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16S rRNA Gene-Based Identification of Midgut Bacteria from Field-Caught *Anopheles gambiae* Sensu Lato and *A. funestus* Mosquitoes Reveals New Species Related to Known Insect Symbionts

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Field-collected mosquitoes of the two main malaria vectors in Africa, *Anopheles gambiae* sensu lato and *Anopheles funestus*, were screened for their midgut bacterial contents. The midgut from each blood-fed mosquito was screened with two different detection pathways, one culture independent and one culture dependent. Bacterial species determination was achieved by sequence analysis of 16S rRNA genes. Altogether, 16 species from 14 genera were identified, 8 by each method. Interestingly, several of the bacteria identified are related to bacteria known to be symbionts in other insects. One isolate, *Nocardia corynebacterioides*, is a relative of the symbiont found in the vector for Chagas' disease that has been proven useful as a paratransgenic bacterium. Another isolate is a novel species within the γ-proteobacteria that could not be phylogenetically placed within any of the known orders in the class but is close to a group of insect symbionts. Bacteria representing three intracellular genera were identified, among them the first identifications of *Anaplasma* species from mosquitoes and a new mosquito-*Spiroplasma* association. The isolates will be further investigated for their suitability for a paratransgenic *Anopheles* mosquito.

Malaria remains the parasitic disease that kills the most people in the world. *Anopheles gambiae* sensu lato and *Anopheles funestus* mosquitoes are the main vectors in Africa, where 90% of malaria-related deaths occur. An approach to stop malaria transmission is paratransgenics. In this approach, suitable symbiotic bacteria are genetically modified to produce an antiparasitic factor and then reintroduced into the insect gut, where they kill or inhibit the development of the parasites (4).

A few studies have been performed to investigate bacterial species in field-collected *Anopheles* mosquitoes, all using culturing techniques. Jadin et al. (22) found *Pseudomonas* sp. in the midgut of mosquitoes from the Democratic Republic of the Congo. Straif et al. (31) identified 20 different genera of midgut bacteria from *A. gambiae* sensu lato and *A. funestus* mosquitoes caught in Kenya and Mali. They identified *Pantoea agglomerans* (synonym *Enterobacter agglomerans*) as the most frequently isolated bacterium, apart from *Escherichia coli* (31). Gonzalez-Ceron et al. (14) isolated *Enterobacter amnigenus*, *Enterobacter cloacae*, *Enterobacter* sp., *Serratia marcescens*, and *Serratia* sp. from *Anopheles albimanus* mosquitoes caught in southern Mexico.

To identify bacterial candidates for a paratransgenic mosquito, we conducted a screen for uncultured and cultured midgut bacteria from wild-caught *A. gambiae* and *A. funestus* mosquitoes.

MATERIALS AND METHODS

Field site, mosquitoes, and dissections. Indoor-resting, blood-fed female *A. gambiae* sensu lato and *A. funestus* mosquitoes were caught in Lwanda, 12 km east of Mbita Point Research and Training Centre, ICIPE, Suba district, Western Kenya. In total, 116 *Anopheles* mosquitoes were caught on eight different occasions (A2 to H2). Living mosquitoes were anesthetized with chloroform, the species were determined by morphology and PCR (*A. gambiae* sensu lato) (30a). The mosquitoes were dissected in a sterile hood. Individual midguts were mashed in 50 μl of sterile saline (0.9% NaCl); this suspension was later used for isolation of bacteria and cloning of the 16S rRNA gene from bacteria. Controls for the efficiency of sterilization were treated like the other samples.

Bacterial isolation and phenotypic characterization. The midgut suspension was streaked on Luria-Bertani agar (LA) plates and incubated for 48 h at room temperature. All bacteria were restreaked and preserved as deep-stick cultures during transport to Sweden. The morphology of the bacteria was examined using visual investigation and a light microscope. Motility tests were performed using the hanging-drop technique and motility medium plates (1% nutrient broth, 5.3% gelatin, 0.3% agar, 0.1% KNO_3, pH 7.2) that were incubated overnight at 30°C. Anaerobic growth was determined by incubating LA plates overnight at 30°C in bioMerieux GENbox anaer generators. Optimum growth temperatures were determined in LB by shaking at 160 rpm and spectrophotometric reading. The isolates were sent to the Culture Collection, University of Gothenburg (CCUG), for classical phenotyping; different types of analyses were used depending on the bacterial genus.

