JOURNAL OF BACILRIOLOCV, Jan. 1994, p. 388-394 Vol. 176, No. 2
0021-9193/94/$04.00+0

Rickettsial Relative Associated with Male Killing in the Ladybird Beetle *(Adalia bipunctata)*

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Received 16 August 1993/Accepted 1 November 1993

A cytoplasmically inherited microorganism associated with male killing in the two-spot ladybird beetle, ***Adalia bipunctata, is*** shown **to be closely related to bacteria in the genus** *Rickettsia.* Sequencing of a PCR-amplified product of the 16S genes coding for rRNA (16S rDNA) shows the organism associated with male killing in ladybirds to share a common ancestry with the *Rickettsias* relative to other genera (e.g., *Anaplasma, Ehrlichia,* and Cowdria). The rickettsial 16S rDNA product is found in four strains of ladybird beetle showing male embryo lethality and is absent from two uninfected strains and an antibiotic-cured strain. In addition, a revertant strain that had naturally lost the male-killing trait failed to amplify the rickettsial 16S rDNA product. Use of PCR primers for a 17-kDa protein antigen which is found only in rickettsias also resulted in an amplified product from infected strains. Uninfected, cured, and revertant strains and insect species infected with related bacteria (cytoplasmic-incompatibility bacteria from *Nasonia* wasps) failed to amplify the product. Discovery of a close relative of rickettsias associated with sex ratio distortion in insects has **implications for the evolution** and population dynamics of this bacterial genus.

Microorganisms that alter sex determination and the sex ratio of their hosts are widespread among arthropods. Various mechanisms of microbe-induced sex ratio distortion include feminization of genetic males (25), induction of parthenogenesis (27), alteration of the primary sex ratio (24), and killing of male (but not female) offspring (16, 32). Most microorganisms causing **these effects are cytoplasmically inherited, typically being** passed **to the next generation** through **the eggs** (but not sperm) of their hosts. Sex ratio distortion is believed to be advantageous for the microorganisms because it distorts the sex ratio toward the sex (females) that transmits the bacteria to future generations (8, 31).

Sex ratio distortion induced by microorganisms has evolved independently numerous times and is caused by organisms belonging to a wide range of microbial taxa (16, 32). For example, microsporidia (protozoans) cause male killing in mosquitoes (4) and feminization in marine amphipods (10), spiroplasmas cause male killing in *Drosophila* species (34), enteric bacteria induce male killing in a parasitoid wasp (13, 32), and rickettsia-like bacteria cause parthenogenesis in parasitoid wasps (27) and feminization in isopods (25).

Microorganisms belonging to *Rickettsia* and related genera are obligatory intracellular bacteria. Several genera *(Rickettsia, Anaplasma, Cowdria,* and *Ehrlichia)* are transmitted by arthropods and cause disease in mammals. Characterizing such fastidious bacteria has presented special challenges because of problems in culturing them outside their hosts. However, the advent of modern molecular techniques, including PCR, has greatly facilitated genetic characterization without the necessity of culturing the bacteria independently of the host. This has assisted in the determination of phylogenetic relationships among rickettsia-like bacteria. For example, recent work has shown that cytoplasmically inherited bacteria associated with reproductive incompatibility and parthenogenesis in a diverse range of arthropods actually form a monophyletic group of bacteria distinct from, but related to, species in the genera *Ehrlichia* and *Anaplasma* (22, 25, 27).

Recently, cytoplasmically inherited bacteria have been found to be associated with male killing in the two-spot ladybird beetle, *Adalia bipunctata (15).* Here, we report that these bacteria are closely related to those in the genus *Rickettsia,* which includes several causative agents of disease in vertebrates. The relevance of this finding to the evolution and population genetics of this genus is discussed.

MATERIALS AND METHODS

Insect strains. Total genomic DNA was isolated from the following lady beetle strains either expressing or not expressing the male-killing trait. Presence of the male-killing bacteria has been confirmed by cytological detections of infections in hemolymph using 4',6-diamidino-2-phenylindole staining (14) as well as by the phenotypic assay (50% mortality of eggs and female-biased sex ratios [14]).

