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Symbiotic Bacteria Associated with Stomach Discs of Human Lice[†]

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The symbiotic bacteria associated with the stomach disc, a large aggregate of bacteriocytes on the ventral side of the midgut, of human body and head lice were characterized. Molecular phylogenetic analysis of 16S rRNA gene sequences showed that the symbionts formed a distinct and well-defined clade in the *Gammaproteobacteria*. The sequences exhibited AT-biased nucleotide composition and accelerated molecular evolution. In situ hybridization revealed that in nymphs and adult males, the symbiont was localized in the stomach disc, while in adult females, the symbiont was not in the stomach disc but in the lateral oviducts and the posterior pole of the oocytes due to female-specific symbiont migration. We propose the designation “*Candidatus Riesia pediculicola*” for the louse symbionts.

Sucking lice (Insecta: Phthiraptera: Anoplura), embracing over 500 described species in the world, are ectoparasitic insects that feed exclusively on mammalian blood (9). There are two closely related species of human lice: the body louse, *Pediculus humanus*, lives in clothes and feeds from the body, and the head louse, *Pediculus capitis*, lives in the hair and feeds from the scalp. The head louse and the body louse are morphologically and genetically very similar, and some researchers regard them as subspecies or ecotypes of the same species (7, 16, 22).

Vertebrate blood is certainly nutritious, but it is deficient in some nutritional components, such as B vitamins, which is probably the reason why insects exclusively living on vertebrate blood throughout their lives, including tsetse flies, louse flies, bedbugs, assassin bugs, and lice, are generally in close association with endosymbiotic microorganisms (6). In the 1920s, human lice were first reported to possess a large aggregate of bacteriocytes, called the stomach disc, on the ventral side of the midgut, in which rod-shaped symbiotic bacteria are harbored (5, 23). Since then, a number of histological (11, 20), embryonic (4, 20), experimental (1, 2, 3, 10), and nutritional (18, 19) studies have been conducted on the endosymbiotic system of human lice. These studies demonstrated that the symbiont is vertically transmitted from the maternal stomach disc to developing oocytes through a peculiar passage (11, 20), is essential for the survival and growth of the host (3), and provides the host with B vitamins that are lacking in the blood meal (19). Despite the substantial body of early work on louse endosymbiosis, the microbial nature of the symbionts is still

unknown. Hence, we characterized the symbiotic bacteria of human lice by using molecular phylogenetic and histological approaches.

We mainly used a long-established inbred line of the human body louse, strain NIID, which has been maintained in the laboratory since 1954 (26). The insects were kept in an evaporating dish with pieces of felt sealed in a plastic container with silica gel, reared at 30°C in constant darkness, and fed with human blood once a day on the arm of one of the authors (M.M.). The insects at different developmental stages were sampled and preserved in acetone until molecular and histological analyses were performed (13). Samples of body lice from 12 different sources and head lice from 4 different sources were also collected in either Japan or Nepal.

DNA was individually extracted from adult females of strain NIID by using a QIAamp DNA minikit (QIAGEN). A 1.5-kb segment of the eubacterial 16S rRNA gene was amplified with the primers 16SA1 (5'-AGAGTTTGATCMTGGCTCAG-3') and 16SB1 (5'-TACGGYTACCTTGTTACGACTT-3') and subjected to cloning, restriction fragment length polymorphism genotyping, and DNA sequencing as previously described (14).

More than 10 clones of the 16S rRNA gene segment from each of the samples exhibited identical restriction fragment length polymorphism patterns (data not shown), suggesting that a single bacterial species is dominant in the insects. The nucleotide sequences of some of these clones, which were 1,482 bp in size and identical to each other, were determined. A BLAST search clearly showed that the sequence belongs to the *Gammaproteobacteria* in the family *Enterobacteriaceae*. No closely related sequences were identified in the DNA databases: the highest hits were *Providencia stuartii* (AF008581; 89.6% [1,141/1,274] sequence identity) and *Enterobacter hormaechei* (AJ853889; 89.0% [1,119/1,258] sequence identity).

