Two Intracellular Symbiotic Bacteria from the Mulberry Psyllid *Anomoneura mori* (Insecta, Homoptera)

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We characterized the intracellular symbiotic bacteria of the mulberry psyllid *Anomoneura mori* by performing a molecular phylogenetic analysis combined with in situ hybridization. In its abdomen, the psyllid has a large, yellow, bilobed mycetome (or bacteriome) which consists of many round uninucleated mycetocytes (or bacteriocytes) enclosing syncytial tissue. The mycetocytes and syncytium harbor specific intracellular bacteria, the X-symbionts and Y-symbionts, respectively. Almost the entire length of the bacterial 16S ribosomal DNA (rDNA) was amplified and cloned from the whole DNA of *A. mori*, and two clones, the A-type and B-type clones, were identified by restriction fragment length polymorphism analysis. In situ hybridization with specific oligonucleotide probes demonstrated that the A-type and B-type 16S rDNAs were derived from the X-symbionts and Y-symbionts, respectively. Molecular phylogenetic analyses of the 16S rDNA sequences showed that these symbionts belong to distinct lineages in the \( \gamma \) subdivision of the Proteobacteria. No 16S rDNA sequences in the databases were closely related to the 16S rDNA sequences of the X- and Y-symbionts. However, the sequences that were relatively closely related to them were the sequences of endosymbionts of other insects. The nucleotide compositions of the 16S rDNAs of the X- and Y-symbionts were highly AT biased, and the sequence of the X-symbiont was the most AT-rich bacterial 16S rDNA sequence reported so far.

Many insects have established highly elaborate symbiotic associations with specific microorganisms. At all times these specific microorganisms are harbored in the gut lumen, in caeca connected to the gut, inside specialized gut epithelial cells, in the hemocoel, or inside highly developed symbiotic organs called mycetomes in the body cavity (6). Because the microbes are always found in the host insect and are passed from generation to generation by vertical transmission and because the host usually suffers sterility or death when it is deprived of the microbes, the relationships between insects and their specific microorganisms are thought to be obligate and mutualistic in many cases (4, 12).

The Homoptera, including cicadas, planthoppers, aphids, scale insects, psyllids, etc., is an insect group whose endosymbiotic systems are highly developed (6). Because homopteran insects live on nutritionally unbalanced diets consisting of plant sap throughout their lives, it is believed that they need the help of endosymbiotic microorganisms to compensate for nutritional deficiencies. In fact, it has been demonstrated that endosymbiotic microbes of homopterans are involved in metabolic processes, such as the synthesis of essential nutrients and recycling of nitrogenous wastes (5, 11, 12, 32). The endosymbiotic microorganisms have not been cultured in common media, probably because they are highly adapted to special environments inside the host organisms and cannot live outside the hosts (4). Since conventional microbiological methods have been based on isolation of microorganisms, the biological nature of the endosymbionts has been unclear for a long time.

However, recent innovations in molecular phylogenetic techniques have revealed the systematic affinities of fastidious endosymbionts of members of the Homoptera (9, 17, 26–28, 34). In general, microbial DNA fragments, putatively derived from the symbionts, have been amplified by PCR and sequenced from the total DNA of the host insects. Such an approach is, however, often complicated by the diversity and complexity of the endosymbiotic microbiota. When a microbial species is the major symbiont in an insect body, this approach works quite well. However, multiple microbial species commonly coexist in an insect body not only in members of the Homoptera but also in members of many other insect groups (6, 16, 18, 19). Practically, we frequently encounter situations in which 16S ribosomal DNA (rDNA) fragments amplified and cloned from whole insect DNA contain a number of different sequences, which might come from multiple endosymbionts, gut microbes, pathogens, occasional contaminating bacteria, or debris adhering to insect surfaces. In addition, possible biases inherent in PCR amplification and DNA cloning may sometimes result in serious artifacts. Therefore, the microbial DNA sequences obtained must be interpreted in connection with morphological data obtained by using, for example, in situ hybridization with specifically designed probes (3).