(i) Male-killing lines of *A. **bipunctata.*** Male-killing lines of *A. bipunctata* were Cambridge (collected in Cambridge, England, and maintained in laboratory culture since 1991), Sussex (collected in Sussex, England), Dutch (collected in Leiden, The Netherlands), and L1-12 (collected in Cambridge, England).

(ii) Non-male-killing lines of *A. bipunctata.* Unl and J3 are both a wild-type lines collected in Cambridge, England.

Ll-12C7 is a line derived from L1-12 (infected) that was cured of the male-killing trait by tetracycline treatment for two generations as described by Hurst (14). The initial female (uncured line) was allowed to lay eggs for 14 days. The female was then allowed to feed daily on sugar syrup containing 100 mg of tetracycline per g for 4 h. Eggs were collected after 10 days of this treatment, and resulting larvae were bred at

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maturity. The F, females were fed aphids for 7 days and then treated as described above before the eggs used for the F, generation were collected. The F, progeny produced normal sex ratios, indicating that the bacterium was no longer causing male lethality. The second-generation treatment was employed to increase chances for complete curing, since this has been found to be necessary with related intracellular bacteria (8). The template was prepared from ovaries of the F, generation beetles. This line subsequently remained free of the male-killing phenotype for two generations, at which time the stock was terminated.

Ll-12R is a subline from LI-12 (infected) in which the male-killing trait spontaneously reverted to the wild type. This reversion was stably inherited. F, progeny from the original revertant line were used for genomic DNA isolation.

J3C is a subline from J3 treated with tetracycline in the same fashion as the LI-12C lines, used as a control for antibiotic treatment.

DNA isolation. DNA was isolated for PCR by our previously published methods (9). Ovaries from adult females were dissected in sterile distilled water and then thoroughly rinsed with sterile water. The tissue was transferred into a 0.5-ml Microfuge tube containing 0.3 ml of sterile zirconium beads (BioSpec Products), 200 R,l of Tris *(pH 8.0),* 10 [,1 of 20% sodium dodecyl sulfate, and 200 Rl of phenol. The solution was shaken in a mini-Bead Beater (BioSpec Products) for 3 min. Phases were then separated by a 10-min microcentrifugation, and DNA was ethanol precipitated and resuspended in 20 g,l of sterile Tris-EDTA buffer. Extreme care was taken throughout the procedure to avoid bacterial contamination. All solutions used were filter sterilized, and sterile techniques were used during dissections.

DNA amplification and isolation. Amplification of the bacterial 16S genes coding for rRNA (16S rDNA) was accomplished with primer pairs fDI and rPI, which are general prokaryotic primers that will amplify 16S rDNA from a wide range of bacteria (29). A volume of 1 to 2 g,l of a DNA sample was added to 5 [,1 of 10X buffer (Promega)-1 R1 of a nucleotide mix (10 mM each)-0.625 [,1 of 20 RM each primer-0.5 g,l of Taq polymerise (Promega)-distilled, deionized H_2O to a final volume of 50 [,1. Some reactions were performed with 25 R1 as the final volume, with other solutions adjusted proportionally. PCR cycling conditions in an Ericomp thermal cycler were 1 cycle of 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C; 35 cycles of 15 s at 92°C, 1 min at 55°C, and 3 min at 72°C; and I cycle of 15 s at 92°C, l min at 55°C, and 10 min at 72°C. All solutions were filter sterilized, and amplification controls were run for the PCR cocktail and for DNA prepared from known uninfected insects (strain AsymC from the parasitoid wasp *Nasonia vitripennis [8]).*

PCR products were detected on 1% agarose gel by ethidium bromide staining following electrophoresis. Under some circumstances, the product was restriction digested to detect 16S rDNA variants or heterogeneous 16S rDNA amplification from more than one bacterial type.

The 17-kDa protein gene was amplified by using the primer sequences and PCR cycling conditions reported by Williams et al. (33). As an additional control, DNA templates from the ovaries of *N. vitripennis* (AsymC) known to be uninfected and *N. vitripennis* (LabII) known to be infected with bacteria that are associated with cytoplasmic incompatibility in this wasp (8) were also used. These bacteria are phylogenetically related to those in the genus *Rickettsia* on the basis of 16S rDNA sequences (7).