Amplification, cloning, and sequencing of 16S rRNA genes. Chromosomal DNA from the remaining midgut suspension was prepared using a guanidine-thiocyanate method (21). PCRs were performed to amplify 1.3 to 1.5 kb of the 16S rRNA gene from all the DNA samples by using PCR beads (0.5-ml Ready-To-Go PCR beads; Amersham Pharmacia Biotech). As the forward primer, 8f (5′-AGAGTTTGATIITGGCTCAG-3′; I = inosine) was used, and as the reverse primer, 1401r (5′-CGGTGTGTACAAGACCC-3′) was used for clones from sampling occasions G2 and H2 and 1501r (5′-CGGITACCTTGTTAC GAC-3′) was used for all other samples. The PCR program was as follows: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 58 to 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, followed by a final extension step at 72°C for 20 min. To construct a gene library with the 16S rRNA genes amplified from the DNA preparation, the PCR products of the expected size were cloned into TOPO 2.1 vectors utilizing TA cloning (Invitrogen).

For 16S rRNA gene cloning of cultured isolates, templates were prepared by boiling a bacterial colony for 10 min in a Tris-EDTA buffer (20 mM Tris, 2 mM EDTA, 1% Triton). PCR with primers 8f and 1501r and cloning were performed as described above. The 16S rRNA gene inserts in the plasmids were sequenced at Macrogen, Korea, using M13 primers.

Sequence analysis. For preliminary identifications, the 16S rRNA gene sequences were analyzed in BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) and the Ribosomal Database Project II (RDP II) (http://rdp.cme.msu.edu). Chimeric sequences were searched for using the Ribosomal Database Project II

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TABLE 1. Phylogenetic affiliations of the uncultured bacteria based on 16S rRNA gene analysis[a]

Clone[b]	GenBank accession no.	*Anopheles* sp.	Closest relative according to BLASTn (% identity)	Closest relative(s) according to phylogenetic analysis
B2.3.17	AY837725	*arabiensis*	*Acidovorax temperans* AF078766[c](99)	*A. temperans* AF078766, bromate-reducing bacterium AF442523
B2.5.31	AY837724	*arabiensis*	*Mycoplasma wenyonii*[d] AF016546 (96)	*M. wenyonii*[d] AF016546
B2.3.14	AY837726	*arabiensis*	*Stenotrophomonas maltophilia* AB180661 (99)	*S. maltophilia* AJ131913, *Pseudomonas hibiscicola*[e] AB021405
B2.13.13	AY837727			
B2.15.35	AY837728	*gambiae* sensu stricto	*Stenotrophomonas maltophilia* AB180661 (99)	
B2.8.27	AY837729	*gambiae*	*Stenotrophomonas* sp. strain AJ002814[c](99)	*S. maltophilia* AJ131910
B2.18.23	AY837730			
D2.2.2–3[f]	AY837745–46	*funestus*	*Spiroplasma* sp. strain AB048263 (99)	See Fig. 1B[g]
D2.2.12–14	AY837731–33	*funestus*	*Spiroplasma* sp. strain AJ245996 (98)	See Fig. 1B[g]
G2.9.23	AY837734	*arabiensis*	*Paenibacillus* sp. strain AY382189 (93)	*Paenibacillus* sp. strain AF290916
G2.12.2, -25, -46	AY837735, -36, -37	*arabiensis*	*Anaplasma ovis* AF414870 (99)	See Fig. 1A
G2.12.7B, -31, -35	AY837738, -39, -40	*arabiensis*	*Ehrlichia* sp. strain Bom Pastor AF318023[h] (99–100)	See Fig. 1A
H2.26.2	AY837741	*gambiae* sensu stricto	*Aeromonas hydrophila* X87271 (99)	*A. hydrophila* X87271
H2.26.11	AY837742	*gambiae* sensu stricto	*Aeromonas* sp. strain U88656 (99)	*Aeromonas* sp. strain AF099027, *Aeromonas caviae*[i] X60409, *Aeromonas* sp. strain U88656
H2.26.29	AY837743	*gambiae* sensu stricto	*Aeromonas* sp. strain AF099027 (99)	*Aeromonas* sp. strain AF099027, *A. caviae*[i] X60409, *Aeromonas* sp. strain U88656

