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A CONSIDERATION ABOUT THE ORIGIN OF APHID INTRACELLULAR SYMBIONT IN CONNECTION
WITH GUT BACTERIAL FLORA

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In an attempt to investigate the origin of the intracellular symbiont of the aphid (Buchnera), aphid gut aerobic bacteria were isolated, and their phylogenetic relations to other prokaryotes were examined based on nucleotide sequences of 16S rDNA. It turned out that there are seven aerobic bacterial groups which constitute major flora of the aphid's gut. As three of the isolated bacteria were identified as members of the family Enterobacteriaceae, and share the common ancestor with the intracellular symbiont, the nucleotide sequences of 16S rDNA were determined for 15 representative strains of the family Enterobacteriaceae. One of the gut microbes belonging to the family Enterobacteriaceae was identified as Erwinia herbicola that is found mainly on plant surfaces. This fact may suggest that the intracellular symbiont of aphid is derived from a habitant
of plant on which host insects feed.

Pea aphid, Acyrthosiphon pisum, harbors prokaryotic intracellular symbionts in the mycetocytes, huge cells in the abdomen, which are differentiated specifically to accommodate the symbionts (1). The date of establishment of the symbiotic association is estimated to be 160-280 million years ago based on 16S rDNA molecular clock calibrated by aphid fossils (12). The aphid and its intracellular symbionts, which are often referred to as Buchnera species (14), are in a closely mutualistic relationship, and neither of them is able to propagate without the other (8,15). Buchnera, essential for the normal growth and reproduction of the aphid, are maternally inherited through generations of the host insect, and have not been successfully cultivated outside the host mycetocyte.

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In view of the location and nutritional role of the intracellular symbionts in insects, it is generally believed that they originated from gut microbes that are descendants of free-living bacteria taken in with diet by the host insect in the evolutionary past (1). According to the 16S rDNA phylogeny of bacteria, Buchnera species of various aphids constitute a monophyletic group (13), belong to the y subdivision of the class Proteobacteria, and have a close relationship with members of the family Enterobacteriaceae (19).
In the previous study, we demonstrated based on the analysis of RFLP of several genes, that pea aphid contains, at least, two groups of gut microbes, one of which was a close relative of Escherichia coli, a member of the family Enterobacte-riaceae (S). In an effort to better understand the evolutionary relationship of the gut microbes and Buchnera, we further raked aphid gut microbes and determined almost the entire sequences of their 16S rDNAs using the polymerase chain reaction (PCR) direct sequencing method for molecular phylogenetic analyses. The roots of Buchnera and mechanism of establishing the symbiotic relationships were discussed from a microbial ecological point of view.

MATERIALS AND METHODS

Aphids. Apterous parthenogenetic pea aphids, Acyrthosiphon pisum (Harris), were reared on young broad bean plants, Vicia faba (L.), at 20°C under a long-day regime of 18 h light and 6 h dark.
Bacteria. Aerobic bacteria were isolated from aphids as follows. Whole bodies of the insect which were surface-sterilized by dipping in 70% ethanol for 5 min, were washed in Carlsons' solution (0.7% NaCI (w/v), 0.02% KCl (w/v), 0.02% CaC12. 2H2O (w/v), 0.01% MgCl2. 6H2O (w/v), 0.02% NaH2PO4 (w/v), 0.012% NaHCO3 (w/v), 0.8% glucose (w/v)), and lightly homogenized in the same solution. The homogenate was spread on L broth agar media and incubated overnight at 28°C to form colonies of the gut bacteria. Bacteria were also isolated from the digestive tracts surgically isolated from aphids by the same procedure. Amplification of 16S rDNA. The region of rDNA encoding 16S rRNA (16S rDNA) was amplified by the PCR method. The primers used for amplification were OF (5'-AGA GTT TGA TCA TGG CTC AGA TTG-3' nt 8-31) and 15R (5'-TAC CTT GTT ACG ACT TCA CCC CAG-3' nt 1513-1490). Numerals following the oligonucleotide sequence designate Escherichia coli rRNA nucleotide (nt) numbers. The reaction mixture (50,u1) consisted of 10 mM Tris-HC1(pH 8.3), 50 mM KCI, 0.01% gelatin, 5 mM MgCl2, 0.2 mM each of dATP, dCTP, dGTP, dTTP, and 0.5 mM each of primers. A trace amount of bacteria was added to the reaction mixture, followed by incubation at 94°C for 10 min. One unit of Taq DNA polymerase (Takara, Inc., Ohtsu, Japan) was added, and the mixture was overlaid with mineral oil and subjected to PCR. Each cycle consisted of 30s at 94°C, 60s at 55°C and 120s at 70°C, and a total of 35 cycles were performed. RFLP analysis of 16S rDNA. Aliquots of PCR products were digested with

