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Original Article

Isolation and Classification of Intracellular Symbiotes from the Rice Brown Planthopper, *Nilaparvata lugens*, Based on Analysis of 18S-ribosomal DNA

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Seven morphologically different yeast-like symbiotes were isolated from eggs of the rice brown planthopper, *Nilaparvata lugens*. To determine their taxonomic affiliation, molecular analysis of DNA encoding 18S-ribosomal RNA was carried out. PCR amplifications of their DNA preparations using the well conserved regions of *Saccharomyces cerevisiae* 18S-rDNA as primers produced a single main band of DNA having the predicted size of 1.3 and 1.6 Kbp on electrophoresis. Digestion of these DNAs with restriction endonucleases gave different band patterns on electrophoresis, indicating that these seven yeast-like microorganisms belong to different taxonomic affiliations.

INTRODUCTION

The rice brown planthopper, *Nilaparvata lugens*, harbors yeast-like symbiotes in the fat body cells.[1,2] The endosymbiotes are transmitted to the next generation *via* the ovary as a mass of organisms, which is called symbiont ball, located at the posterior pole of the egg. Then the symbiont ball moves to the anterior end as the embryo develops. On the 4th day after oviposition, the symbiont ball moves back to the posterior end and the blastokinesis completes.[3] Physiological roles of symbiotes in embryonic development remain unclear, because elimination of symbiotes from eggs is impossible. Exposure of newly hatched larvae to 32–35°C for 3 days (heat-treatment) or chemical treatment with antibiotics reduced the number of symbiotes.[4,5] In the eggs laid by heat-treated females, the embryo did not undergo blastokinesis and the symbiont ball remained at the anterior end. These eggs failed to hatch due to the lack of differentiation of the abdominal segment (head embryo). The eggs ligated by nylon threads between the symbiont ball and the yolk at the posterior end also resulted in head embryo.[6] Difficulty in *in vitro* culture of symbiotes outside the host has prevented the determination of their taxonomic affiliation. Mitsuhashi[7] first succeeded in the *in vitro* culture of yeast-like symbiotes isolated from *Laodelphax striatellus*, the smaller brown planthoppers. Then, two different yeast-like symbiotes, Ls-1 and Ls-2, were isolated from the eggs of *L. striatellus*,[8] sucessively Nl-1 and Nl-2 from *N. lugens*.[9] These symbiotes have been successively cultured *in vitro*. Immunological experiments confirmed that these symbiotes were all originated from insects but not from contamination. It is unclear, however, whether Ls and Nl symbiotes

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are same or different, although these two symbiotes immunologically react in the same manner. Recent development of molecular biology has made possible to determine the taxonomic classification of microorganisms through molecular analysis of the genes encoding 16-18S-ribosomal RNA. Campbell *et al.* have determined the taxonomic affiliation of weevil symbiotes by analyzing 16S-ribosomal DNA.[10]

This paper reports the isolation of yeast-like symbiotes from the eggs of *N. lugens* and the taxonomic classification based on analysis of 18S-rDNA.

MATERIALS AND METHODS

1. *Insects*

The rice brown planthoppers, *Nilaparvata lugens*, were obtained from the Nissan Chemical Industry Lab. in 1989. Colonies of the planthoppers had been maintained at 25°C under a 12L:12D photoperiod on rice seedlings. Newly oviposited eggs were collected and kept at 25°C for 4 days before isolation of symbiotes.

2. *Isolation and Culture of Symbiotes from the Eggs of N. lugens*

Four-day-old eggs were sterilized by immersion in 0.1% Hyamine solution for 3 min or in 0.01% Hyamine solution for 10 min, followed by washing three times with sterile distilled water. The surface sterilized eggs (200 eggs) were homogenized in 100 μl sterile medium (KH_2PO_4 4.5 g, Na_2HPO_4 6.0 g, L-cystein HCl 0.5 g, Tween 80 0.5 g, Agar 1.0 g/*l* dis. water) in a teflon homogenizer. Five microliter aliquots of the homogenate were placed on agar plates at 25°C for 3 days. Bacto-YM broth (Difco) and Potato dextrose agar(Difco) were used for isolation and culture of the symbiotes.