In the Homoptera, psyllids (Psylloidea) constitute the well-defined group Sternorrhyncha together with aphids (Aphidoidea), scale insects (Coccoidea), and whiteflies (Aleyrodioidea). Only a few histological descriptions are available for the endosymbionts of psyllids. According to previous reports, psyllids have a large, yellow, bilobed mycetome in its abdomen. The mycetome is a complex of the following three types of cells: many round uninucleated mycetocytes, a syncytial tissue surrounded by these cells, and an envelope composed of many flattened cells encasing the whole mycetome. The cytoplasm of the mycetocytes is full of a specific bacterium, called the Y-symbiont (6, 8, 30, 36). Although molecular phylogenetic studies of the intracellular symbiotic bacteria of aphids, scale insects, and whiteflies have been performed (9, 26, 27), no such study has been conducted on the endosymbiotic bacteria of psyllids.

In this study, we characterized the intracellular symbiotic...
bacteria of the mulberry psyllid *Anoplophora mori* by using a molecular phylogenetic approach combined with an in situ hybridization technique.

**MATERIALS AND METHODS**

**Materials.** Nymphs of *A. mori*, which had formed colonies covered with plenty of wax, were collected from the undersides of mulberry leaves at Tsukuba, Ibaraki, in May 1997 and were preserved in acetone.

**DNA extraction, PCR, and cloning of 16S rDNA.** The insects were repeatedly washed with acetone to remove wax and possible contamination. After the insects were placed on clean tissue paper for a while to remove the preservative, they were subjected to DNA extraction with a DNA extraction kit (QIAGEN). From the whole-insect DNA, the entire length of bacterial 16S rDNA (about 1.5 kb) was amplified by PCR using primers 16SA1 (5'-AGAGTTT GATCMTGCGCTCAG-3') and 16SB1 (5'-TACGGYTACCTTGTTACGACTT-3') and the following temperature profile: 94°C for 2 min, 50°C for 1 min, and 70°C for 2 min. The PCR product was purified with a GeneClean II kit (Bio 101, Inc.) and was cloned with TA cloning vector pT7Blue (Novagen) and *Escherichia coli* JM109 competent cells (Takara) by using ampicillin and the X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) blue-white selection system.

**Typing of cloned 16S rDNA by restriction fragment length polymorphism (RFLP).** White colonies that were expected to contain the inserted plasmids were directly subjected to in situ hybridization for the screening of the hybridizing clones (primers U19 [5'-GTGTTCCCCCACTACAGCCTGAC-3'] and B7 [5'-TAATACGACTCACTATAGGG-3']) in order to check the length of the inserted DNA fragment. If the PCR product was the expected size (about 1.5 kb), it was digested with four base-recognizing restriction endonucleases (HindIII, *RsaI*, *Sau3A1*, and *TaqI*) and electrophoresed in agarose gels in order to type the cloned 16S rDNA.

**DNA sequencing.** The white colonies that were determined to contain a 16S rDNA clone were isolated and cultured in 1.5 ml of Luria-Bertani medium supplemented with ampicillin overnight and subjected to plasmid extraction with a QIAprep Spin miniprep kit (QIAGEN). The purified plasmids, which were eluted with 30 μl of TE buffer, were used as the template DNA for sequencing. A dye terminator-labelled cycle sequencing reaction was performed with a type D-galactopyranoside blue-white selection system.

**Histological preparation, in situ hybridization, and enzymatic probe detection.** The probe was detected by using a *DIG*-nucleic acid detection kit (Boehringer Mannheim) essentially as recommended by the manufacturer. Each tissue section was washed with buffer 1 (0.1 M maleic acid–NaOH [pH 7.5]), 0.15 M NaCl) and incubated with buffer 2 (blocking solution) for 30 min. An anti-DIG-AP conjugate solution (150 mU of anti-digoxigenin–alkaline phosphatase conjugate per ml in buffer 2) was applied to the slide to stain the bound digoxigenin-labelled probe deep blue.

**RESULTS**

**Detection of digoxigenin-labelled probe.** The probe was detected by using a DIG-nucleic acid detection kit (Boehringer Mannheim) essentially as recommended by the manufacturer. Each tissue section was washed with buffer 1 (0.1 M maleic acid–NaOH [pH 7.5]), 0.15 M NaCl) and incubated with buffer 2 (blocking solution) for 30 min. An anti-DIG-AP conjugate solution (150 mU of anti-digoxigenin–alkaline phosphatase conjugate per ml in buffer 2) was applied to the slide, which was then incubated overnight. After the preparation was washed with buffer 3 (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM MgCl₂), a nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate substrate solution was added to the slide to stain the bound digoxigenin-labelled probe deep blue. The tissue section was washed with distilled water, mounted in glycerol, and observed with a differential interference microscope.