Cloning and sequencing. PCR products were purified with the Geneclean kit (Bio 101, Inc., La Jolla, Calif.) and dissolved in 20 B,l of distilled, deionized H,O. The 16S rDNA product was restriction digested with *Sall* and *BamHI* and ligated into a similarly cut Bluescript Il KS vector (Stratagene). Sequencing was performed by using the Sequenase version 2.0 kit (U.S. Biochemical), and the samples were run on buffer gradient gels. Two clones were sequenced to detect potential PCR and sequencing artifacts.

The 17-kDa gene was similarly purified and then directly cloned into a T-tailed M13mp18 derivative (9a). Four clones were sequenced.

Restriction analysis. Restriction profiles were determined for the 16S rDNA sequences of the male-killing bacteria and 11 other bacteria *(Escherichia coli, Proteus vulgaris, Wolbachia persica, Rickettsia rickettsii, Rickettsia prowazekii, Rickettsia typhi, Ehrlichia ristici, Auaplasma marginale, Brucella abortus, Rochalimaea quintana,* and the cytoplasmic-incompatibility bacterium of *N. vitripennis).* The restriction enzyme *Xbal* was found to give a characteristic pattern for the male-killing bacterium and related rickettsias. *Xbal* cuts the 16S PCR product of the male-killing bacteria once, giving fragments 559 and 876 by in length. This enzyme was used to determine whether the PCR product and clone inserts contained the expected 16S product and whether PCR amplifications were homogeneous or heterogeneous for the 16S product. The PCR product was restricted with *Xbal,* electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and examined under UV fluorescence to detect the banding pattern.

Phylogenetic analysis. The 16S rDNA and 17-kDa sequences were manually aligned. Sequence similarity for each sequence pair was determined as the number of common base pair positions divided by the total number of positions. The estimated evolutionary distance was based on the number of substitutions per position (17).

Phylogenetic trees were constructed by the neighbor-joining method using the software package of Tamura (NJBOOT2 version 1.10) and by the branch and bound method of PAUP (28). The 16S rDNA sequences of the male-killing bacteria were manually aligned according to the information in the rDNA data base (22) by using conserved regions and secondary structure as guides (20). The aligned sequences were 1,797 positions long (including gaps). A gap was **treated as a fifth base in the phylogenetic analysis.** Inclusion or exclusion of gaps did not change the phylogenetic position of the lady beetle male-killing bacterium relative to the genus *Rickettsia* or related taxa. The data **set contained 650 informative sites after elimination of constant sites.** The aligned data set was analyzed with PAUP version 3,1 (28) to find the most parsimonious tree. In addition, 100 heuristic bootstrap replicates were run to obtain indices of support for particular nodes on the tree.

Nucleotide sequence accession numbers. The 16S rDNA and 17-kDa protein nucleotide sequences for the *A. bipunc*tata-associated (AB) bacterium are available in the GenBank data base under accession numbers U04163 and U04162, respectively.

RESULTS

Sequencing of the male-killing bacterium 16S rDNA. An initial PCR amplification was performed with genomic DNAs extracted from ovaries of an infected (Cambridge) and an uninfected (Unl) lines. General prokaryotic primers that amplify 16S rDNA from a wide range of bacteria were used. (18, 29). These primers encompass the majority of the 16S rDNA gene, from positions 27 to 1492 in the E. coli 16S rDNA numbering system (35). A product of the expected size was amplified from the infected template, whereas no product was

