The malaria burden in Brazil is estimated to 600,000 cases per year (Coura et al. 2006). Both Plasmodium falciparum and Plasmodium vivax infections are prevalent, and the principal malaria vector in the Amazon region is Anopheles darlingi Root (Diptera: Culicidae) (Deane 1986, Tadei and Dutary-Thatcher 2000). Release of genetically engineered adult mosquitoes resistant to parasite infections has been proposed as a way to control malaria transmission (James 2003). However, the use of transgenic An. darlingi to control malaria transmission in the Amazon region has been hampered by the failure to rear this species in captivity. To circumvent this problem, an alternative would be to use paratransgenesis where bacteria are modified genetically to produce antiparasitic factors and then introduced into the insect midgut (Beard et al. 2002). To search for bacteria suitable for paratransgenesis, investigations of cultured and uncultured bacteria have been performed in Culex quinquefasciatus Say (Pidiyar et al. 2004) and in African Anopheles species (Lindh et al. 2005). Both studies used captured blood-fed mosquitoes for gene amplification of bacterial 16S rRNA. Despite the sensitivity of this method, rather few culture-independent bacteria were detected. In an attempt to increase the proportion of mosquitoes from which 16S rRNA sequences can be retrieved, we explored the outcome of controlled blood feeding of host-seeking An. darlingi. In this article, we also present a first survey of the uncultured bacteria associated with An. darlingi and show that they mainly belong to the Enterobacteriaceae family.

**Materials and Methods**

Host-seeking An. darlingi females were captured when attempting to feed on humans, during August 2006, 6–9 p.m., in Manaus (3° 08’ S, 60° 01’ W), Amazonas, Brazil. They were identified using morphological characters (Faran and Linthicum 1981, Forattini 1962) and confined individually in plastic cups. The mosquitoes were allowed to feed on domestic ducks (Cairina moschata) within 2 h of capture, or they were given access to a 10% sucrose solution. Twenty-six hours after capture, mosquitoes were immersed in isopropanol and stored at room temperature. DNA was extracted individually from nine whole mosquitoes (Wizard Genomic DNA Purification kit, Promega, Madison, WI). To avoid contamination, filtered tips were used throughout the process, and the DNA and the primers were dissolved in DNA-free water (Ambion, Austin, TX). The 16S rRNA gene amplifications were performed in 25-μl reactions (Supermix, Invitrogen, Carlsbad, CA) containing 50 ng of DNA and the primers 8f (5’-AGAGTTTGATCCTTGGCTCAG-3’) and 1501r (5’-CGGTTACCTGTAGACGAC-3’) (Lindh et al. 2005). The DNA amplification program was as follows: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 58–48°C for 30 s (the temperature was decreased by 1°C every cycle for 10 cycles and then held at 48°C for 20 cycles), 72°C for 1 min 30 s, followed by a final extension step at 72°C for 25 min (Lindh et al. 2005). Amplification products (~1.5 kb) were cloned into TOPO 2.1 (Invitrogen), and 10 16S rRNA clones from each mosquito were sequenced (Laguna Scientific, Laguna Beach, CA) by using M13 forward and reverse primers. The 16S rRNA gene
sequences were analyzed in BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) by using GenBank as the reference library. Chimerical sequences were eliminated after analysis with the Ribosomal Database Project II Chimera Check program (Cole et al. 2005). The percentage of coverage of the sequence analysis was calculated by Good’s method, with the formula \[ \left[ 1 - \left( \frac{n}{N} \right) \right] \times 100, \] where \( n \) is the number of molecular species represented by one clone (single-clone operational taxonomic units [OTUs]), and \( N \) is the total number of sequences (Good 1953). An OTU was defined as consisting of all sequences with <2% divergence from the aligned homologous nucleotides (Pidiyar et al. 2004).

**Results and Discussion**

The bacteria colonizing midguts of insect disease vectors have drawn special attention for their interactions with both the insect hosts and the pathogenic organisms (for review, see Azambuja et al. 2005). Based on these interactions, paratransgenesis has been developed as a strategy to control Chagas disease transmission by triatomine bugs (Beard et al. 2005). It is anticipated that paratransgenesis also can be used to deliver anti-Plasmodium effector molecules to wild mosquito populations, resulting in the control of malaria transmission. So far, no true mosquito symbionts have been found, but recent research has provided the basis for mosquito paratransgenesis (Riehle and Jacobs-Lorena 2005), and it has been shown that it is possible to enhance the capability of a gut bacterium to remain in the mosquito gut (Riehle et al. 2007). Many of the bacteria shown to inhabit the guts of mosquitoes are environmental and isolated commonly from soil or plants. For example, a species of the genus *Asaia*, isolated mainly from tropical flowers in Asia can stably inhabit the gut of the Asian malaria vector *Anopheles stephensi* Liston (Favia et al. 2007). The identification of 16S rRNA sequences from bacterial populations found in the gut of vector mosquitoes was performed previously on mosquitoes from Asia (Pidiyar et al. 2004) and from Africa (Lindh et al. 2005). Here, we present results of a polymerase chain reaction (PCR)-based approach to identify bacteria associated with *An. darlingi*, a major malaria vector in Latin America.

From the nine mosquitoes analyzed, we retrieved 56 bacterial 16S sequences from six mosquitoes, corresponding to a coverage of 93% by using the criteria set in Pidiyar et al. (2004). The coverage is the probability of the next cloned sequence falling in a novel (not yet observed) OTU or molecular species. 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 the used PCR conditions and primer set). A coverage of 93% indicates that sequencing more clones would result in only 7% of the samples being novel.

