STUDIES ON CULTURED AND UNCULTURED MICROBIOTA OF WILD CULEX QUINQUEFASCIATUS MOSQUITO MIDGUT BASED ON 16S RIBOSOMAL RNA GENETIC ANALYSIS

VYANKATESH J. PIDIYAR,* KAMLESH JANGID,* MILIND S. PATOLE, and YOGESH S. SHOUCHE
Molecular Biology Unit, National Centre for Cell Science, University of Pune, Pune, India

Abstract. To describe the midgut microbial diversity and the candidate bacteria for the genetic manipulation for the generation of transgenic mosquitoes refractory to transmission of diseases, the microbiota of wild Culex quinquefasciatus mosquito midgut was studied using a conventional culture technique and analysis of a 16S ribosomal RNA (rRNA) gene sequence library. The culturable microbiota was identified as Acinetobacter junii, Ac. calcoaceticus, Aeromonas culicicola, Bacillus thuringiensis, Microbacterium oxydans, Pantoea agglomerans, Pseudomonas aeruginosa, Staphylococcus epidermidis, Stenotrophomonas maltophilia and an unidentified bacterium from the host Drosophila paulistorum. The 16S rRNA gene library was composed of 46% unidentified and uncultured bacteria, 41% Acinetobacter spp., and 13% Lactococcus spp. The coverage calculated for the 150 clones was 83.3%. Thus, the probability of the next cloned sequence falling in a novel operational taxonomic unit (not yet observed) was 16.7%. The majority of the cultured isolates and the 16S rRNA gene library clones belonged to the γ-proteobacteria class. Most of the bacteria have been previously reported to inhabit the midgut of different mosquito species. Therefore, the results of this study indicate that different mosquito species harbor common representatives of the microbiota that may be the potential candidates for genetic manipulation to control the disease transmission capabilities of the host.

INTRODUCTION

Mosquitoes are medically important arthropod vectors. Culex quinquefasciatus is a mosquito vector for Wuchereria bancrofti, the filarial worm that causes filariasis, which is one of the six important tropical diseases. Approximately 80 million people are infected globally, and of these 100 million chronic cases manifest as typical elephantiasis.1 The disease is highly prevalent in developing countries such as India. Moreover, Japanese encephalitis virus has been isolated from field-collected Cx. quinquefasciatus. This makes Cx. quinquefasciatus an important vector species.

Mosquito control still remains the primary strategy for controlling mosquito-borne diseases. Insecticide resistance of mosquitoes, drug resistance of parasites, cost of new drug development, limitations of vaccines, and environmental hazards of pesticide application all necessitate the need for the development of novel disease control strategies. There have been attempts to generate transgenic mosquitoes refractory to transmission of diseases, but the genetic mechanisms of refractoriness are poorly understood and are at times multigenic.2–6 An alternative approach to manipulating the mosquito genome might be to use the normal bacterial symbionts of the mosquito midgut. Genetic manipulation could be done in these species for which well-studied gene transfer mechanisms are available. Cecropin expressed in the endosymbiont of different mosquito species for which well-studied gene transfer mechanisms are available. Cecropin expressed in the endosymbiont of different mosquito species may be to use the normal bacterial flora in the mosquito midgut increased the microbial community structure of the mosquito midgut. The microbial community of the mosquito midgut might be to use the normal bacterial symbiont of the mosquito midgut. Genetic manipulation could be done in these species for which well-studied gene transfer mechanisms are available. Cecropin expressed in the endosymbiont bacterium Rhodococcus rhodnii by genetic manipulation was found to block transmission of Trypanosoma in sand flies.3 For such strategies to be successful, an understanding of the microbial community structure of the mosquito midgut is necessary, which will enable us to identify the organisms that participate significantly in the maintenance of the community and are predicted to play significant roles in the environmental chemistry. It was previously reported that a reduction in the normal bacterial flora in the mosquito midgut increased Plasmodium falciparum infection rates in experimentally infected Anopheles mosquitoes.7 A better understanding of interactions between midgut bacteria and malaria parasites in wild mosquito populations could explain how the vector potential for malaria parasite transmission is modulated by environmental factors such as acquisition of different types of bacteria.