In order to confirm whether the 16S rRNA gene sequence was derived from the symbiotic bacteria in the stomach disc, we designed an oligonucleotide probe specific to the sequence, Cy3-Lice1255R (5'-Cy3-TTGGCTCGCTTACGAGT-3'),

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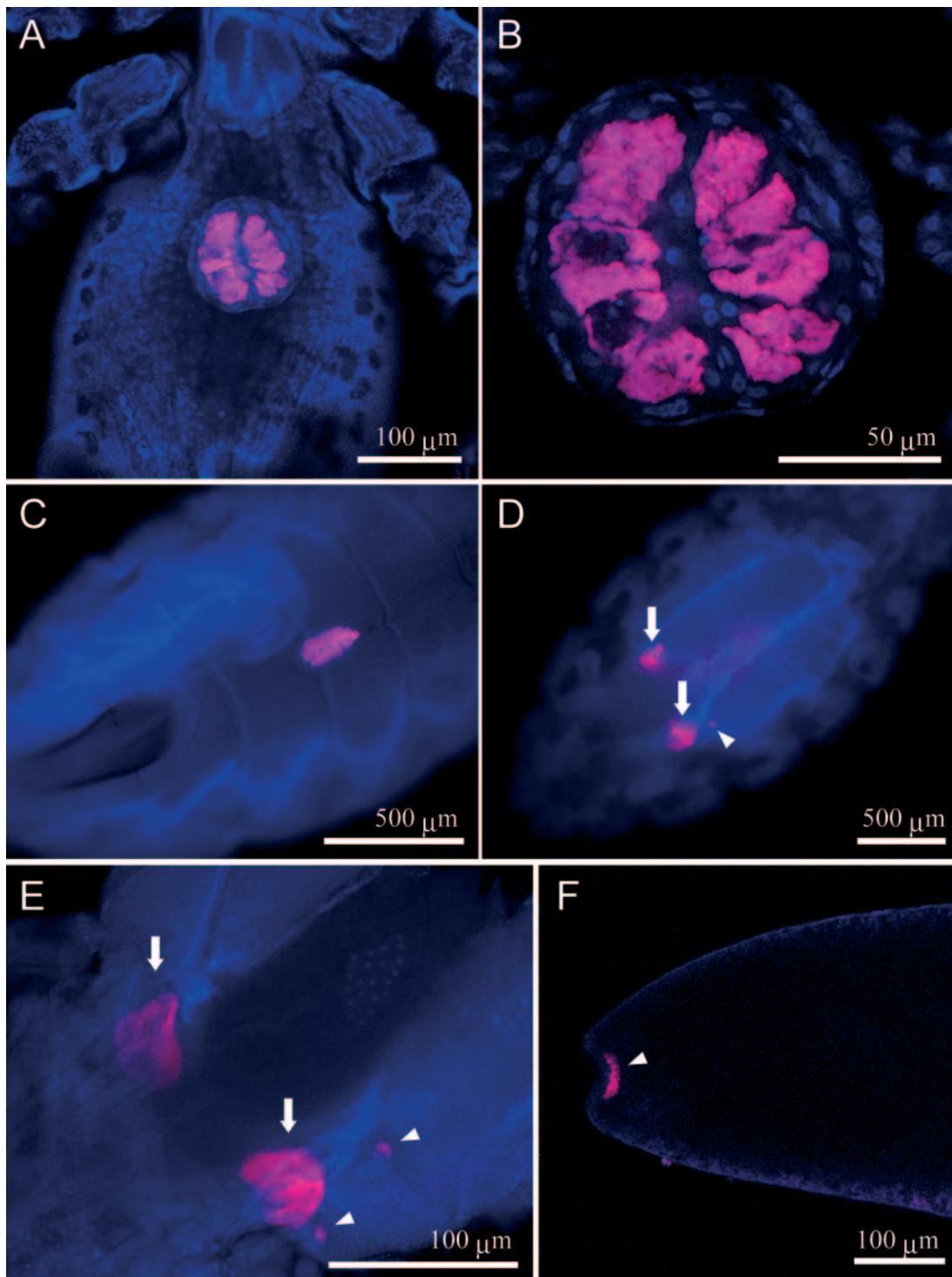


FIG. 1. Whole-mount *in situ* hybridization of symbiotic bacteria in human body lice. (A) A first-instar nymph with a large round stomach disc at the center of the ventral abdomen. The symbiont cells are specifically detected in the stomach disc. (B) An enlarged image of the stomach disc. The location of the symbiont cells exhibited radial compartment-like structures. (C) An adult male, in which the symbiont is localized in the stomach disc. (D) An adult female, in which the symbiont is not detected in the stomach disc but in the lateral oviducts and the posterior poles of oocytes. (E) An enlarged image of the female reproductive organs. (F) An enlarged image of an oocyte. The arrows and arrowheads indicate the lateral oviducts and the posterior poles of oocytes, respectively.