3. *Genomic DNA Isolation*

The isolated yeast-like microorganisms were cultured with shaking in 10 ml Bacto-YM broth medium at 25°C for 12 hr. Methods for isolation of DNA were adapted from Cryer *et al.*[11] with slight modifications. One gram of the isolated yeast-like microorganisms was suspended in a mixture of 0.5 ml EDTA-Na solution (EDTA 0.1 M, NaCl 0.15 M, pH 9.0) and 2 ml pK buffer ($Na_2HPO_4 \cdot 12H_2O$ 22 g, $NaH_2PO_4 \cdot 2H_2O$ 6.2 g, KCl 59.6 g/*l*). Three hundred microliters of the suspension was moved to a microtube, to which 5 μl mercaptoethanol and 7 μl Zymolyase-20T (10 mg/ml) were added. The microtube was shaken at 37°C for 1–2 hr to digest the cell walls. After centrifugation at 3000 rpm for 10 min, the precipitate was resuspended in 280 μl EDTA-Na solution to which 7 μl Protease K (20 mg/ml) and 42 μl 10% SDS solution were added. The suspension was kept for 30 min and extracted with 300 μl each of phenol, phenol/chloroform (1:1) and chloroform/isoamylalcohol (25:1) successively. DNA was precipitated by adding a mixture of sodium acetate (30 μl) and ethanol (900 μl), and pelleted by centrifugation. The pellet was washed with 70% ethanol and dried. The DNA obtained was redissolved in 100 μl SSC(NaCl 150 mM, Sodium citrate 15 mM) and kept overnight at 25°C. Then, the aqueous phase was incubated with 5 μl Ribonuclease A (1 mg/ml) at 37°C for 90 min to remove RNA from the DNA and extracted with 100 μl phenol. DNA was precipitated by adding 10 μl sodium acetate and 300 μl ethanol, washed with 70% ethanol twice and dried.

4. *PCR Amplification*

Polymerase chain reaction (PCR) was used to amplify double-strand 18S-rDNA from genomic DNA preparations. PCR was performed, as recommended in the Gene Amp DNA Amplification Reagent Kit (Takara), using Taq DNA polymerase as the amplifying enzyme. Each reaction included 5 μl (20 μM) each of primers A and B. The PCR thermal program consisted of 35 cycles at 94°C for 1 min, 37°C for 2 min, 72°C for 3 min and 72°C for 7 min as a final extension after the last cycle. The well conserved regions of 5′- and 3′-ends of *Saccharomyces cerevisiae* 18S-rDNAs were synthesized and used as primers, primer A(TCTCAAAGATT-AAGCCATGC) and primer B(GCGACGGGC-GGTGTGTACAA) corresponding to regions 35–54 and 1625–1644 of the *S. cerevisiae* 18S-rDNA, respectively.

5. *DNA Digestion*

The DNA amplified by PCR was digested with restriction endonucleases, HaeIII, HhaI, HinfI, MspI and Sau3AI. Each reaction included 9 μl (*ca.* 10 ng DNA/μl) of the amplified

DNA solution (10 mM Tris-HCl, pH 7.5) and 0.5 μl(*ca*. 5 units) of endonuclease and was kept at 37°C for 90 min. The products were size-fractionated on 2.0% agarose gel electrophoresis.