Little is known about the normal midgut microbiota of mosquitoes. In early 1960s, a few studies were carried out on midgut microbiota of laboratory-bred species of Culex.8–10 The presence of oxidase-positive bacteria from the midgut of anopheline mosquitoes has been reported.11,12 The successful colonization of Serratia marcescens in laboratory-bred Anopheles stephensi has also been reported.13 A study of wild Aedes triseriatus, Cx. pipiens, and Psorophora columbiae using routine laboratory bacteriologic techniques indicated the presence of S. marcescens, Klebsiella ozonae, Pseudomonas aeruginosa, and Enterobacter agglomerans.6 A study of the midgut of Cx. quinquefasciatus larvae indicated the presence of bacteria represented by Bacillus spp., Staphylococcus spp., and Pseudomonas spp., while Aspergillus and Streptomyces spp. represented the fungal and actinomycete inhabitants, respectively.9,14 The available conventional culture techniques limit the isolation and identification of all the components of the microbiota inhabiting any niche or any environmental sample since it is not possible to simulate the conditions for their growth under laboratory conditions.15 Ribosomal RNA (rRNA)–based sequence studies of environmental organisms can identify the abundant organisms in the environments studied. All necessitate the need to apply rRNA-based analyses for this purpose.

We previously reported the isolation and taxonomic characterization of a new species, Aeromonas culicicola Microbial Type Culture Collection (MTCC) 3249 from the midgut of Cx. quinquefasciatus, and two strains of A. culicicola (SH and SLH) from Aedes aegypti during the same study,16 indicating that different mosquito species in the same environment may harbor common representatives of the microbiota. In this report, we studied the midgut microbiota of wild Cx. quinquefasciatus using both culture-dependent and culture-independent techniques.
MATERIALS AND METHODS

Collection of mosquito species and isolation of bacterial flora from midgut. Wild *Cx. quinquefasciatus* were collected from surroundings of the National Centre for Cell Science (Pune, Maharashtra, India) and brought live to the laboratory in cages. Only the females were selected for further studies. The species identity of the collected mosquitoes was confirmed using sequencing of the mitochondrial 12S and 16S rRNA genes as previously described. Those females containing blood bolus within the midgut were considered to have taken a blood meal prior to collection because the blood bolus is visible for hours after a blood meal. During three independent collections, 20–30 mosquitoes were collected each time. Mosquitoes were fed (time not exceeding 2–3 hours) on a sterile glucose solution until dissected. The dissection was done under sterile conditions after surface sterilization with 70% ethanol as previously reported. The midgut contents were suspended in 500 μL of 0.85% (w/v) NaCl. A 100-μL aliquot of these contents was serially diluted up to 10^{-6} and plated onto blood agar base (HiMedia, Mumbai, India) with 5% (v/v) human blood and incubated at 30°C for 18–24 hours. The sterility of all reagents was checked during the entire procedure.

Amplification and cloning of 16S rRNA gene. Chromosomal DNA was isolated from the remaining midgut contents by the standard phenol/chloroform extraction method. The 1.5-kilobase partial sequence of the 16S rRNA gene was amplified from the pooled chromosomal DNA representing all the samples using a polymerase chain reaction (PCR) and universal Eubacteria-specific primers 16F27 (5'-CCA GAG TTT GAT CMT GGC TCA G-3') and 16R1525XP (5'-TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC-3'). The PCR conditions used were an initial denaturation at 94°C for two minutes, followed by 35 cycles of denaturation at 95°C for one minute, annealing at 55°C for one minute, and extension at 72°C for one minute, and a final extension at 72°C for 10 minutes.