[a] Sequence analyses are based on 1.4 to 1.5 kb, where nothing else is stated, and were performed in November 2004.
[b] The mosquito label consists of two parts: first, the sampling occasion (A2 to H2), and second, a number in order of dissection. Clones retrieved from a mosquito have the same label as the mosquito plus an additional number to separate them.
[c] Best hit with a sequence having a species name.
[d] Synonym *Eperythrozoon wenyonii.*
[e] Synonyms *Xanthomonas maltophilia*, *Stenotrophomonas maltophilia.*
[f] Sequence analysis based on 800-bp sequence.
[g] The clones group together with D2.2.12 in the phylogenetic tree (not shown).
[h] Belongs to the clade of *Ehrlichia* species renamed *Anaplasma* (Dumler et al. [9] and PAUP analysis [not shown]).
[i] Synonym *Aeromonas hydrophila* subsp. *anaerogenes.*

Chimera Check program (http://rdp8.cme.msu.edu/cgis/chimera.cgi). The ARB system (26) was used for phylogenetic analysis with the ssujun02 database (http://www.arb-home.de). The 16S rRNA gene sequences were imported into the database and aligned using the ARB tool Fast Aligner, and the alignment was then checked manually. The aligned sequences were inserted into the main tree using the parsimony insertion tool of ARB to show their approximate positions; these positions were verified using distance (neighbor joining) and parsimony (100 bootstrap replicates) analyses with default settings in ARB and the ARB filter corresponding to the respective class or phylum of bacteria.

RESULTS AND DISCUSSION

In this study, two different detection pathways were used to screen for bacteria in *Anopheles* mosquito midguts, one culture independent and one culture dependent. A total of 116 mosquitoes (91 *A. gambiae* sensu lato and 25 *A. funestus* mosquitoes) and 19 sterility controls were examined. Four bacterial species that were identified in both mosquito samples and sterility control samples and two chimeric clones, found by the RDP II Chimera Check program, were removed from the data set.

Sixteen species of bacteria were identified as habitants of *Anopheles* mosquito midguts. They represent 14 genera, 7 genera obtained using the culture-independent pathway (Table 1) and 7 other genera obtained with the culture-dependent pathway (Table 2). Since streaks on LA plates and DNA isolation were performed on each midgut, it is surprising that the PCR-based method did not retrieve the genera found with the culture-dependent method. One explanation might be that remnants from the midgut cells or human blood interfere with the PCR. Another explanation could be competition between the DNAs from different bacteria favoring the ones with higher abundance. All previous studies of midgut flora of *Anopheles* mosquitoes exclusively utilized cultivation methods for screening. By including a culture-independent method, we obtained a broader picture of the mosquito midgut flora. The first study describing identification of uncultured and cultured microbiota in mosquitoes, investigating wild-caught *Culex quinquefasciatus*, was recently published by Pidiyar et al. (27). Similar to our study, different bacteria were found with the culture-dependent and the culture-independent methods.