6. *Electrophoresis*

Two microliters of stain marker solution (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was added to 10 μl of the amplified DNA solution. This preparation was electrophoresed on 0.7% agarose gel containing ethylenbromide (0.5 μg/ml) under constant

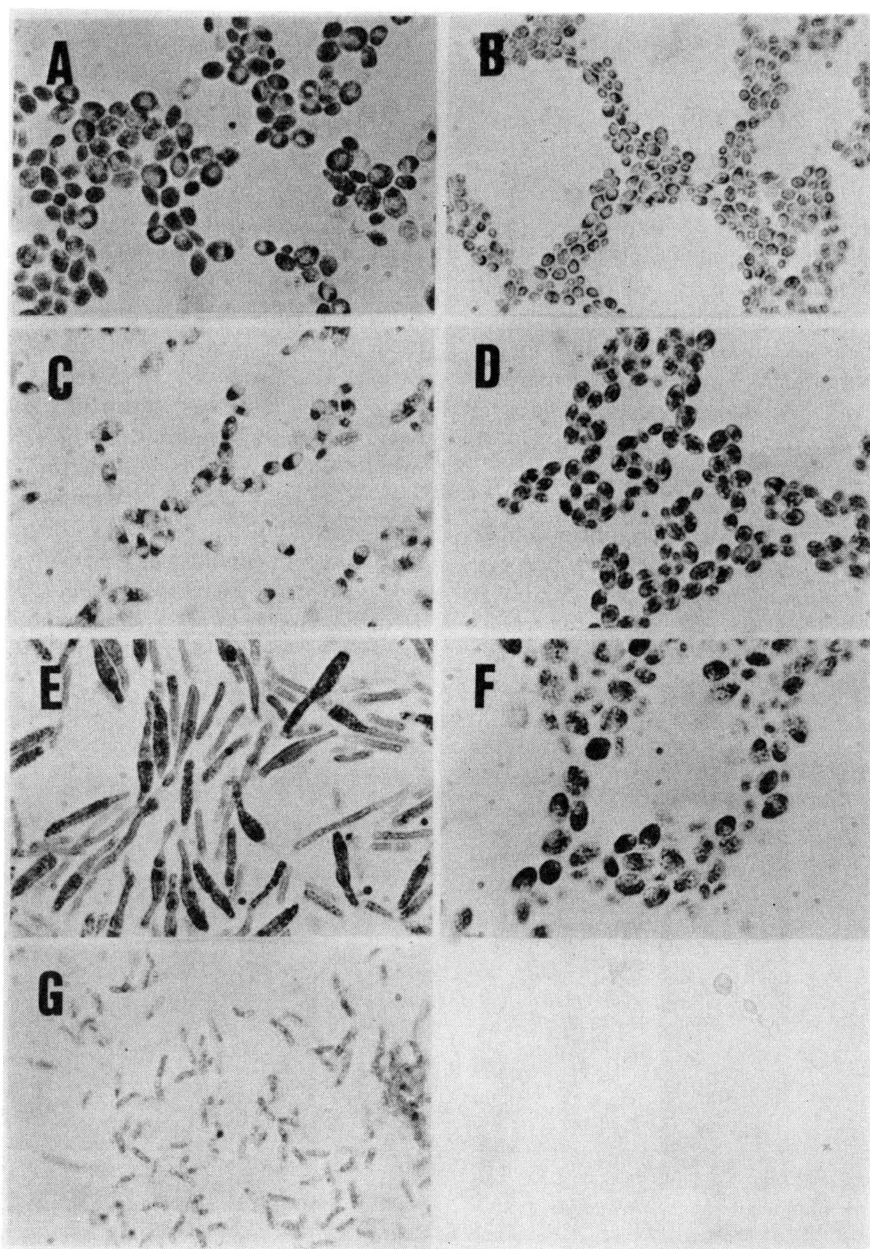

Fig. 1 Yeast-like symbiotes isolated from the eggs of *N. lugens* (×1000). Microorganisms A to G belong to groups A to G respectively.

voltage condition (100 V for 45 min). Molecular weight standards, marker 1 (λDNA was digested with EcoT14I) and marker 2 (λDNA was digested with BstPI), were coelectrophoresed with experimental samples. After the amplified DNA was digested with restriction endonucleases, 2 μl of the stain marker was added to the reaction volume. Then, this preparation was size-fractionated on 2.0% agarose gel containing ethylenbromide (0.5 μg/ml) under the same condition as above.