**Nucleotide sequence accession numbers.** The 16S rDNA sequences of the X- and Y-symbionts of *A. mori* reported in this paper have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession nos. AB013086 and AB013087.

**Identification of two types of 16S rDNA.** Almost the entire length of 16S rDNA was successfully amplified from the whole-insect DNA by PCR. Since it was expected that more than one sequence, derived from different microorganisms, would be in the product, the amplified DNA fragments were subjected to cloning. RFLP analysis of the cloned fragments revealed two sequences, tentatively designated A-type and B-type. The A-type clones were obtained more frequently than the B-type clones (Fig. 1).

**General observations on the endosymbiotic system.** A large part of the *A. mori* abdomen was occupied by a large, orange, bilobed mycetome which was easily recognized under a dissecting microscope. When examined histologically, the mycetome was found to be composed of a number of round mycocytes and a syncytial cytoplasm surrounded by them. These observations are in agreement with a previous report (36).

**FIG. 1.** RFLP analysis of bacterial 16S rDNAs amplified and cloned from total DNA of *A. mori*. Lanes 1 through 7 contained cloned 16S rDNA fragments digested by *RsaI* (left) or *Sau3A1* (right) and resolved on a 2.5% agarose gel. Lanes 1, 3, 4, 6, and 7 contained A-type clones, whereas lanes 2 and 5 contained B-type clones. Lanes M contained DNA size markers, whose sizes (in base pairs) are indicated on the left. RFLP profiles of *HorI* and *TaqI* digests also agreed with the typing data (data not shown).
FIG. 2. 16S rDNA sequences of the X-symbiont (A-type) and the Y-symbiont (B-type) aligned with the sequence of E. coli. The nucleotide regions complementary to the probes used for in situ hybridization are highlighted. Asterisks represent matched nucleotide sites, and dashes represent alignment gaps.
FIG. 3. In situ hybridization of intracellular symbiotic bacteria in the mycetome of *A. mori*. (A) Probe DIG-Kiji16SA targets the A-type sequence in the round mycetocyte, where the X-symbionts are located. (B) Probe DIG-Kiji16SB targets the B-type sequence in the syncytium, where the Y-symbionts are harbored. Bar = 20 μm.
were 1,463 bases for the A-type clones and 1,471 bases for the B-type clones. The RFLP profiles expected from the sequences agreed with the patterns observed.

In situ hybridization. It seemed likely that the two types of 16S rDNA were derived from the X- and Y-symbionts of *A. mori*. To confirm this, we performed 16S rRNA-targeted in situ hybridization with oligonucleotide probes DIG-Kiji16SA and DIG-Kiji16SB, which were specifically designed for A-type and B-type, respectively (Fig. 2).

Figure 3 shows the in situ hybridization results. When probed with DIG-Kiji16SA, the cytoplasm of the round mycetocytes was specifically visualized (Fig. 3A). The syncytial cytoplasm surrounded by the mycetocytes was not stained at all, indicating that the 16S rDNA sequence of A-type was derived from the intracellular symbiotic bacterium of the round mycetocytes, the so-called X-symbiont. In contrast, when hybridized with DIG-Kiji16SB, the syncytium was stained, whereas the round mycetocytes gave no signal (Fig. 3B), indicating that the sequence of B-type could be attributed to the endosymbiotic bacterium of the syncytium, the so-called Y-symbiont. A series of control experiments confirmed the specificity of these results (data not shown). Under stringent hybridization-wash conditions under which *Escherichia coli* and *Buchnera* sp. of the pea aphid were clearly detected, probe EUB338 gave no signal with the tissue sections of *A. mori* (data not shown).

Molecular phylogenetic analysis. Figure 4 is a neighbor-joining tree showing the phylogenetic positions of the X- and Y-symbionts of *A. mori*. The 16S rDNA sequences of the X- and Y-symbionts, representatives of the Proteobacteria, and two gram-positive bacteria (as an outgroup) were analyzed by the neighbor-joining method by using Kimura's two-parameter correction. A total of 1,141 unambiguously aligned nucleotide sites were subjected to the analysis. The bootstrap values obtained with 1,000 resamplings are shown at the nodes. The numbers in brackets are accession numbers.