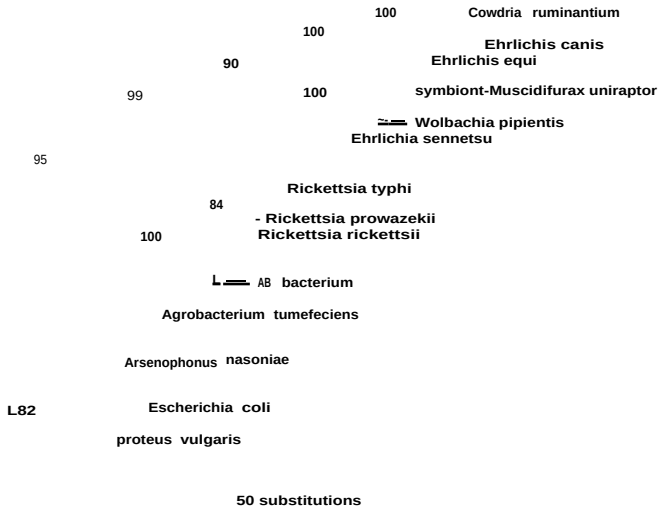


FIG. 1. Most parsimonious phylogenetic tree of the AB bacterium 16S rDNA using the branch and bound method of PAUP (28). Only variable sites for the shortest common sequence were included. Numbers indicate the levels of support for individual nodes based upon 100 bootstrap (branch and bound) replicates. Included in the phylogenetic tree is the cytoplasm ic-incompatibility bacterium *of Culex pipiens (W. pipientis)* and the bacterium associated with parthenogenesis in the parasitoid wasp *M. uniraptor.*

initially amplified from the uninfected (Unl) strain. The 16S product was cloned, and two clones were completely sequenced. The clones showed only one difference over the 1,435 bases.

On the basis of the 16S rDNA sequence, it is clear that this AB bacterium belongs to the alpha subdivision of *Proteobacteria (35).* The alpha proteobacteria include rhizobacteria (involved in nitrogen fixation in plants), agrobacteria (plant pathogens), and intracellular parasites such as the rickettsias. As in other alpha proteobacteria, the AB bacterium 16S rDNA sequence shows a reduced stem-loop structure at (E. *coli)* positions 200 to 218 and 450 to 490 and a characteristic thymine in place of guanine at position 648 (35).

A phylogenetic analysis comparing the AB bacterium sequence with the 16S rDNA sequences of 11 alpha subdivision and 3 gamma subdivision bacteria was performed by the branch and bound method of PAUP (28). As can be seen in Fig. 1, the most parsimonious tree places the AB bacterium in a monophyletic unit with other bacteria in the genus *Rickettsia.* In agreement with this tree, the AB bacterium 16S rDNA sequence is considerably more similar to those of *Rickettsia* than to those of other related clades (30). For example, the genetic distance between the AB bacterium and R. *rickettsii* (with each gap treated as a fifth base) is 0.068 versus 0.369 between the AB bacterium and *Wolbachia pipientis,* a member of a related sister clade (22, 27). All 100 heuristic runs place the AB bacterium within *Rickettsia* relative to other alpha bacteria.

A second major branch of rickettsia-like bacteria is apparent in the 16S rDNA phylogenetic tree. It includes species of *Ehrlichia* and *Cowdria* (mammalian disease agents vectored by arthropods) and bacteria associated with reproductive incompatibility and parthenogenesis in various insects. For example, the symbiont of *Muscidifurax uniraptor* shown in Fig. 1 is associated with parthenogenesis in that parasitoid wasp (27), and *W. pipientis is* associated with reproductive incompatibility in culex mosquitoes (36).

It should be pointed out that the bacterial genus *Wolbachia* appears to be polyphyletic. On the basis of 16S rDNA sequencing, Weisburg et al. (30) place W. *persica* (an associate of ticks) in the gamma subdivision, whereas 16S rDNA sequences amplified from culex mosquitoes known to be infected with W. *pipientis* place this bacterium in the alpha subdivision (22, 25) (Fig. 1). In addition, cytoplasmic incompatibility and parthenogenesis bacteria from diverse insects have been found to be phylogenetically close to *W. pipientis* on the basis of 16S rDNA (7, 22, 25) and other sequenced regions (3l a).