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following restriction endonucleases: BglII, EcoRI, EcoT14, EcoRV, HindIII, HinfI, MspI, electrophoresed on 2% agarose gels, and stained with ethidium bromide. Sequencing of the amplified DNA. Amplified DNA was purified by gel fil-tration using the MicroSpinTM S-400 HR column (Pharmacia Biotech, Inc., Tokyo, Japan) and used as a template. The nucleotide sequence was directly determined by the dideoxy chain-termination method (I8) using either the Taq Dye DeoxyTM Terminator or Dye Primer Cycle Sequencing Kit (Perkin Elmer, Chiba, Japan) according to the manufacturer's recommended protocol in combination with the ABI Model 373A automatic sequencer. In addition to primers OF and 15R, primers 5F (5'-CAG GAG TGC CAG CAG CCG CGG-3'), 5R (antisense analog of prim-er 5F), 8R (5'-CAG GAC TAC CAG GGT ATC TAA T-3'), 9F (5'-GCA CAA GCG GTG GAG CAT GTG G-3'), 11 F (5'-CAG GAG CAA CGA GCG CAA CCC-3'), and 11 R (antisense analog of primer 11 F) were used for sequencing. Ap-proximately 1,410 nucleotides were sequenced from each amplified gene product. Sequence analysis. The sequences of 165 rDNA obtained from the GenBank database were as follows: Escherichia coli (J01859), Yersinia enterocolitica (M59292), Serratia marcescens (M59160), Proteus vulgaris (JO1874), Buchnera sp. (M27039), Actinobacillus hominis (LO6076), Haemophilus ducreyi (M63900), Pasteurella betti (L06088), Haemophilus influenzae (M35019), Pasteurella maini (L06089), Vibrio cholerae (X74695), V. logei (X74708), V. furnisii (X76336), V. natriegens (X74714), Aeromonas jandaei (X74678), A. hydrophila (X60404), A. salmonicida (X74681), Pseudomonas aeruginosa (X06684), P. mendocina (M59154), P. putida (D37924), P. flavescens (U01916), Comamonas testosteroni (M11224), Burkholderia cepacia (M22518), Erwinia carotovora (M59149), Citro-bacter freundii (M59291), Photorhabdus luminescens (X82248), Xenorhabdus beddingii (X82254), Staphylococcus aureus (L37597), St. epidermidis (L37605), St. warneri (L37603), St. haemolyticus (L37600), St. hominis (L37601), St. sapro-phyticus (L37596), St. sciuri (S83569), Bacillus thuringiensis (D16281), B. cereus (D16266), B. cohnii (X76437), B, megaterium (D16273), B. macroides (X70312), Lactobacillus thermophilus (MS 8832), Sporolactobacillus dextrus (D16282), and Mycoplasma capricolum (U26041). The numbers in parentheses following the bacterial species' names are the respective accession numbers.
The sequences were aligned with the aid of the Clustal V program (6). Phylogenetic analyses were based on the neighbor joining method (17) using the program from MEGA version 1.0 (9). Reliability of clustering patterns was assessed by bootstrapping (4). The trees were obtained after 500 bootstraps. DNA-DNA hybridization. To prepare genomic DNA of bacterium T and En. carotovora, cells were grown in nutrient broth at 30°C for 24 h with shaking. DNA was prepared by the method of Marmur (11). The other bacterial DNAs listed in Table 1 were the gift of Y. Kosako. Aliquots of DNA of bacterium T (strain 191) were labeled with photobiotin, and hybridization studies were done by the method reported by Ezaki et al. (3). The fluorescence was read using a fluorometer (Scanning densitometer CS-9300PC; Shimadzu, Kyoto, Japan).

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RESULTS

Isolation of gut aerobic bacteria from pea aphid and RFLP analysis of 16S rDNA It was observed that the whole body extract of surface-sterilized pea aphids contains many groups of bacteria. A preparatory experiment revealed that extract of digestive tracts surgically isolated from aphids also contained the same kinds of bacteria (confirmed by RFLP analysis) as those isolated from the whole body. Then, the bacteria isolated from the whole body were supposed to be mostly derived from the aphid gut, and the bacteria from the whole body were submitted to further characterization. A trace amount of bacteria was picked up from each colony, and used as the template for PCR to amplify 16S rDNA. The PCR product was digested with one of seven restriction enzymes and electrophoresed. The restriction fragments obtained from l6S rDNA of each bacterium showed a group-specific pattern.
Gut bacteria were isolated from a total of 20 aphids. According to the results, seven different groups of bacteria were detected from the aphid population used. We tentatively named them bacterium T (2 strains), U (2 strains), V (3 strains), W (3 strains), X (12 strains), Y (13 strains) and Z (3 strains). Figure 1 represents a comparison among the patterns of restriction fragments from these seven 16S

Table 1. Levels of intraspecies DNA-DNA relatedness of strain 191
to selected members of the Enterobacteriaceae.