**FIG. 4.** Phylogenetic positions of the X- and Y-symbionts of *A. mori*. The 16S rDNA sequences of the X- and Y-symbionts, representatives of the Proteobacteria, and two gram-positive bacteria (as an outgroup) were analyzed by the neighbor-joining method by using Kimura's two-parameter correction. A total of 1,141 unambiguously aligned nucleotide sites were subjected to the analysis. The bootstrap values obtained with 1,000 resamplings are shown at the nodes. The numbers in brackets are accession numbers.
biont formed a monophyletic group with the symbionts of whiteflies, which was supported by a bootstrap value of 74.0%. The Y-symbiont formed a cluster with the intracellular symbionts of aphids and ants, although the level of bootstrap support was very low. The maximum-likelihood analysis gave essentially the same results (data not shown).

**AT-biased nucleotide composition.** The 16S rDNA sequences of the X- and Y-symbionts were extremely AT rich. The AT contents were 63.6% for the X-symbiont and 55.0% for the Y-symbiont. Figure 5 shows a histogram of the AT contents of 3,745 prokaryotic 16S rDNA sequences longer than 500 bases that have been deposited in the RDP database along with the AT contents of the X- and Y-symbionts. Notably, the sequence of the X-symbiont was the most AT-rich sequence reported to date.

**DISCUSSION**

Because multiple endosymbiotic bacteria coexist in the psyllid *A. mori*, simple PCR amplification and sequencing procedures were not sufficient to distinguish and characterize them. We identified two types of bacterial 16S rDNA sequences from the total DNA of the insect (Fig. 1 and 2) and performed in situ hybridization with highly specific oligonucleotide probes under stringent conditions. The 18-mer probes DIG-Kiji16SA and DIG-Kiji16SB were designed for a specific region of the A-type and B-type sequences, respectively. The high level of specificity of these probes was confirmed when they were subjected to a database homology search. When the RDP database was examined, no sequence exhibited 100% identity to DIG-Kiji16SA. Only three sequences exhibited 100% identity to DIG-Kiji16SB; one of these was from a Euglenozoa chloroplast (accession no. X14386), and two were from unidentified eubacteria (accession no. U05662 and X84607). These matches can be regarded as matches that occurred by chance. Under stringent hybridization and wash conditions that did not permit even a single base mismatch, both probes revealed specific localization in tissue sections of the insect. The signal obtained with DIG-Kiji16SA was coincident with the localization of the X-symbiont in the mycetocytes, whereas the signal obtained with DIG-Kiji16SB was coincident with the location of the Y-symbiont in the syncytium (Fig. 3). From these lines of evidence, taken together, we concluded that we successfully cloned and sequenced the 16S rDNAs of the X- and Y-symbionts of *A. mori*. This is the first report of molecular characterization of the endosymbionts of a psyllid.

When preparations were probed with EUB338 under stringent conditions, the X- and Y-symbionts were not detected. This result was expected from the 16S rDNA sequences, because both the X-symbiont and the Y-symbiont contained nucleotide substitutions in the EUB338 target region. Considering that not only the mycetome but also the other tissues were not stained with EUB338 and that RFLP analysis revealed only two 16S rDNA sequences, it is likely that there are no major bacterial endosymbionts in the psyllid other than the X- and Y-symbionts, although the possible presence of minor microbial associates cannot be ruled out. In fact, when some 50 inserted 16S rDNA clones were subjected to *RsaI* digestion, we found two clones that were neither A-type nor B-type clones (data not shown).