The majority of 16S sequences detected are from bacteria belonging to the *Enterobacteriaceae* family (Fig. 1). The *Enterobacter hormaechei*-cluster and the ant lion gut bacterium 755-cluster contain bacteria obtained from most of the specimens analyzed. This differs from the results from other malaria vectors where only a single bacterial species was found to be present in more than one specimen of 91 *Anopheles gambiae* and 25 *Anopheles funestus* Giles mosquitoes examined (Lindh et al. 2005). Our result is encouraging, because the use of paratransgenesis requires a bacterium that could be introduced to the majority of the mosquitoes in a vector population. Many of the 16S rRNA sequences retrieved from *An. darlingi* are similar to those of gut bacteria found in other mosquitoes, but some are related to bacteria from insects as evolutionary distance increased (thrips (Thysanoptera) and ant lions (Neuroptera). The first *Aeromonas* species, *Aeromonas culicicola*, isolated from a mosquito was found in *Cx. quinquefasciatus* (Pidiyar et al. 2002, Huys et al. 2005). *Aeromonas* species were found subsequently in African anophelines (Lindh et al. 2005), and now in this study. Representatives from the previous studies (Fig. 1) reveal that one of the sequences from *An. darlingi* (3.13) is closely related to the *Aeromonas* sp. clone H2.26.29 (*AY837743*) from *An. gambiae* (Lindh et al. 2005), whereas the other sequence is closer to, although different from, *Aeromonas culicicola* (*Aeromonas veronii*). Similarly, the *Pseudomonas* sequences retrieved from *An. darlingi* are related closely to a *Pseudomonas putida* relative isolated from *An. gambiae* s.s. from Kenya (Lindh et al. 2005)

Three clades of sequences belong to potentially new species of bacteria, based on their sequence similarities being below 98% to other sequences in GenBank. The 4.5–2.3 clade is 96–97% similar to uncultured *Enterobacter* relatives, the 3.2–4.2 clade is 97–98% similar to *Enterobacter* species, and the 5.1–5.2 clade is only 96% similar to *P. putida*. None of these sequences were found in the DNA from the nonblood-fed mosquito; therefore, they could have their origin from the ducks that were used for blood feeding.

Gene amplification of all (five) blood-fed mosquitoes generated a 16S rDNA amplification product, whereas only one of four nonblood-fed mosquitoes resulted in any product. These data indicate that any bacteria, possibly existing on the cuticle, were in too low numbers to yield any PCR product and that the bacterial DNA originated from either bacterial populations increased by a bloodmeal or the bloodmeal itself. The results are in agreement with previous work on *Anopheles* mosquitoes that found 11–40-fold increase of bacteria 24 h after a bloodmeal (Pumpani et al. 1996). That this increase is sufficient for genetically modified bacteria to affect malaria parasite development has been shown in two studies where *Escherichia coli* were fed to *An. stephensi*. In the first article on successful paratransgenesis in mosquitoes, Yoshida et al. (2001) used a single-chain antibody targeting *Plasmodium berghei* ookinetes Pbs21 linked to the lytic peptide Shiva-1 and obtained 95.6% transmission blockage. Recently, Riehle et al. (2007) showed that *E. coli* expressing the anti-*Plasmodium* effector molecules phospholipase-A (2) and SM1 inhibited oocyst formation by 23 and 41%, respectively. That we obtained 16S rRNA...
Fig. 1. Phylogenetic tree of all 16S rRNA clones in the study (shaded) and selected database sequences. The tree is based on ClustalW alignment (Thompson et al. 1994) of sequences ~1,500 bp in length with Kimura-2 distance parameters and was generated using neighbor-joining with 2000 bootstrap replicates in MEGA version 3.1 (Kumar et al., 2004). Branch points supported by bootstrap values of >90% are represented by black circles, >70% are represented by gray circles, and >50% are represented by white circles. Branches without circles are unresolved (bootstrap values of ≤50%). Sequence ID consists of mosquito number followed by clone number (N, non-blood fed). EF179808 to EF179845 refer to the GenBank accession numbers. If two or more sequences from one specimen are >99.5% similar, the total number of clones represented is written in parentheses. The bar indicates 1% difference.
sequences from all blood-fed mosquitoes, whereas Lindh et al. (2005) only detected uncultured bacteria in 9% of the mosquitoes analyzed using the same PCR conditions, suggests that the controlled blood feeding increases the likelihood to retrieve bacterial DNA. However, because the number of specimens analyzed is small, the generality of our finding remains to be confirmed.

From this pilot investigation, we can conclude the following:

1. Human land catches, without the mosquitoes biting or touching the skin of the human volunteer (to avoid contamination), select for the subpopulation of female mosquitoes most attracted to humans. Therefore, bacteria obtained from these mosquitoes are especially suitable for paratransgenesis.

2. Controlled blood feeding, 24 h before DNA extraction, enhances the recovery of 16S rRNA sequences from mosquitoes. The mosquitoes should preferably be fed sterile blood to avoid contamination of the blood with bacteria. As a control, the sterility of the blood should be assessed by using the same methods of DNA extraction and gene amplification as for the mosquitoes.

3. Nonblood-fed mosquitoes in general did not generate any product indicating that bacteria on the exoskeleton of the mosquitoes were too few to affect the results.

4. There is a great diversity of bacteria in An. darlingi, including potentially new species. This justifies further search and isolation of bacteria useful for paratransgenesis.

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