(bacterium T)

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rDNAs. Bacteria X, T, W, and U were Gram-negative, while bacteria Y, Z, and V were Gram-positive. Among these, bacteria X and Y were the dominant groups in the gut of the pea aphid.

Phylogenetic analysis of Gram-negative bacteria
In an effort to further characterize these gut bacteria, we determined almost the entire region of their 16S rDNA sequences, and carried out molecular phylo-genetic analyses. Since a preparatory analysis suggested that the four groups of newly isolated Gram-negative bacteria belong to the r subdivision of the class Proteobacteria, we analyzed this group minutely and constructed a typical tree shown in Fig. 2. It turned out that bacteria X, T, and W are members of the family Enterobacteriaceae, sharing the closest common ancestor with the pea aphid Buchnera. The results also showed that there are no extant bacteria, including aphid gut bacteria, that make a common cluster with Buchnera. Bacterium U made a cluster with the fluorescent Pseudomonas such as P. aeruginosa.

Further studies on the family Enterobacteriaceae
Since the results in Fig. 2 showed that bacteria T, W, and X share the closest common ancestor with Buchnera, further studies were focused upon these groups of bacteria.

Because the results of biochemical tests using the API 50 CHE system (bioMerieux-Vitek Japan, Ltd., Tokyo, Japan) indicated the close relationship of

Fig. 1. RFLP of 16S rDNA of (a) bacterium T, (b) bacterium X, (c) bacterium
W, (d) bacterium U, (e) bacterium V, (f) bacterium Y, and (g) bacterium Z.
Seven groups of 16S rDNA were amplified by PCR, and digested with 7 restriction
enzymes, electrophoresed in 2% agarose gel, and stained with ethidium bromide. 1,
Bg1II; 2, EcoRI; 3, EcoTl4; 4, EcoRV; 5, Hindu!; 6, HinfI; 7, MspI.

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these bacteria to either of the genera, Enterobacter, Serratia, Klebsiella and Erwinia (data not shown), the sequences of 16S rDNA were determined for representative enterobacteria strains, Enterobacter asburiae JCM (Japan Collection of Microor-ganisms) 6051, En. sakazakii JCM 1233, En. intermedium JCM 1238, En. gergoviae JCM 1234, En. amunigenus JCM 1237, En. aerogenes JCM 1235, Serratia rubidaea JCM 1240, S. liquefaciens JCM 1245, S. ficalia JCM 1241, Klebsiella pneumoniae JCM 1662, K. oxytoca JCM 1665, K. planticola JCM 7251, K. ornithinolytica JCM 6069, Erwinia herbicola JCM 7000, and Er. ananus JCM 6986, molecular phylogeny of these three bacteria and related species was analyzed (Fig. 3). It turned out that bacterium T was closely related to Er. herbicola JCM 7000. The sequence identity between their 16S rDNAs was 99.4%; the result of DNA-DNA hybridization test (Table 1) confirmed the identity of these bacteria. Er, herbicola JCM 7000 exhibited 100% DNA binding to bacterium T (strain 191). The results of API 50 CHE testing showed a close relationship of bacteria X and W to the genera Enterobacter, Serratia, Klebsiella and Erwinia, however, no species showed a higher DNA-DNA homology index than 30% (data not shown).

Phylogenetic analysis of Gram positive bacteria
The 16S rDNA nucleotide sequences of aphid Gram-positive gut bacteria were compared with homologous sequences of Bacillus and Staphylococcus species listed

Fig. 2. A consensus neighbor joining tree of y subdivision of the class
Proteobacteria based on 16S rDNA sequences.
The numerals at the forks indicate the percentage of times the species to the right
of the fork grouped together in bootstrap trees. The branch length is scaled below the
tree. 1, Enterobacteriaceae; 2, Pasteurellaceae; 3, Vibrio; 4, Aeromonas; 5, Pseudo-monadaceae.

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Fig. 3. A consensus neighbor-joining tree based on 16S rDNA sequences of
bacteria T, W, X and selected members of the family Enterobacteriaceae.
The branch length is scaled below the tree. The numerals in each tree indicate the
bootstrap (%) scores.

Fig. 4. A consensus neighbor joining tree based on 16S rDNA sequences of
bacteria V, Y, Z and Gram-positive bacteria selected from the GenBank database based on homology search.
The branch length is scaled below the tree. The numerals in each tree indicate the bootstrap (%) scores.

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in the GenBank database. The phylogenetic tree of Gram-positive bacteria (Fig. 4) demonstrated that bacteria V, Y, and Z were related to Bacillus thuringiensis (99.8% (sequence identity)), Staphylococcus saprophyticus (98.2%), St. sciuri (99.8%), respectively.