Based on the 16S rDNA phylogeny data, both the X- and Y-symbionts are members of the γ subdivision of the *Proteobacteria*, although they belong to distinct lineages. No 16S rDNA sequence in the database was closely related to the 16S rDNA sequences of the psyllid endosymbionts. However, it should be noted that the sequences that were relatively closely related to the sequences of the psyllid endosymbionts were sequences of endosymbionts from homopteran and other insects. The X-symbiont constituted a monophyletic group along with endosymbionts of whiteflies, whereas the Y-symbiont formed a clade with endosymbionts of aphids and ants (Fig. 4). At a glance, these results suggest interesting phylogenetic inferences. Since psyllids (Psyllioidea), whiteflies (Aleurodioidea), and aphids (Aphidoidea) constitute the well-defined group Sternorrhyncha and all of these insects have mycetocyte endosymbiotic bacteria (6, 7, 35), it is tempting to assume that their common ancestor possessed two types of intracellular symbiotic bacteria, one of which descended to whiteflies and psyllids and the other of which passed to aphids and psyllids in the evolutionary course of the Sternorrhyncha. However, we should be careful in interpreting the molecular phylogenetic results. For instance, the statistical support for the phylogenetic affinities was far from satisfactory. In addition, the highly AT-biased nucleotide compositions of the 16S rDNAs of the psyllid symbionts (Fig. 5) might lead to misinterpretation (21).

Although the X-symbiont of the psyllid formed a monophyletic group with the endosymbionts of whiteflies, with a bootstrap probability value of 74%, the 16S rDNA sequences of these organisms are among the most AT-biased 16S rDNA sequences reported so far (63.6% for the X-symbiont, 52.3% for the Y-symbiont). The Y-symbiont formed a monophyletic group along with endosymbionts of whiteflies, whereas the Y-symbiont formed a clade with endosymbionts of aphids and ants (Fig. 4). The Y-symbiont is related to the sequences of the psyllid endosymbionts. However, it should be noted that the sequences that were relatively closely related to the sequences of the psyllid endosymbionts were sequences of endosymbionts from homopteran and other insects. The X-symbiont constituted a monophyletic group along with endosymbionts of whiteflies, whereas the Y-symbiont formed a clade with endosymbionts of aphids and ants (Fig. 4). At a glance, these results suggest interesting phylogenetic inferences. Since psyllids (Psyllioidea), whiteflies (Aleurodioidea), and aphids (Aphidoidea) constitute the well-defined group Sternorrhyncha and all of these insects have mycetocyte endosymbiotic bacteria (6, 7, 35), it is tempting to assume that their common ancestor possessed two types of intracellular symbiotic bacteria, one of which descended to whiteflies and psyllids and the other of which passed to aphids and psyllids in the evolutionary course of the Sternorrhyncha. However, we should be careful in interpreting the molecular phylogenetic results. For instance, the statistical support for the phylogenetic affinities was far from satisfactory. In addition, the highly AT-biased nucleotide compositions of the 16S rDNAs of the psyllid symbionts (Fig. 5) might lead to misinterpretation (21).
the phylogenetic tree, the branch lengths appear to be elongated in the lineages of the X- and Y-symbionts (Fig. 4), which could reflect accelerated nucleotide substitution rates in these lines. It has been suggested that a small population size and a lack of effective recombination in vertically transmitted endosymbiotic microorganisms result in the accumulation of mildly deleterious mutations, which could be detected as faster sequence evolution and a shift in base composition that reflects mutational bias (25). The aphid symbiont Buchnera sp. has only a single copy of the 16S rRNA gene, which might reflect slow growth of the endosymbiotic bacterium (5). Although it is not known how many copies of rRNA genes there are in the psyllid symbionts, it seems possible that these organisms also have only one, considering that the growth and reproduction of psyllids are much slower than the growth and reproduction of aphids (22). If this is true, mutations cannot be corrected by gene conversion. The extraordinary molecular features of the 16S rDNAs of psyllid endosymbionts might be explained in this context.

The biological functions of the mycetome endosymbionts of psyllids have not been investigated. However, the highly developed mycetome is conserved in all of the psyllids that have been examined (6, 8, 30, 36), suggesting that the endosymbionts may play an essential physiological and nutritional role in the host psyllids, as has been demonstrated in aphids, planthoppers, cockroaches, and other insects (5, 10–12, 32).

Profft (30) examined the endosymbiotic systems of 18 species of psyllids histologically. He found that the majority of these psyllids possess two types of intracellular symbiotic bacteria, one in round, uninucleated mycocytes and the other in the syncytium. Later, Chang and Musgrave (8) arbitrarily designated the former X-symbionts and the latter Y-symbionts in their electron microscopic study of the pear psyllid Psylla pyricola. Waku and Endo (36) confirmed that the same endosymbiotic bacteria (Homoptera: Aleyrodoidea) constitute a lineage distinct from the endosymbionts of aphids and mealybugs. Curr. Microbiol. 25:123–129.