RESULTS

1. Isolation of Yeast-Like Symbiotes from Eggs of *N. lugens*

Seven different groups of colonies, groups A to G, were isolated on agar plates containing YM or PDA medium. Each colony was composed of morphologically different yeast-like microorganisms (A to G) as shown in Fig. 1. These microorganisms have remained morphologically unchanged over 1 year on YM agar slants.

2. PCR Using *Saccharomyces cerevisiae* DNA

PCR was carried out to amplify double-strand 18S-rDNA from genomic DNA preparations of *S. cerevisiae* using primers A and B. As shown in Fig. 2, a single electrophoretically resolvable band of DNA having the predicted size of 1.6 Kbp was obtained. This DNA fragment was digested with HaeIII, HhaI, HinfI, MspI and Sau3AI, and it produced 4, 2, 5, 3 and 2 bands, respectively, when electrophoresed on agarose gel. The DNA size of each band agreed well with the theoretical DNA fragment size. These results indicate that the 1.6 Kbp DNA fragment obtained was originated from 18S-rDNA regions of *S. cerevisiae*.

3. PCR Using the Isolated DNA from Yeast-Like Microorganisms

PCR amplifications of yeast-like microorganism DNA preparations produced a single main band of each DNA of 1.6 Kbp size, except for group G of 1.3 Kbp size, as seen in Fig. 3. When four different colonies in group A were used as DNA preparations for PCR amplification, the produced DNAs were all of the same 1.6 Kbp size as shown in Fig. 4. These results strongly support that the DNAs amplified by PCR were originated from 18S-rDNA regions of the yeast-like symbiotes, because they were almost as large as 18S-rDNA of *S. cerevisiae* and three other eucaryotes (shown in DISCUSSION). They also suggest that these DNAs have the same or similar base sequences as primers A and B at their both ends.

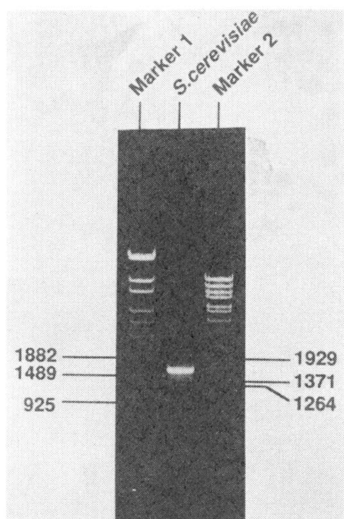

Fig. 2 PCR amplification of *S. cerevisiae* DNA preparations.

Marker 1: λDNA was digested with EcoT14I, Marker 2: λDNA was digested with BstPI.

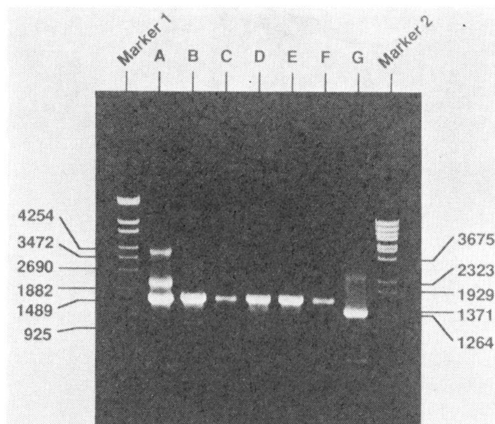

Fig. 3 PCR amplifications of yeast-like symbiote DNA preparations (A to G).

Markers 1 and 2 are the same as those in Fig. 2.