In the present study, bacteria were found in 15% of the mosquitoes. Few of the mosquitoes harbored more than one bacterial species, possibly reflecting internal competition among the bacteria, and only one species (*Stenotrophomonas* sp.) was found in more than one mosquito. We consider the number of mosquitoes in our study too small to be representative of field-caught *Anopheles* mosquitoes from this area; however, another study performed in Kenya showed similar results (31). The low prevalence may reflect one of two things, either that mosquitoes in nature do not

TABLE 2. Phenotypic data[a] and phylogenetic affiliations (based on 16S rRNA gene analysis[b]) of the cultured bacteria

Isolate[c]/*Anopheles* sp.	GenBank accession no./CCUG no.	Growth temp interval (°C) (growth temp optimum [°C]/GT [min])[d]	Phylogenetic affiliation	Closest relative according to BLASTn (% identity)	Closest relative(s) according to phylogenetic analysis
B2.1B/*arabiensis*	AY837746/49717	10–40, (35/32)	*Bacillaceae*	*Bacillus simplex* AJ628747 (99)	*Bacillus* sp. strains AJ315059 and AJ315062
E2.5/*arabiensis*	AY837747/49718	10–40, (35/20)	*Vibrionaceae*	*Vibrio metschnikovii* X74712 (98)	*V. metschnikovii* X74711 and X74712
H2.1/*arabiensis*	AY837748/49520	15–45, (30/100)	γ-Proteobacteria	*Serratia odorifera* AJ233432 (91)	See Fig. 2A
H2.3/*arabiensis*	AY837749/49711	20–40, (40/104)	*Nocardiaceae*	*Nocardia corynebacterioides*[e] AF430066 (99)	See Fig. 3
H2.5/*arabiensis*	AY837750/49712	10–40, (35/92)	*Bacillales*	*Bacillus silvestris* AJ006086 (99)	*B. silvestris* AJ006086
H2.14/*arabiensis*	AY837751/49713	10–40, (35/28)	*Enterobacteriaceae*	*Escherichia senegalensis* AY217654[f] (98)	See Fig. 2B
H2.16B/*arabiensis*	AY837752/49715	20–40, (35/44)	*Intrasporangiaceae*	*Janibacter limosus* Y08539 (98)	*Janibacter* sp. strain AF170746
H2.26/*gambiae* sensu stricto	AY837753/49716	10–35, (30/32)	*Pseudomonadaceae*	*Pseudomonas putida* AF447394 (99)	*P. monteilii* AF181576, *P. mosselii* AF072688

[a] Additional data are available at http://www.ccug.se.
[b] Sequence analyses are based on 1.4 to 1.5 kb and were performed in November 2004.
[c] The mosquito label consists of two parts: first, the sampling occasion (A2 to H2), and second, a number in order of dissection. Isolates retrieved from a mosquito have the same label as the mosquito.
[d] 10°C was the lowest temperature examined. GT, generation time.
[e] Synonyms *Rhodococcus corynebacterioides* and *Nocardia corynebacteroides.*
[f] Best hit with sequence having a species name.

harbor more bacteria or that the methods available for bacterial screening are not sufficient to obtain the whole picture of the mosquito midgut flora.

Interestingly, three genera of intracellular bacteria were identified by the culture-independent method in this study. The bacterial DNA clones from mosquito D2.2 represent the fifth *Spiroplasma* species found in mosquitoes. It does not group with any of the previously known mosquito spiroplasmas, *Spiroplasma culicicola* (19), *Spiroplasma diminutum* (34), *Spiroplasma saubaudiense* (1), or *Spiroplasma taiwanense* (2), in the phylogenetic analysis (Fig. 1B) and phylogenetic analysis using PAUP (not shown). *S. taiwanense* was previously demonstrated to be pathogenic to adult *Aedes aegypti* and *Aedes stephensi* mosquitoes (17) and *A. aegypti* larvae (16), and the potential role of mosquito spiroplasmas as vector control agents has been discussed (16–18). Several species of *Spiroplasma* are male-killing bacteria (3, 20, 23, 25); however, this has not been shown for mosquito-*Spiroplasma* associations. Although *Spiroplasma* spp. most often are considered pathogens (33), they have also been reported to be symbionts in some insects (12, 13). Two different *Anaplasma* species were identified (Fig. 1A), making this the first report of *Anaplasma* in mosquitoes. The genus *Anaplasma*, which is a sister taxon to *Wolbachia* (Fig. 1A), contains several tick-borne species that are pathogenic to ruminants, including *Anaplasma ovis*, a sheep and goat pathogen (5, 24). In 1994, *Anaplasma phagocytophilum* (synonym *Ehrlichia phagocytophila*) (Fig. 1A) was described as a human pathogen for the first time (15). By 2004, over 600 cases had been reported in the United States and 19 in Europe (26). We cannot exclude the possibility that the bacterial DNA recovered from *Anaplasma* in this study was present in the blood ingested by the mosquito, since these bacteria live intracellularly in blood cells. The vectorial capacity of mosquitoes for *Anaplasma* remains to be investigated. Clone B2.5.31, *Mycoplasma wenyonii* (synonym *Eperythrozoon wenyonii*) (Table 1), is a close relative of *Mycoplasma suis* (synonym *Eperythrozoon suis*) that can be mechanically transmitted between pigs by *A. aegypti* mosquitoes, according to Prullage et al. (28).