The amplified PCR product was a mixture of 16S rRNA genes from all the Eubacteria present in the midgut contents of the mosquito. To study these genes individually, a 16S rRNA gene library was constructed using pGEM-T Easy vector (Promega, Madison, WI) in *Escherichia coli* DH5α. Plasmid DNA preparations from all the clones positive for the 1.5-kilobase insert were made as previously described and sequenced on an ABI310 automated DNA sequencer using the Big Dye terminator kit (Applied Biosystems, Inc., Foster City, CA) as previously described. The identification of the isolates from blood agar plates was done by 16S rRNA gene sequencing after preliminary biochemical identification. The amplified 16S rRNA gene PCR products from these isolates were directly sequenced after purification by precipitation with polyethylene glycol and NaCl. The primers used to obtain the complete sequence of 16S rRNA gene of the isolates were the same as for PCR amplification (16F27N and 16R1625XP). An internal primer (16F536, 5'-GTG CCA GCA GCC GCG GTR ATA-3') was also used in addition to the other primers. The sequencing of the 16S rRNA gene insert from the clones was done using the 16F27N primer. The 5'-terminus, a 500-nucleotide region of the 16S rRNA gene, contains variable regions (positions approximately 100-200) that not only discriminate between closely related species, but in most cases will also underestimate the degree of sequence similarity of nearly complete sequences by 1–12% for strains belonging to different phyla.

Sequence analysis. An equal portion (approximately 500 basepairs) of the 16S rRNA gene (E. coli positions 67 to 572, Accession number J01859), was used for sequence analysis at the Ribosomal Database Project (RDP II; Michigan State University, East Lansing, MI) and the National Center for Biotechnology Information (Bethesda, MD) (http://www.ncbi.nlm.nih.gov/BLAST). The similarity matrix was prepared using the similarity matrix calculator at the RDP II site. The presence of chimeric sequences was checked with the RDP Chimera Check program and by comparing independently the alignments at the beginning of each sequence and at the end of each sequence and the alignments of the entire sequence. The alignment of all the 16S rRNA gene sequences was done using CLUSTAL W program at the European Bioinformatics server (European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, United Kingdom) (http://www.ebi.ac.uk/clustalw) to determine similarity value and distance matrix. The phylogenetic tree was constructed using 500 basepair aligned sequences by the neighbor joining method using Kimura 2 parameter distances in MEGA 2.1 software. It was reported earlier that phylogenetic trees based on partial sequences have the same topologies as those based on a complete sequence with the established groups being identical but some deep branches differing slightly. Furthermore, sequences of 500 nucleotides are sufficient for placement if some longer sequence is closely related (> 90% identity in homologous nucleotides) to the query sequence.

An operational taxonomic unit (OTU) or molecular species, as used here, consisted of all sequences with less than 2% divergence from the aligned homologous nucleotides. This threshold was based on earlier conclusion that “the sequence divergence of clones belonging to the same OTU [is] generally low (between 2 and 0%)” and was also generally consistent with the results of the comparison of 16S rRNA similarity and DNA-DNA reassociation values.

Coverage was calculated by Good’s method, according to which the percentage of coverage was calculated with the formula \[ 1 - \frac{n(N-n)}{N(N-1)} \times 100, \] where \( n \) is the number of molecular species represented by one clone (single-clone OTUs) and \( N \) is the total number of sequences. The sequences of the isolates from the culture-dependent study were deposited in GenBank with accession numbers AF170914, AF417863, AF417866, AF417868, AF417870, AF417872, AF417874, AF417876, AF417877, and AY057450, whereas sequences of clones from the library were deposited with accession numbers AY119288 to AY119440, and AY332749 to AY332756.

RESULTS

Isolation and 16S rRNA sequence analysis of strains. Direct plating of the mosquito midgut contents was used for the isolation of the culturable microbiota. The colonies on blood agar were selected on the basis of conventional bacteriological techniques, and initially those with minor variations were also picked for further isolation. The initial number of 32 isolates was reduced to 10 after a first round of screening based on colony characteristics (involving colony size, shape, color, margin, opacity, elevation, and consistency) and the
morphology of isolates studied by Gram staining and motility by the hanging drop technique. To determine the phylogenetic relatedness of the strains, the isolates were subjected to analysis with the complete 16S rRNA gene sequence.