The position of the AB bacterium relative to those of members of the genus *Rickettsia is* not completely resolved by the phylogenetic analysis. The phylogenetic tree shows the AB bacterium branching off prior to *R. rickettsii,* with *R. prowazekii* and *R. typhi* most closely related to each other. However, the relative positions of *R. rickettsii* and the AB bacterium are less certain, since only 84 of 100 branch and bound runs placed the AB bacterium branch prior to *R. rickettsii.* Furthermore, the relative positions of the three other rickettsias within the clade are uncertain, as indicated by the low frequency of occurrences of particular nodes in the replicates. Hence, these relative positions are currently unresolved on the basis of the 16S rDNA sequences.

Restriction analysis of infected and uninfected strains. The 16S rDNA sequence information suggests that the AB bacterium is closely related to members of the genus *Rickettsia* but does not establish that this bacterium is associated with the cytoplasmically inherited male-killing trait in the ladybird beetle. To examine this association, total genomic DNA was prepared from the ovaries of four **male-killing lines and** four non-male-killing lines. Three male-killing lines had been collected from Cambridge, England, one was collected from Sussex, England, and one was collected from Leiden, The Netherlands. These had been confirmed as male killing on the basis of skewed sex ratios, small numbers of egg hatches, and presence of the male-killing bacterium in hemolymph (14). Two non-male-killing lines were natural isolates collected from the vicinity of Cambridge, England. In addition, one non-male-killing line (LI-12C) was a subline derived from MK line L1-12 and then cured of the male-killing trait by antibiotic treatment for two generations. This line was free of the male-killing bacteria on the basis of phenotype and cytological observation (14). A fourth non-male-killing line (L1-12R) was a spontanc-

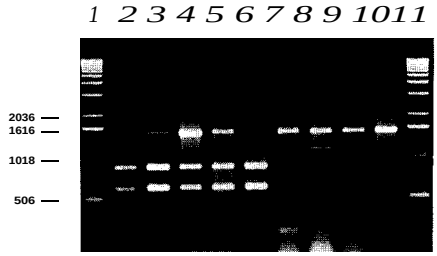


FIG. 2. *Xbal* digests of the 16S rDNA amplification products from ovarian templates of eight ladybird beetle strains. Lane l, standard; lane 2, insert from the sequenced AB bacterium clone; lanes 3 to 6, male-killing strains Sussex, Dutch, Cambridge, and L1-12, respectively; lanes 7 to 10, non-male-killing strains J3, Ll-12C, Unfl, and LI-12R, respectively; lane 11, standard. The characteristic *Xbal* digestion pattern of the AB bacterium is present in each male-killing strain but absent in uninfected, antibiotic-cured, and revertant strains. A different bacterial 16S rDNA product is also present in most strains. Molecular weights are indicated on the left.

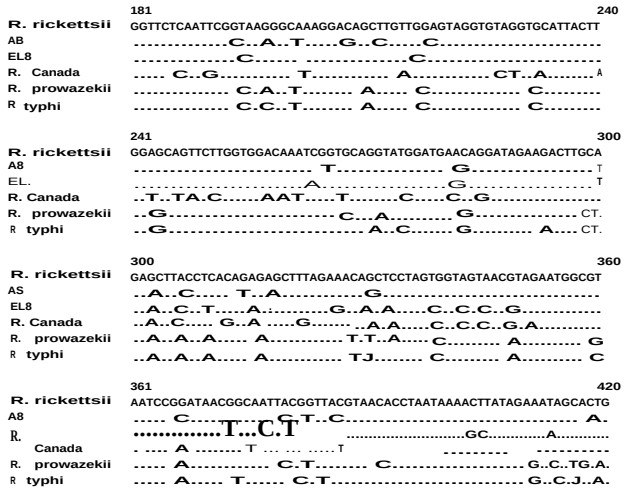


FIG. 3. Sequences of a 241-bp region of the 17-kDa protein gene from the AB bacterium and five *Rickettsia* species.

ous revertant to the non-male-killing phenotype derived from the male-killing line L1-12. The reversion had been stably transmitted for one generation prior to testing by PCR and has been stably inherited for two subsequent generations. This revertant line serves as an excellent control for association of the AB bacterium with the male-killing phenotype.

