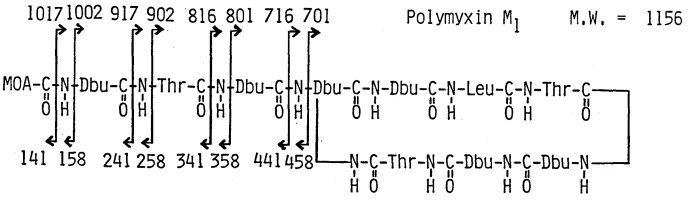
TABLE II. PURIFICATION OF THE ANTIBACTERIAL PEPTIDE FROM THE CULTURE MEDIUM

Purification step	Total protein[a] (mg)	Total activity[b] (KU)	Specific activity (KU/mg · protein)	Yield (%)
Culture filtrate	718	405	0.56	100
CM-Sephadex	53	377	7.11	93
Hydroxylapatite	1.98	318	161	79
Mono S	0.92	244	265	60

[a] The total amount of protein was monitored by measuring $A_{215} \sim A_{225}$, which is an indicator of the absorption due to peptide bonds, as described by Murphy and Kies.[8]

[b] One unit of antibacterial activity was defined as the amount of the peptide that caused 50% inhibition of bacterial growth relative to the control.[9]



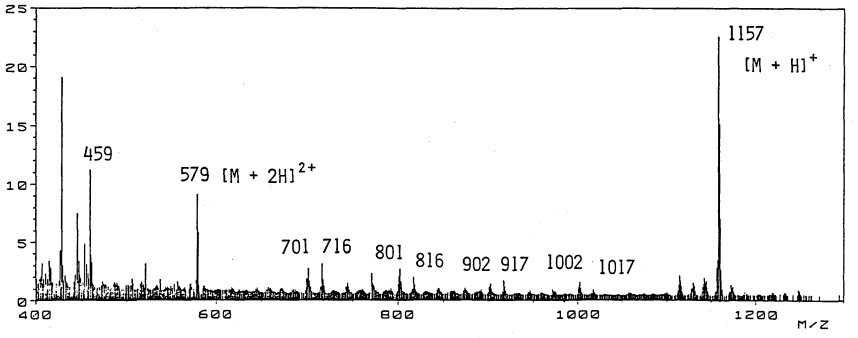


FIG. 1. FAB Mass Spectrum of the Purified Antibacterial Peptide.

activity, from the culture medium by column chromatographies on CM-Sephadex and hydroxylapatite, and HPLC on a Mono S column (Pharmacia) (Table II). The yield of the purified substance from the culture medium was 60%. The homogeneity of the purified substance was examined by SDS–15% (w/v) polyacrylamide gel electrophoresis.[10] A single band corresponding to a 1.6 kilo dalton linear peptide was observed. The purity of the purified substance was also determined on a non-denaturing 16% (w/v)–polyacrylamide slab gel.[11] A single band showing antibacterial activity, on overlaying with soft agar containing *E. coli*, was observed (data not shown).

Amino acid analysis of the active substance revealed threonine, leucine and an abnormal amino acid (X) in a molar ratio of 3.1 : 1.0 : 5.5. The abnormal amino acid (X) was eluted slightly after lysine on amino acid analysis, suggesting that "X" should be more basic than lysine. On reverse phase HPLC (Cosmosil C-18; Nakarai Chem. Co.), the retention time of the phenylthiohydantoin (PTH) derivative of "X" was found to be identical with that of the PTH derivative of authentic α,γ-diamino butyric acid. The properties of the purified substance described above suggested the presence of a peptide antibiotic, a polymyxin. Among known polymyxins reported so far,[12] only polymyxins M_1 and M_2 contain threonine, leucine and α,γ-diamino butyric acid residues in a molar ratio of 3 : 1 : 6, which coincides with the above results. Polymyxins M_1 and M_2, however, differ in molecular weight ($M_1 = 1{,}156$ and $M_2 = 1{,}142$). Polymyxin M_1 contains (+)-6-methyloctanoic acid and polymyxin M_2 contains isooctanoic acid.[13] The FAB (Fast Atom Bombardment) mass spectrum of the purified substance revealed a protonated molecular ion peak at 1,157, which was identical with the molecular weight of polymyxin M_1. Furthermore, as shown in Fig. 1, fragmentation peaks suggested the presence of a chain of a diaminobutyryl-threonyl-

diaminobutyryl structure attached to a ring of seven amino acid constituents. Thus, the purified antibacterial peptide was strongly suggested to be polymyxin M_1 (Fig. 1), although the possibility of a new polymyxin with a different amino acid sequence for the cyclic heptapeptide part cannot be ruled out.

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