for whole-mount fluorescent *in situ* hybridization (FISH). After their legs were removed to facilitate infiltration of reagents, the insects, preserved in acetone, were fixed in Carnoy's solution (chloroform-ethanol-acetic acid [6:3:1]) overnight and incubated with 6% H₂O₂ overnight to quench the autofluorescence of the insect tissues. The insects were thoroughly washed and equilibrated with a hybridization buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 30% formamide), and the Cy3-Lice1255R probe and SYTOX green were added at final concentrations of 100 nM and 0.5 μM, respectively. After overnight incubation, the samples were thoroughly washed in washing buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% sodium dodecyl sulfate) and were observed under an epifluorescent microscope (Axiophot; Carl Zeiss) and a laser confocal microscope (PASCAL5; Carl Zeiss).

In nymphal insects, the probe specifically hybridized with a round stomach disc located in the ventral abdomen (Fig. 1A). In the stomach disc, the signals showed radial compartment-like structures (Fig. 1B), which had been described as the locations of the symbiotic bacteria (4, 11, 20). A three-dimensional FISH movie image of the stomach disc is available in the supplemental material. A probe control experiment and a competitive suppression control experiment with excess unlabeled Lice1255R probe (14) did not identify these signals, confirming the specificity of the FISH detection (data not shown). These results indicated that the 16S rRNA gene sequence was certainly derived from the symbiotic bacteria harbored in the stomach disc.

In adult males, the signals were localized in the stomach disc (Fig. 1C). In adult females, however, the signals were not detected in the stomach disc but in the lateral oviducts and the posterior poles of oocytes in ovarioles (Fig. 1D to F). Ries (20) and Eberle and McLean (11) reported that in the last (i.e., third) nymphal instar, the symbiont cells escape from the stomach disc, migrate to the lateral oviducts, infect the ovarian epithelium, and are transmitted to the oocytes through a specialized tissue organization called ovarian ampullae. The results of whole-mount FISH were in accordance with these histological descriptions.

We also cloned and sequenced the eubacterial 16S rRNA gene segments from four body lice and two head lice from different sources. All of the sequences were very similar to each other, 1,482 bp in size, with sequence identities ranging from 99.1% to 99.9%. The nucleotide compositions of the sequences were remarkably AT biased, ranging from 50.6% to 51.0%. These AT content values were higher than those of related free-living bacteria, such as *P. stuartii* (AF008581; 46.9%) and *E. hormaechei* (AJ853889; 44.9%), and were equivalent to those of obligate endosymbiotic bacteria of other insects, such as *Buchnera* spp. of aphids (e.g., AJ296751; 51.3%) and *Wigglesworthia* spp. of tsetse flies (e.g., AB063521; 51.5%).

These 16S rRNA gene sequences were subjected to molecular phylogenetic analysis, together with the sequences of related gammaproteobacteria that exhibited high BLAST scores in the DNA database search. A multiple alignment of the sequences was generated by using the program package Clustal W (24). Aligned nucleotide sites containing a gap were removed from the data set, and the final alignment was inspected and corrected manually. A neighbor-joining tree, with 1,000

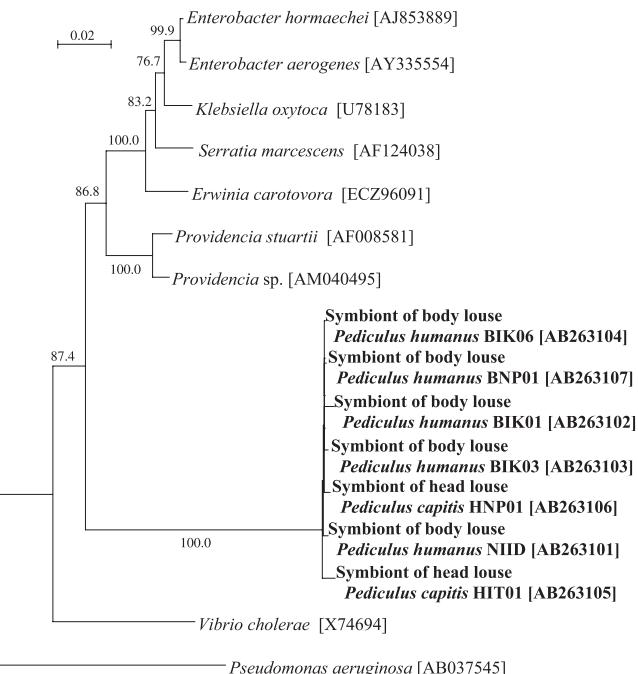


FIG. 2. Molecular phylogenetic analysis of 16S rRNA gene sequences of the stomach disc symbionts from human lice. A neighbor-joining tree is shown; maximum-parsimony analysis gave essentially the same result (data not shown). Bootstrap values higher than 70% are shown at the nodes. In brackets are sequence accession numbers.

bootstrap resamplings, was also constructed by the program package Clustal W (24).