All of the bacteria isolated showed rod-shaped forms of various lengths; however, H2.16B changes shape from rods to cocci as it grows. Isolates H2.14 and E2.5 are facultative anaerobes, and the rest are obligate aerobes. All isolates except H2.3 and H2.16B are motile. The results from the phenotypic characterization performed at CCUG (http://www.ccug.se) correspond well with the phylogenetic results for the isolates.

Our isolate designated H2.1 is a novel species within the γ-proteobacteria. Phylogenetically it is placed outside all families and orders within this class and close to a group of insect symbionts (Fig. 2A); among these are the *Arsenophonus* endosymbionts often found in whiteflies (Hemiptera: Aleyrodidae) (32). Further characterizations of isolate H2.1 is in progress. Isolate H2.14 could be identified only to the family level, *Enterobacteriaceae* (Fig. 2B) (http://www.ccug.se). The family *Enterobacteriaceae* contains species previously identified in mosquito midgut screens (6–8, 14, 27, 29–31) and, in addition, several species that have been described as insect symbionts (35). H2.5 is a close relative of *Bacillus silvestris* (AJ006066) (Table 2) according to the phylogenetic tree; however, RDP II analysis places H2.5 closest to *Caryophanon* sp. (AF385535). The phenotypic analysis identifies H2.5 as a *Bacillus* sp. (http://www.ccug.se). Several *Bacillus* spp. have previously been identified in mosquitoes. Straif et al. (31) found different *Bacillus* species in field-caught *A. gambiae* and *A. funestus* mosquitoes. Fouda et al. (11) concluded that *Bacillus* and *Staphylococcus*, isolated from the midguts of a laboratory colony of *Culex pipiens* mosquitoes and then reintroduced, were essential for high and normal fecundity. Our isolate H2.3, identified as *Nocardia corynebacterioides* (synonyms *Rhodococcus corynebacterioides* and *Nocardia corynebacteroides*), may be important as a candidate to test for paratransgenics, since it is a relative of *Rhodococcus rhodnii*