To determine whether the 16S rDNA sequence amplified from the different products was the AB bacterium sequence and, also, to determine whether the PCR amplification was homogeneous or heterogeneous for 16S rDNA **sequences, restriction profiles of 16S rDNA sequences from the** AB and other bacteria were first determined by using the 16S rDNA sequences in the ribosomal data base (21). *Xbal* was chosen because it gives a characteristic restriction pattern for rickettsias. *Xbal* cuts the AB bacterium product once, giving two fragments of 559 and 876 bp.

PCR amplifications using the general prokaryotic primers were performed on total DNA prepared from ovaries of the four infected and four uninfected strains. The 16S rDNA product was amplified from all strains infected with the male-killing bacterium and, in some cases, also from the uninfected strains, antibiotically cured strains, and the natural revertant strain. However, restriction digests with *Xbal* show that the AB bacterium is present only in strains showing the male-killing trait.

Figure 2 shows the restriction profiles for *Xba1.* As a control for the expected restriction pattern, the clone insert from the sequenced AB bacterium 16S rDNA was used after it was excised from the vector, gel purified, and digested with *Xbal.* As can be seen, L1-12, Sussex, Dutch, and Cambridge each yielded products showing the AB bacterium 16S rDNA digestion pattern. Among the uninfected lines, none contained the AB bacterium, on the basis of the restriction patterns of their amplification products. However, all templates contained at least one additional 16S rDNA product that was not cut with *Xbal.*

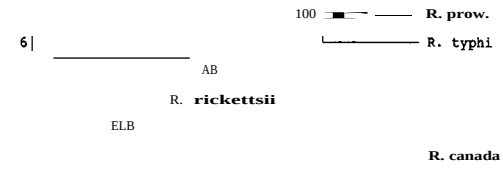
Although a 16S rDNA amplification product is present in L1-12C, the AB bacterium is absent. This result is consistent with the finding that this line was cured of the male-killing trait by antibiotic treatment. Equally interesting is the natural revertant strain (Ll-12R), which spontaneously lost the male-killing trait. This strain now lacks the AB bacterium (on the basis of PCR amplification), even though it was derived from a previously infected line. These findings strongly support the hypothesis that the AB bacterium is the causative agent of male killing in A. *bipuuctata.*

We are uncertain of the nature of the other bacteria from which 16S rDNA sequences were amplified from the ovarian template fromA. *bipunctata.* Although in earlier PCRs the 16S rDNA product was not consistently amplified from uninfected lines, in reactions conducted several months later uninfected lines did consistently yield the product, suggesting template contamination. **Alternatively, these bacteria could represent** a second cytoplasmically inherited microorganism in A. *bipunctata* or an infectious agent present in this species. We sequenced one clone from the 16S product of the Unfl strain. A phylogenetic analysis using neighbor joining (26) indicates that the bacterium is related to *Streptococcus bovis* and *Streptococcus salivarius,* showing 10.2 and 11.2% differences, respectively, over 1,184 base positions compared with the 2.2% difference between S. *bovis* and S. *salivarius* (data not shown).

Amplification and sequencing of the 17-kDa gene. The 17-kDa protein gene codes for an antigen common to members of the genus *Rickettsia (2, 3).* Subsequent studies have shown this protein to be present in members of the genus *Rickettsia* but not in other bacteria (3). Sequences of this gene have been determined from a number of rickettsias (2). Williams et al. (33) and Azad et al. (5) used a primer pair designed from conserved regions of this gene to amplify the product from the ELB agent, a rickettsia found in cat fleas. Using the same primers and PCR protocol as those of Williams et al. (33), we initially performed PCR on a template from one infected strain (Dutch) and one uninfected strain (Unfl). In addition, templates from uninfected N. *vitripennis* strains and those uninfected with cytoplasmic-incompatibility bacteria were used to determine whether the primers could amplify a sequence from this related group of bacteria.

Results yielded an amplification product only from the AB bacterium-infected strain. The product was approximately 430 bp, as expected from other rickettsias. No product was obtained from the incompatibility bacteria of N. *vitripennis,* in agreement with previous findings that this protein is not conserved among proteobacteria (3). An amplification product was cloned from the Dutch strain, and three clones were sequenced.