Figure 2 shows the neighbor-joining phylogeny on the basis of 1,408 unambiguously aligned nucleotide sites. The louse symbionts formed a distinct monophyletic group with 100% bootstrap support, constituting a basal lineage in the γ-subclass of the *Proteobacteria*. The symbionts of body lice could not be phylogenetically differentiated from the symbionts of head lice, probably reflecting the close morphological and genetic relatedness between the body louse and the head louse (7, 16, 22). No endosymbiotic bacteria of other insects showed phylogenetic affinity to the louse symbionts.

In comparison with the related free-living bacteria, the louse symbionts exhibited remarkably elongated branches on the phylogenetic tree (Fig. 2), which was suggestive of accelerated molecular evolution in the lineage of the louse symbionts. Thus, we performed a relative-rate test based on genetic distances estimated under Kimura's two-parameter model (15). Statistical significance was evaluated by using the program package RRTree (21). The following 16S rRNA gene sequences were subjected to the analysis: AB263101 from a symbiont of the body louse, AB263105 from a symbiont of the head louse, AF008581 from *Providencia stuartii*, AM040495 from *Providencia* sp., and X74694 from *Vibrio cholerae* as the outgroup. The evolutionary rate of the 16S rRNA gene in the lineage of the louse symbionts was around 3.1 times faster than those in the lineages of related free-living gammaproteobacteria. The difference was highly significant statistically ($P = 10^{-7}$).

Recent molecular evolutionary analyses revealed that the

lifestyle of obligate insect endosymbionts has strongly affected their genome evolution, causing an AT-biased nucleotide composition, an accelerated rate of molecular evolution, and significant genome reduction. These peculiar genetic traits are hypothesized to be the consequences of attenuated purifying selection due to small population size and a strong bottleneck associated with the endosymbiotic lifestyle (17, 25). The AT bias and the accelerated evolution in the 16S rRNA gene sequences are also suggestive of a stable and intimate host-symbiont association in human lice over evolutionary time.

How prevalent is the symbiont in the louse populations? We examined 57 body lice from 12 different sources and 9 head lice from 4 sources for symbiont infection. For confirmation of successful DNA preparation from each of the samples, the mitochondrial 16S rRNA gene of the host insect was amplified with the primers MtrA1 (5'-AAWAAACTAGGATTAGATA CCCTA-3') and MtrB3 (5'-ACACTTCCAGTACAYTTAC TTTGT-3') under a temperature profile of 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, 48°C for 30 s, and 65°C for 3 min. Diagnostic PCR of the symbiont was performed with the primers 16SA3 (5'-TGCATGGYTGTCGTCAGCTCG-3') and Lice1255R under a temperature profile of 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, and 70°C for 1 min. The diagnostic PCR analysis identified the symbiont in all the louse samples examined. Thus, it was strongly suggested that the symbiont infection is fixed in the louse populations, corroborating the essential biological roles of the symbiont for the host insect (3, 19).

From all these results taken together, it was concluded that the symbiotic bacteria associated with the stomach disc of the human louse constitute a phylogenetically distinct and coherent bacterial group in the *Gammaproteobacteria*. Hence, we propose the designation "*Candidatus Riesia pediculicola*" for the symbiont of human lice. The generic name honors Erich Ries, who first comprehensively investigated the endosymbiotic system in lice (20). The specific name indicates the association with lice.

Human lice are notorious as medical and hygienic pests. The body louse is the vector of the epidemic typhus pathogen *Rickettsia prowazekii*, the relapsing fever pathogen *Borrelia recurrentis*, and the trench fever pathogen *Bartonella quintana* (12). The head louse has recently been reemerging even in advanced countries, particularly among school children, and its resistance to insecticides is increasing (8). Considering the essential roles of the symbiont for the host (3, 19), microbiological studies of the louse symbionts would lead to new means of control for the pests. Other than human lice, a number of lice are known from a wide variety of mammals, and it has been histologically reported that the localizations, morphologies, and life cycles of symbiotic bacteria in animal lice are distinct from those of human lice in many respects (6, 20). Whether the symbionts of animal lice are phylogenetically related to the symbionts of human lice is of evolutionary interest.

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