Analysis with the 16S rRNA gene sequence identified the 10 different bacterial isolates as *Acinetobacter junii*, *Ac. calcoaceticus*, *Bacillus thuringiensis*, *Microbacterium oxydans*, *Pantoea agglomerans*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Stenotrophomonas maltophilia*, and an unidentified bacterium of the host *Drosophila paulistorium*, the details of which are shown in Table 1. The tenth isolate, which showed a 98.2% similarity with close relatedness to *A. jandaei* and an increased count (2,000-fold) after a blood meal, was found to be a novel species of the genus *Aeromonas* and was characterized in detail in a separate study as *A. culicicola*.

The majority of these isolates have been previously reported to inhabit the midgut of *Cx. pipiens* and *Psorophora columbiae*. Furthermore, seven of the 10 isolates belonged to the γ-proteobacteria class, emphasizing that the majority of the mosquito midgut microbiota are composed of bacteria belonging to this class. The percent similarity between the isolated sequences and the type strains in the database was mostly greater than 97%, except for a strain related to *Pantoea agglomerans* JCM 1236 (95.8%). Phylogenetic grouping of the isolates was tested with the respective representatives and the type strains in the database was done using 500-basepair aligned homologous nucleotide sequences. The phylogenetic analysis of the remaining 150 clones (Table 2) was done using 500-basepair aligned homologous nucleotide sequences. The percentage distribution of the clones from the 16S rRNA gene library representing the microbiota of the midgut of *Cx. quinquefasciatus* was determined and is shown in Table 2. On the basis of sequence similarity to the existing GenBank database entries, the clones were clustered together to form three major groups: the *Acinetobacter* group, the *Lactococcus* group, and the unidentified and uncultured bacteria group. The last group included all the uncharacterized and as yet uncultured bacteria. The coverage calculated for the 150 clones was 83.3%. Thus, the probability of the next cloned sequence falling in a novel OTU (not yet observed) was 16.7%. This value gives an estimation of how well the clones analyzed account for the biodiversity within the original sample by the present methodology (i.e., with our PCR conditions and primer set).

Sixty-two clones (41%) belonged to *Acinetobacter* group and all except three clones had similarity values of more than 98%. Of these, 60 clones showed highest similarity to a single *Acinetobacter* strain (AJ410290), whereas the remaining two were found related to another *Acinetobacter* strain (AY055373). Among the clones that showed similarity to the cultured and identified bacteria, this was the largest group. These results correlated with the culture studies since two species of *Acinetobacter* were isolated from the midgut. There were only three one-clone OTUs within the *Acinetobacter* group; thus, the percentage of coverage in this group was high (95.2%). The phylogenetic analysis of the 62 clones along with the two *Acinetobacter* isolates from the culture study and the nearest neighbor database entries is shown in Figure 1.

The unidentified and the uncultured group comprised 46% (69 clones) of the total 16S rRNA gene library with similarity values ranging from 84.8% to 100% with the sequences in the database. An initial taxonomic analysis of all the clones that belonged to this group showed their affiliation to the family Proteobacteria of the domain bacteria with higher relatedness to the γ-proteobacteria class. Four different clusters, depending upon the nearest neighbor in the database, represented the group. The majority of the clones showed highest similarities with the isolates from the deepest sea mud of the Mariana Trench. Thirty-two (22%) clones showed similarities to different isolates of unidentified γ-proteobacteria with similarities between 88% and 100%. Twenty (13%) clones showed similarity to unidentified bacteria from activated sludge and 12 of these clones had more than 98% similarity, whereas for the remaining ones, the values were below 95%. Seventeen (11%) clones showed similarities to sequences representing uncultured bacteria. Eleven of the 17 clones showed maximum similarity to a clone from the marine environment. Of these 11, six clones showed more than 98% sequence similarity and the remaining five clones showed similarities between 97% and 89%. The remaining six clones of the uncultured bacteria group showed similarities to uncultured soil bacteria. In all, there were 21 single-clone OTUs; therefore the coverage for the group was less (70%).