Figure 3 shows a 241-bp region of the AB bacterium



Distance Matrix

	Rr	AB	ELB	RC	Rp
AB	0.0747				
ELB	0.0788	0.0705			
RP	0.1494	0.1743	0.1411		
	0.1286	0.1120	0.1286	0.1992	
Rt	0.1245	0.1120	0.1328	0.2033	0.0373

FIG. 4. Phylogenetic tree of the AB bacterium and five other rickettsial 17-kDa protein sequences constructed on the basis of the region between base positions 181 and 420 (5). Also shown is the proportion difference matrix. Rr, *R. ricketsii;* Rc, *R. canada;* Rp, *R. prowazekii;* Rt, *R. typhi.*

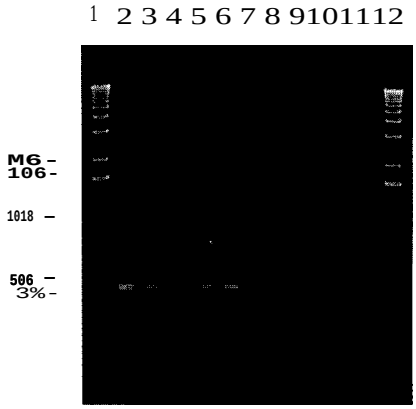


FIG. 5. Products of PCR amplification using the 17-kDa primers for the rickettsial common antigen gene. Lanes are the same as in Fig. 2 except for the following: lane 2 contains the amplification product from the original Cambridge strain template used for amplification and sequencing of the AB bacterium 16S rDNA, lane 11 contains the PCR solution control, and lane 12 contains the standard. All male-killing strains amplify the rickettsial product, whereas uninfected, cured, and revertant strains do not. Molecular weights are indicated on the left.

sequence aligned to those of other rickettsias: *R. rickettsii* (causative agent of rock mountain spotted fever), *R. typhi* (causative agent of murine typhus), *R. prowazekii* (causative agent of epidemic typhus), *R. canada* (isolated from ticks), and ELB agent (associated with cat fleas). The region chosen is one for which sequence data are available for all these bacteria (5).

The AB bacterium clearly falls among the rickettsias and actually has a higher sequence similarity to *R. rickettsii* than does *R. canada. A* phylogenetic analysis using both neighbor joining and parsimony was performed. The neighbor-joining tree constructed by using the genetic distance method of Jukes and Cantor (17) is shown in Fig. 4. As previously found, *R. prowazekii* and *R. typhi* form a closely related group. The neighbor-joining tree suggests that the AB bacterium is more closely related to this group than is *R. rickettsii,* in contrast to the 16S rDNA phylogenetic tree. However, as with the 16S rDNA tree, there is less confidence in the relative positions of these taxa. Of 100 replications (bootstrap runs) of the tree, these relative branch points occurred in only 56 and 53 replications, respectively. According to the neighbor-joining tree, *R. canada is* the most divergent of the bacteria. This is due primarily to the accumulation of base changes that are not present in any of the other taxa. Results clearly indicate that the AB bacterium is closely related to bacteria of the genus *Rickettsia.*

The parsimony analysis (PAUP) gave similar results, except that *R. canada* was grouped with the ELB agent. This difference in the trees is due primarily to the fact that PAUP excludes from the analysis base differences that are unique to a particular taxon.

To confirm an association of the AB bacterium with male killing, PCR amplification of the various templates was performed with the 17-kDa primers. These primers are much more specific for rickettsias than are the general prokaryotic 16S rDNA primers previously used. As can be seen in Fig. 5, only strains expressing the male-killing trait amplified a 17-kDa gene product, confirming that the AB bacterium is closely associated with the male-killing trait. *As* with the 16S rDNA product, the AB bacterium is absent from the antibiotic-cured strains and from the natural revertant strain.