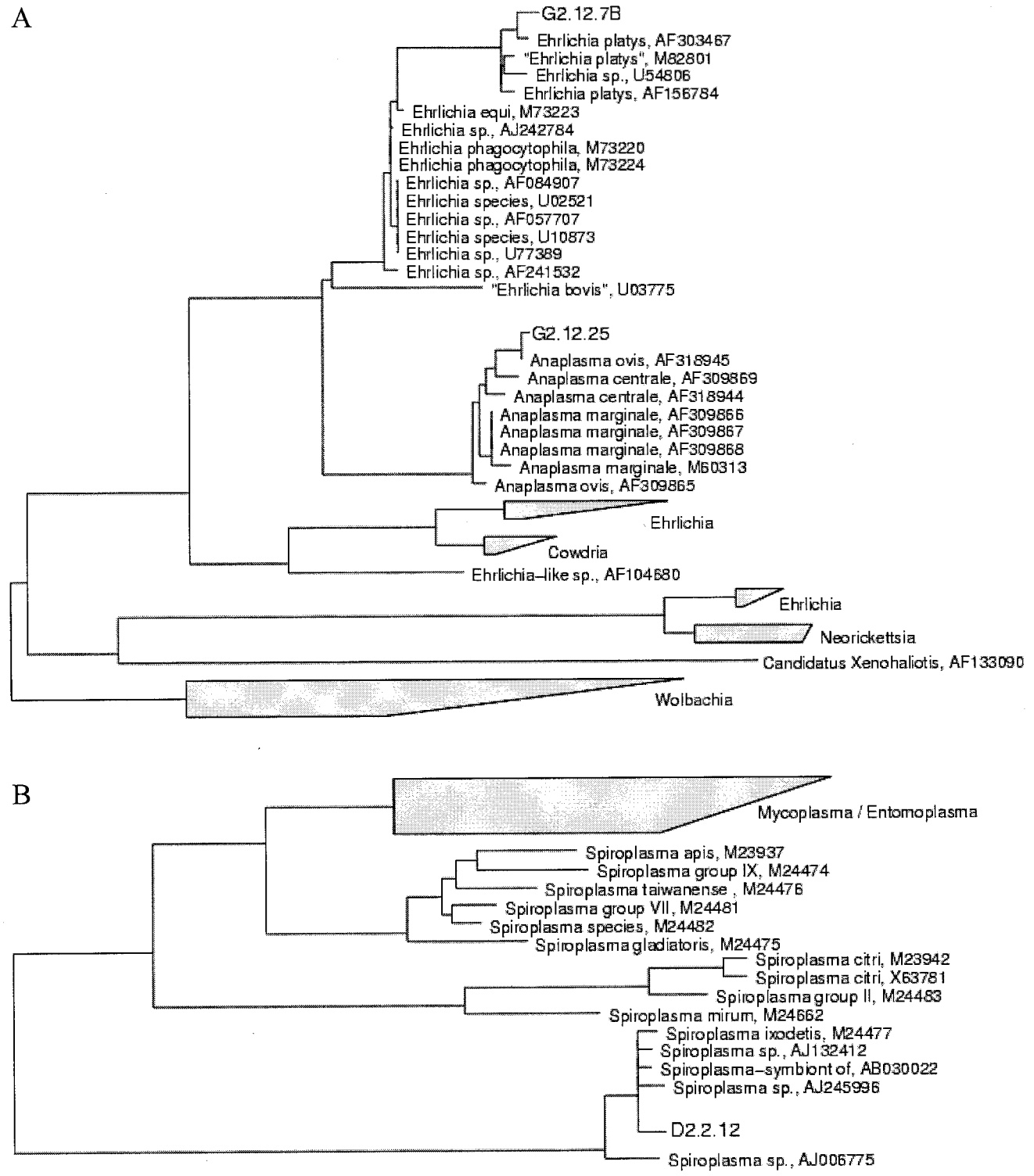

FIG. 1. Phylogenetic dendrograms constructed in ARB based on 16S rRNA gene sequences (1,350 to 1,500 bp). (A) Bacterial DNA clones from mosquito G2.12. The clones belong to the α-proteobacteria class, and their positions within the genus *Anaplasma* are shown. The *Ehrlichia* spp. in the upper clade have recently been renamed *Anaplasma* spp. (10). (B) Position of the bacterial DNA clone D2.2.12 within the genus *Spiroplasma* belonging to the class *Mollicutes*.

(Fig. 3). The last is a true symbiont found in *Rhodnius prolixus* (Hemiptera: Reduviidae) and has been successfully used in a paratransgenic approach (4). This isolated bacterium was reintroduced into the *Rhodnius* gut and killed the trypanosome causing Chagas' disease after it had been transformed with a plasmid expressing a cecropin gene (10). Isolate H2.16B is the first reported bacterium of the family *Intrasporangiaceae* to be isolated from mosquitoes. Further

FIG. 2. Phylogenetic dendrograms constructed in ARB based on 16S rRNA gene sequences (1,400 to 1,500 bp). (A) Position of bacterial isolate H2.1 within the γ-proteobacteria class. (B) Position of bacterial isolate H2.14 within *Enterobacteriaceae*. Groups marked "JL group" have been created to simplify the overview of the tree.