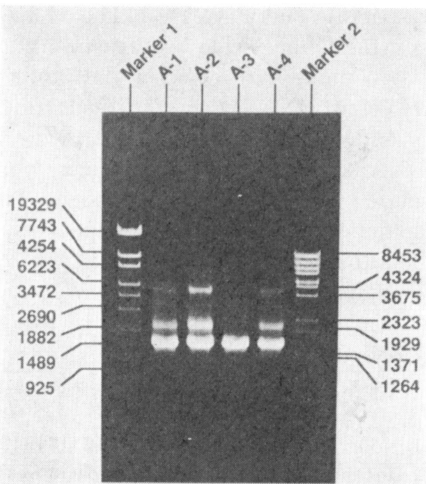

Fig. 4 PCR amplifications of yeast-like symbiote DNA preparations from four colonies (A-1 to A-4) of group A.

Markers 1 and 2 are the same as those in Fig. 2.

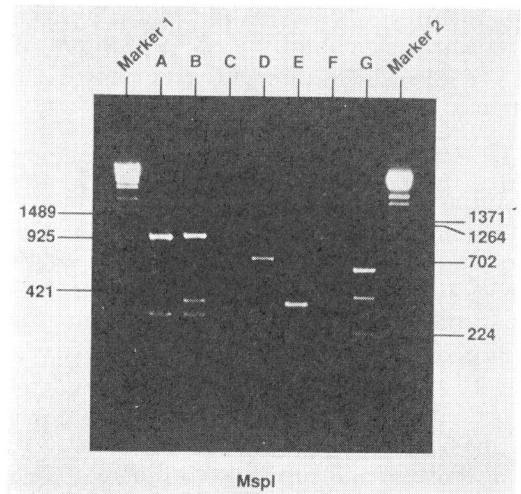

Fig. 6 Band patterns on electrophoresis when amplified DNAs, A to G, were digested with MspI.

Markers 1 and 2 are the same as those in Fig. 2.

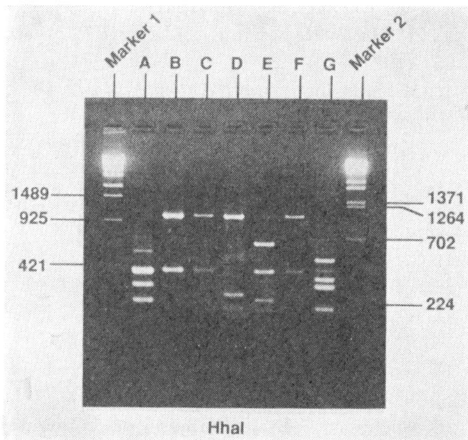

Fig. 5 Band patterns on electrophoresis when amplified DNAs, A to G, were digested with HhaI.

Markers 1 and 2 are the same as those in Fig. 2.

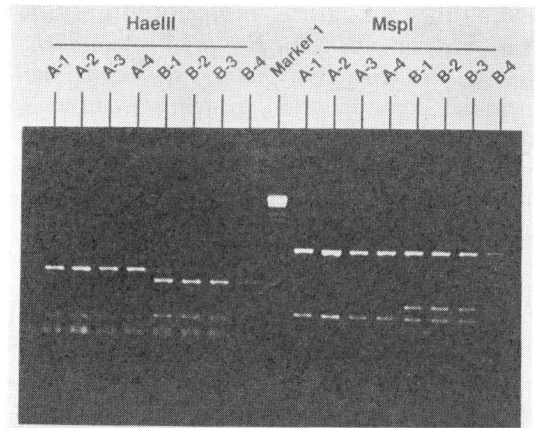

Fig. 7 Band patterns on electrophoresis when amplified DNAs of A-1 to A-4 and B-1 to B-4 were digested with HaeIII and MspI.

Marker 1 is the same as that in Fig. 2.