DISCUSSION

Endosymbioses with microorganisms are virtually universal phenomena that are observed widely in eukaryotes, though the degrees of interdependency vary greatly. For example, mitochondria and chloroplasts are considered to be the most successful bacterial symbionts (10). There is no more dynamic mechanism that promotes evolution than endosymbiotis, because one organism can acquire a new way of metabolism owned by a distinct organism, which, in turn, enables it to push into novel ecological niches (1). Therefore, to investigate mechanisms underlying the establishment of the endosymbiotic association is very important in order to understand the origin and diversity of eukaryotes.
Although the endosymbiotic partnership of the aphid and its symbionts seems
to be very stable, and the symbionts, Buchnera, are no longer able to live outside the aphid cells, their ancestor must have been a free-living bacterium. In addition, this stable partnership can be frequently challenged by other microorganisms. Addi-tional bacterial, or "secondary" symbionts, are found in many lineages of aphids (1), suggesting repeated acquisition of free living microbes through gut. In this context, it is significant to look into the microbial flora of the aphid gut for a better understanding of insect-microbe interactions.
In the present study, we found seven bacterial groups (groups T, U, V, W, X,
Y, and Z) as major flora of aphid gut. Bacteria T, X, W, and U were Gram-negative rods, whereas Y, Z, and V were Gram-positive bacteria. Among Gram-negative bacteria groups T, W, and X were identified as members of the family Enterobacteriaceae based on the physiological characterization. The three groups were included in the same cluster together with the members of the family Enterobacteriaceae in the phylogenetic trees (Figs. 2 and 3). Buchnera were also closely related to the family Enterobacteriaceae (19). However, Buchnera formed a quite unique branch which is located intermediate between the Enterobacteri-aceae and Vibrionaceae-Pasteurellaceae clusters. This result suggested that there are no close relatives to Buchnera among extant bacteria including aphid gut bacteria, one of which is supposed to share a common ancestor with Buchnera when judged from its habitat. This result also supports the idea that an ancestor of Buchnera was acquired by the host after the common ancestor of the family Enterobacteriaceae and Buchnera had diverged from the ancestors of the family Pasteurellaceae and Vibrionaceae, but before the family Enterobacteriaceae diversified.
Precise identification was carried out by the DNA-DNA hybridization method
for groups T, W, and X. From DNA-DNA hybridization, group T was definitively identified as Er. herbicola. However, there were no close relatives to the groups W

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and X, which showed homology indices higher than 30%. These results suggested that new species should be established for the two groups.
Er. herbicola is generally found on plant surfaces, suggesting that Buchnera have been derived from a habitant of plants on which host insects feed. Although we did not examine the nitrogen-fixing ability of group T strains (Er. herbicola), it is known that some strains of Er. herbicola are capable of nitrogen fixation (16). Er. herbicola is also known to produce herbicolin (20), an antibiotic against fungi. Those special abilities of Er. herbicola must be profitable for the host insects. Actually termites contain nitrogen fixing bacteria in their gut that belong to En terobacter (2).
Both bacteria X and T, in common, are able to reinfect aphids which have been maintained free of microbes (H. Harada and H. Ishikawa, unpubl.). This ability is probably essential to establish the symbiotic association with the host insects. In spite of these similarities between bacteria X and T, the former is a predominant species in the aphid gut while the latter is not. In view of its predominancy, bacterium X seems to be adapted better than bacterium T to the gut environment. Among Gram-positive bacteria, groups Y and Z were identified as Staphylococ-cus species. Most aphids contained bacterium Y (Staphylococcus sp.) together with bacterium X (member of enterobacteria), although the number of bacterium Y in the gut is much smaller than that of bacterium X (5). It was observed that bacte-rium X, when reinfected by itself, grows infinitely in the aphid gut and leads to the death of the host (data not shown). Since aphids found in nature look normal with a large number of bacterium X in their gut, it is supposed that the balance of various groups of bacteria may be very important in the micro flora of the aphid gut to keep the host insect normal. It is known that, in the termite gut, Staphylo-coccus represents a major bacterial genus together with Enterobacter (2). While the reason for predominancy of Staphylococcus in the insect gut is not clear, the inter-action between gut Staphylococcus spp. and insects is a very interesting subject. Bacterium V was shown to be a close relative of Bacillus thuringiensis (Fig. 4),
the sequence identity between their 16S rDNAs being 99.8%. Although we did not perform the DNA-DNA homology test between bacterium V and B. thuringiensis, judging from its very high sequence identity of 16S rDNA, it is quite reasonable to identify the bacterium V as B. thuringiensis. B. thuringiensis is well known for its production of b-endotoxins that break the gut of various insects. It is also known that those toxins show specificity to certain insect groups (7). If bacterium V produces a d-endotoxin, this ability would have a very important ecological role in that the toxin kills predatory insects to aphid.

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