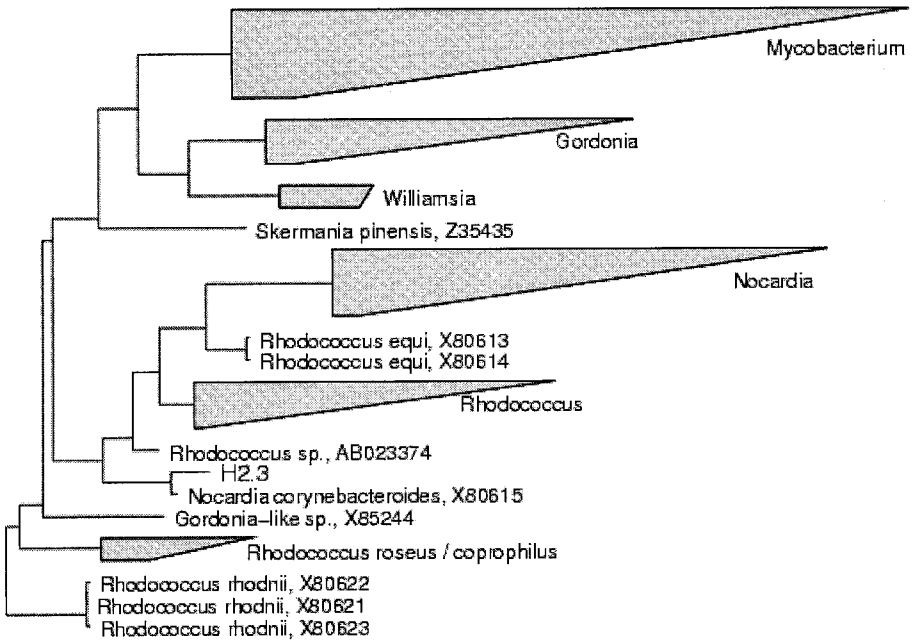

FIG. 3. Phylogenetic dendrogram constructed in ARB based on 16S rRNA gene sequences (~1,500 bp). Shown is the position of the bacterial isolate H2.3 within the genus *Rhodococcus*.

analyses of this isolate revealed that it is a novel species within the genus *Janibacter* (P. Kämpfer, J. M. Lindh, O. Terenius, and I. Faye, unpublished data).

All of the bacterial isolates from this study will be further evaluated for their suitability as paratransgenic tools. A first step will be to study sustainability in mosquito midguts after reintroduction. A second step is to assess the immune response induced by the bacteria. The survival of a reintroduced bacterium will depend on its level of tolerance to the immune response mounted by the mosquito and putative antagonistic effects from other midgut bacteria. Several studies have shown that gram-negative midgut bacteria can suppress *Plasmodium* parasites (14, 29, 30), possibly by acting as elicitors of the mosquito immune response affecting *Plasmodium* development (30). Hence, an ideal bacterium for paratransgenics would be one that elicits a strong immune response that suppresses other bacteria and *Plasmodium* parasites but does not affect its own survival. In addition, genetic modification of this bacterium by introducing a gene expressing an antiparasitic molecule could achieve total clearance of *Plasmodium* parasites from the mosquito midgut. From this point of view, it is promising that several of the isolates are gram-negative γ-proteobacteria, for which there are means of genetic modification.

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ADDENDUM IN PROOF

The cultured isolates H2.1 and H2.16B have now been further classified and named *Thorsellia anophelis* (P. Kämpfer, J. M. Lindh, O. Terenius, S. Hagdost, E. Falsen, H. J. Busse, and I. Faye, Int. J. Syst. Evol. Microbiol., in press) and *Janibacter anophelis* (P. Kämpfer, O. Terenius, J. M. Lindh, and I. Faye, Int. J. Syst. Evol. Microbiol., in press).

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