DISCUSSION

Results clearly show that the AB bacterium is a close relative of bacteria in the genus *Rickettsia,* on the basis of both the 16S rDNA and the 17-kDa protein nucleotide sequences. The AB bacterium is associated with male killing in the two-spot ladybird beetle, A. *bipunctata.* Most known bacteria in the genus *Rickettsia* are arthropod-vectored causative agents of disease in vertebrates. *R. rickettsii is* the causative agent of rocky mountain spotted fever and is vectored by ticks. *R. prowazekii* causes epidemic typhus and is vectored by fleas. *R. typhi* causes murine typhus and is also vectored by fleas. *Rickettsia tsutsugamushi is* carried by mites and causes scrub typhus. *R. canada* was isolated from ticks. The ELB agent is found in cat fleas (1, 23).

In contrast, the AB bacterium occurs in ladybird beetles. It is cytoplasmically inherited through females and induces lethality of male, but not female, embryos. Other rickettsias also show cytoplasmic inheritance through the arthropod host. *R. typhi* shows low levels of transovarial transmission in fleas (11). The ELB agent appears to have significant rates of transovarial transmission in cat fleas (34).

It is interesting that the AB bacterium, which is a transovarially transmitted agent of disease in ladybird beetles, clusters with a number of causative agents of disease in mammals. There is a strong ascertainment bias toward detecting bacteria that cause disease in mammals. We expect that this detection bias plus the fact that few 16S rDNA sequences have been determined for strictly arthropod-associated bacteria explain the apparent high incidence of mammalian pathogens in this genus. The genus *Rickettsia* and closely related genera probably contain numerous other (strictly) arthropod-associated bacteria that do not cause disease in mammals.

Whether the AB bacterium can cause disease in vertebrates is unknown. We know of no cases of rickettsial infections in humans or other mammals being associated with ladybird beetles. Biting by ladybird beetles is rare (19). In addition, individuals who have worked closely with lady beetles for many years (e.g., M. E. N. Majerus and G. D. D. Hurst) report no unusual infections. However, the possibility of occasional infections by this bacterium cannot be ruled out at this time. If AB is proven to be apathogenic in mammals, it could prove useful for comparative studies of the development of pathogenicity in rickettsias.

Bacterially induced distortion of the sex ratio towards females is expected whenever bacteria have high rates of transovarial transmission through their hosts (9, 31). This is selectively favored by the bacterium because female hosts become a major vehicle for transmission of the bacterium to new hosts, i.e., their progeny. In the case of the AB bacterium, the selective advantage of male killing to the bacterium is believed to derive from the habit of egg cannibalism in ladybird beetles (15). Female progeny in infected lineages consume the unhatched eggs of their male siblings, and evidence strongly suggests that this egg cannibalism gives a significant survival advantage to neonate ladybird larvae in the first few days following hatching (14). Egg cannibalism may also provide a vehicle for transmission of the AB bacterium infections to other lineages, although this route of transmission has not yet been established whereas maternal transmission of the male-killing trait has been firmly demonstrated (15).

Only one *Rickettsia* species is known to cause sex ratio distortion in its arthropod host. *R. tsutsugamushi* has been shown to cause female-biased sex ratios in the scrub typhus mite, through which it is transovarially inherited (24). In this case, the bias appears to be due to a shift in the primary sex ratio towards females rather than to male embryo lethality. However, as with the AB bacterium the precise mechanism of sex ratio distortion is not known.

A closely related group of bacteria also cause sex ratio distortion in arthropods. *W. pipientis is* a cytoplasmically inherited bacterium that causes reproductive incompatibility among culex mosquitoes (36). Close relatives of this bacterium (on the basis of 16S rDNA sequences) have been shown to be associated with parthenogenesis in several species of wasps (27) and feminization in an isopod (25).

It is important to recognize that obligatory intracellular microorganisms such as rickettsia-like bacteria are likely to have significant levels of transovarial transmission in their arthropod hosts. This will not only have an important effect upon their population dynamics (and disease-causing potential), which is widely recognized (12, 34), but can also select for the bacterium to cause sex ratio distortion in its host arthropod (31). Sex ratio distortion can give a significant transmission advantage to such bacteria and, thus, enhance their maintenance in arthropod host populations. This effect has not been widely considered by epidemiologists studying arthropod-vectored pathogens.

ACKNOWLEDGMENTS

Thanks are extended to Bryant McAllister and Kent Reed for comments on the manuscript.

This research was supported by a USDA grant to J.H.W.

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