### Table 1

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>Accession number</th>
<th>Nearest phylogenetic neighbor</th>
<th>Accession number</th>
<th>% Similarity</th>
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<tbody>
<tr>
<td>A</td>
<td>AF417863</td>
<td><em>Acinetobacter junii</em></td>
<td>X81664</td>
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<tr>
<td>E</td>
<td>AF417877</td>
<td>Unidentified bacterium</td>
<td>U20277</td>
<td>99.0</td>
</tr>
<tr>
<td>F</td>
<td>AY057450</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>D83632</td>
<td>99.3</td>
</tr>
<tr>
<td>G</td>
<td>AF417866</td>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>AJ293470</td>
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<td>H†</td>
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<td><em>Aeromonas jandaei</em></td>
<td>AF099025</td>
<td>98.2</td>
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<tr>
<td>P</td>
<td>AF417868</td>
<td><em>Microbacterium oxydans</em></td>
<td>Y17227</td>
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<td>AF417870</td>
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<td>AF417872</td>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>AF159045</td>
<td>97.8</td>
</tr>
<tr>
<td>7c</td>
<td>AF417874</td>
<td><em>Bacillus thuringiensis</em></td>
<td>AF155955</td>
<td>98.7</td>
</tr>
<tr>
<td>9</td>
<td>AF417876</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>AF094713</td>
<td>98.9</td>
</tr>
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</table>

* The sequence analysis was done at the Ribosomal Database Project (Michigan State University, East Lansing, MI) and the National Center for Biotechnology Information (Bethesda, MD) (http://www.ncbi.nlm.nih.gov/BLAST) and is based on the complete 16S rRNA gene sequence.

† Isolate H was completely characterized as *Aeromonas culicicola* Microbial Type Culture Collection 32497 in a separate study.
FIGURE 1. Phylogenetic affiliation of the members of the Acinetobacter group from the midgut of *Culex quinquefasciatus* based on a partial sequence of the 16S ribosomal RNA gene. The sequence alignment was performed using the CLUSTAL W program and the tree was generated using the neighbor joining method with Kimura 2 parameter distances in MEGA 2.1 software. Only positions 67-572 (*Escherichia coli* numbering) were considered for the alignment. Numbers at nodes indicate percent bootstrap values above 50 (1,000 replicates). The bar indicates the Jukes-Cantor evolutionary distance. Names in **bold** represent isolated strains and the nearest neighbor obtained in Blast analysis.

FIGURE 2. Phylogenetic affiliation of the members of the γ-proteobacteria group from the midgut of *Culex quinquefasciatus* based on a partial sequence of the 16S ribosomal RNA gene. The sequence alignment was performed using the CLUSTAL W program and the tree was generated using the neighbor joining method with Kimura 2 parameter distances in MEGA 2.1 software. Only positions 67-572 (*Escherichia coli* numbering) were considered for the alignment. Numbers at nodes indicate percent bootstrap values above 50 (1,000 replicates). The bar indicates the Jukes-Cantor evolutionary distance. Names in **bold** represent isolated strains and the nearest neighbor obtained in Blast analysis. *Aeromonas culicicola* is isolate H from the present study reported elsewhere. (10)
The phylogenetic analysis of the group along with the isolates from the culture-based study belonging to the γ-proteobacteria class is shown in Figure 2.

The third group of the 16S rRNA gene library along with the remaining three isolates from the culture studies belonged to the Firmicutes group (Gram-positive bacteria) of the domain bacteria with higher relatedness to the family Bacilli. Nineteen (13%) clones belonged to the Lactococcus group, with a majority of them showing 100% similarity with two different L. garvieae 16S rRNA sequences in the database. Seven of the 19 clones showed similarities greater than 98% to Enterococcus seriolicida (AF061005). There was only one single-clone OTU in the group; thus, the coverage was very high (94.7%). The isolates F, P, and 7c from the culture-based study showed the highest similarity to members of the same class to which these 19 clones belonged. The phylogenetic analysis of these clones is shown in Figure 3.