4. Digestion of the Amplified DNA with Restriction Endonucleases

When the PCR-amplified DNA fragments from yeast-like symbiote DNA preparations were digested with five kinds of restriction endonucleases, they all had different band patterns as shown in Figs. 5 and 6, which are just two examples when the amplified DNAs were digested with HhaI and MspI. The other endonucleases gave different band patterns. The amplified DNAs from four colonies in the same groups produced the same band patterns on electrophoresis, however, when digested with the endonucleases. Figure 7 shows an example of the groups A and B when the amplified DNAs were digested with HaeIII and MspI. The other groups and digestion with the other endonucleases gave the similar results (data

not shown), which indicate that the seven yeast-like microorganisms isolated from *N. lugens* belong to different taxonomic affiliations.

DISCUSSION

Intracellular symbiotes have been observed in various kinds of insects,[12] but their physiological roles remain unclear, because of the difficulty in culturing them outside their hosts. Many investigators have tried to isolate and culture them *in vitro*, but only a few have been successful. In planthoppers, yeast-like symbiotes are dominantly found in the eggs and fat bodies of larval and adult abdomens. Nasu *et al.* isolated and cultured two different yeast-like symbiotes from *N. lugens* eggs.[9] We also isolated seven kinds of yeast-like microorganisms from the eggs of the same species under milder condition to sterilize the egg surface, under which no microorganism was observed without homogenizing the eggs before isolation and all the eggs hatched normally. In preliminary experiments, some bacterial colonies were seen on the same agar plates, and to eliminate bacterial contamination, primary isolation of yeast-like microorganisms was done in the presence of chloramphenicol (100 mg/*l*) and sodium propionate (2.5 g/*l*). These seven yeast-like microorganisms were isolated under aerobic condition, and group A was isolated under either aerobic or unaerobic condition. No additional yeast-like microorganisms were isolated on the agar plates supplemented with the host haemolymph and a rice-plant extract. Determination of their taxonomic affiliation is difficult on microorganisms, especially on yeasts. We tried to classify them by analyzing 18S-rDNA, which is conserved in a wide range of organisms. The base sequences of 17-18S-rDNAs of four eucaryotes, *S. cerevisiae, Neurospora crassa, Drosophila melanogaster* and *Dictyostelium discoideum*, were compared in database search, and primers A and B, which are conserved sequences in the 18S-rDNAs of the above four eucaryotes, were selected as PCR primers. The 1.6 Kbp DNA fragment was amplified from genomic DNA preparations of *S. cerevisiae* by PCR. Nucleotide sequencing of this DNA fragment was not conducted in this experiment. Instead this, DNA was digested with restriction endonucleases. This technique is easier than nucleotide sequencing and can be used to make sure whether the product is the predicted DNA, when the nucleotide sequence of DNA is completely analyzed. The 18S-rDNA fragments were obtained from all of the isolated yeast-like microorganisms. Each DNA had a different nucleotide sequence in the digestion analyses. The question is which one is a dominant symbiote in insects. We are now trying to isolate symbiotes directly from the adult fat body.

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要　約

トビイロウンカの細胞内共生微生物の分離と18S-rDNA 解析による分類

加賀山慶一，白神伸江，永峰俊弘
梅原利之，満井　喬

トビイロウンカ (*Nilaparvata lugens*) 卵より形態的に異なる7グループの酵母様共生微生物が分離された．これらの酵母株から全 DNA を抽出し，これを鋳型として PCR 法により 18S-rRNA をコードする rDNA 領域を増幅し，増幅された DNA を比較することによっ

てこれらの酵母の分類を試みた．PCR のプライマーとしては，18S-rDNA の生物間で保有性の高い領域を用いた．PCR の結果，6 グループからは約 1600 塩基対の，1 グループからは約 1300 塩基対の DNA 断片が増幅された．次に増幅された DNA を 5 種の制限酵素で切断したところ，各グループでの切断パターンはそれぞれ異なっていた．これらの結果から，この 7 種の酵母株は分類学的に別種に分類されることが明らかとなった．