**DISCUSSION**

Members of the genera Aeromonas, Acinetobacter, and Pseudomonas have been reported in the mosquito midgut in previous studies and our results are consistent with those of these earlier reports. One isolate had a similarity to an unidentified bacterium isolated from a semi-species comprising the D. paulistorum complex, and it was reported to be infectious in lepidopterans when introduced by inoculation. The tenth isolate, which was characterized as A. culicicola, showed an increased count after a blood meal. A 70–16,000-fold increase in bacterial counts 24 hours after a blood meal...
Phylogenetic affiliation of 16S ribosomal RNA (rRNA) gene clones in different clusters of domain bacteria based on a partial 16S rRNA gene sequence

Table 2

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Clusters</th>
<th>No. of clones</th>
<th>16S rRNA % similarity</th>
<th>% distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Acinetobacter group</td>
<td>62</td>
<td>88.49–99.4</td>
<td>41.3</td>
</tr>
<tr>
<td>Acinetobacter sp. EV4 14 (AJ10290)</td>
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<td>Acinetobacter sp. aerobic (AY05573)</td>
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<td>2.</td>
<td>Unidentified and Uncultured bacteria group</td>
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<td>84.8–100</td>
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<td>2a.</td>
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<td>Isolate HTA527 (AB002657)</td>
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<td>95.7–100</td>
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<tr>
<td>Isolate HTA554 (AB002656)</td>
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<td>91.4–100</td>
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<td>Isolate HTA580 (AB002659)</td>
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<td>2b.</td>
<td>Uncultured bacteria group (Z93992)</td>
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<td>Uncultured bacteria clone CD3D6 (AY038379)</td>
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<td>89–100</td>
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<td>2d.</td>
<td>Uncultured soil bacteria</td>
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<td>95.1–99.8</td>
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<tr>
<td>Clone 816-1 (AF423294)</td>
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<td>96.9–98.9</td>
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<td>Firmicutes cluster</td>
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<td>3.</td>
<td>Lactococcus group</td>
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<td>Enterococcus seriolicida (AF061005)</td>
<td>7</td>
<td>98.6–100</td>
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</table>

* The sequence analysis was done at the Ribosomal Database Project, Michigan State University, East Lansing, MI and the National Center for Biotechnology Information (Bethesda, MD) (http://www.ncbi.nlm.nih.gov/BLAST) and is based on partial 16S rRNA gene sequence (Escherichia coli positions 67–572). Sequence showing more than 98% similarity were considered to be a single operational taxonomic unit (OTU) or molecular species based on the conclusion that “the sequence divergence of clones belonging to the same OTU [is] generally low (between 2 and 0%).”

has been previously reported. It was suggested that the rapid growth of midgut bacteria after a blood meal is fueled by the iron and protein-rich bolus of blood. The β-hemolytic character of A. culicicola might help in blood digestion in the midgut and might play a major role in symbiotic association.

In the 16S rRNA gene library, the majority of the clones showed sequence similarities to either the cultured or the uncultured members of γ-proteobacteria group, which correlates with the cultivable bacterial flora in which seven of 10 isolates belonged to this group. Moreover, two isolates were of the genus Acinetobacter and 40% of the clones showed the highest similarity to this genus, indicating that γ-proteobacteria, especially those related to Acinetobacter, may form a significant proportion of the mosquito midgut microbiota. We were unable to isolate any clones related to A. culicicola. In the earlier studies on human gut microbiota, investigators failed to identify any rRNA sequences corresponding to the most commonly observed bacteria in the human gut. If one considers breeding water to be the source of bacteria in the midgut, the presence of sequences related to activated sludge or soil clones was not surprising.

In the 16S rRNA gene library, the majority of the clones showed a maximum similarity to sequences related to activated sludge or soil clones was not surprising. Some of these sequences had not been identified and presented a challenge for characterization and further research. The availability of reliable expression systems and transposable elements for the genus Acinetobacter provides an easy tool for the manipulation of the bacteria to produce anti-parasitic proteins. Once established in the mosquito midgut using artificial means, these modified bacteria can thus be used for the generation of transgenic mosquitoes refractory to transmission of diseases. Thus, γ-proteobacteria, especially those related to Acinetobacter, could be considered as the candidate bacteria for the genetic manipulation of mosquitoes.
REFERENCES


