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Genotype Characterization of the Bacterium Expressing the Male-Killing Trait in the Ladybird Beetle *Adalia bipunctata* with Specific Rickettsial Molecular Tools

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The male-killing ladybird beetle (LB) bacterium (AB bacterium) was analyzed with specific rickettsial molecular biology tools in the LB *Adalia bipunctata* strains. Eight phenotype-positive LB strains showing mortality of male embryos were amplified with rickettsial genus-specific primers from the gene for citrate synthase (CS) and the gene for a 17-kDa protein and spotted fever group-specific primers from the gene for the 120-kDa outer membrane protein (*ompB*). The specificity of amplification was confirmed by Southern hybridization and the absence of the above-listed gene products in three phenotype-negative LB strains. Restriction polymorphism patterns of three examined amplicons from the CS gene, 17-kDa-protein gene, and *ompB* gene were identical among the eight phenotype-positive LB strains and were unique among all known rickettsiae of the spotted fever and typhus groups. Amplified fragments of the CS genes of the AB bacterium, *Rickettsia prowazekii* Breinl, *Rickettsia typhi* Wilmington, *Rickettsia canada* 2678, and *Rickettsia conorii* 7 (Malish) were sequenced. The greatest differences among the above-listed rickettsial and AB bacterium CS gene sequences were between bp 1078 and 1110. Numerical analysis based on CS gene fragment sequences shows the close relationships of the AB bacterium to the genus *Rickettsia*. Expanding of knowledge about rickettsial arthropod vectors and participation of rickettsiae in the cytoplasmic maternal inheritance of arthropods is discussed.

Various microorganisms living in endocellular symbiosis with arthropods cause sex ratio distortion and alteration of sex determination of their hosts, such as parthenogenesis (31), female-biased sex ratios (27), and sterility of offspring of crosses between infected individuals or between infected males and uninfected females (8, 23, 42). Alterations of arthropod sexuality are maternally (cytoplasmically) inherited. This correlates with the presence of bacterial endosymbionts in the gonadal tissue of arthropods (7, 16, 18, 21, 42), and the normal sex ratio can be restored by treatment with tetracycline antibiotics (8, 15, 17, 32, 42). This feature seems to be an evolutionary advantage for the survival of these microorganisms, as it increases the number of females who can transmit the bacteria to their offspring or favors female progenies which carry them (37).

Sequence analysis of the 16S rRNA gene has shown that these symbiotic bacteria belong to the α subdivision of the class *Proteobacteria* (22). They are most closely related to members of the genus *Rickettsia* and their relatives, are vectored by arthropods, and cause mammalian diseases. *Wolbachia pipientis* is the type species of the genus *Wolbachia* (36). It was the first bacterium associated with cytoplasmic incompatibility in the mosquito *Culex pipiens* as a result of interference with paternal chromosome incorporation in fertilized eggs and disruption of the first mitotic divisions (8). As yet unnamed bacteria are close to *Wolbachia* species in their 16S rDNA sequences and are associated with parthenogenesis in parasitoid wasps (31) and feminization in isopods (27). *Rickettsia tsutsugamushi*, which causes scrub typhus, is known to modify the sex ratio to female in the mites *Leptotrombidium fletcheri* and *Leptotrombidium arenicola* (25, 26).

Recently a cytoplasmically maternally inherited agent (AB bacterium) associated with male-killing expression in the ladybird beetle (LB) *Adalia bipunctata* (17, 19) was placed into a monophyletic unit with other bacteria in the genus *Rickettsia* (38). Identification of AB bacterium was based on phylogenetic analysis of its 16S rRNA gene sequence and the sequence of the conserved region of the 17-kDa-protein gene common to the members of genus *Rickettsia*.

The purpose of this study was to extend the genotype analysis of the newly described AB bacterium responsible for the male-killing trait in the LB *A. bipunctata*. The genus- and species-specific rickettsial molecular tools PCR-restriction fragment length polymorphism (RFLP) analysis, Southern blot hybridization, and sequencing of the citrate synthase (CS) gene amplicon followed by numerical analysis were used.

MATERIALS AND METHODS

Insects. LBs (*A. bipunctata*) either expressing [LB(+)] or not expressing [LB(−)] the male-killing trait were a gift from M. E. N. Majerus (Cambridge, England). Eight insects from an LB(+) line and three insects from an LB(−) line used in this study were preserved in ethanol and kept at room temperature. In preparation for study, the insects were dried on a filter paper and then in an airstream to eliminate ethanol residues. Dried insects were triturated in Eppendorf tubes, washed three times with 500 μl of sterile distilled water, and then centrifuged at 12,000 × *g* for 10 min. Then the pellet was suspended in 100 μl of water and boiled for 10 min. These samples were used for PCR amplification.

***Rickettsia* strains, cultivation, and purification.** *Rickettsia prowazekii* Breinl, *R. typhi* Wilmington, *R. canada* 2678, and *R. sibirica* K-1 (246) were obtained from the Rickettsial National Collection of Gamaleya Research Institute of Epidemiology and Microbiology (Moscow, Russia). The following strains were obtained

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TABLE 1. Oligonucleotide primers used for genotypic identification of rickettsial species

Species	Gene	Primer	Nucleotide sequence (5′ → 3′)	Amplified fragment size (bp)[a]	Author or reference[a]
R. prowazekii	CS	RpCS.877p	GGGGGCCTGCTCACGGCGG	381	Regnery et al. (24, 41)
		RpCS.1258n	ATTGCAAAAAGTACAGTGAACA		
R. rickettsii	17-kDa genus-common antigen	Rr17.A25p	GAATTCTAAAAACCATATACTATT	534	Baird et al. (2, 5)
		Rr17.C508n	CCAATTCACAACTTGCCAT		
		Rr17.61p	GCTCTTGCAACTTCTATGTT	434	Williams et al. (2, 39)
		Rr17.492n	CATTGTTCGTCAGGTTGGCG		
	120-kDa protein antigen (*ompB*)	RrBG.1-21	GGCAATTAATATCGCTGACGG	650	B. E. Anderson (13)
		RrBG.2-20	GCATCTGCACTAGCACTTTC		
		RrBG.3-19	GGGGTTAATGTTGATACTG	615	B. E. Anderson (13)
		RrBG.4-18	CACCTAAAACAACCACGG		
		RrBG.5-23	GCTATCGGTATCACTAAAACTG	710	B. E. Anderson (13)
		RrBG.6-22	CGGGAGCGATTAGAAGTTTACACGG		
	190-kDa protein antigen (*ompA*)	Rr190.70p	ATGGCGAATATTTCTCCAAAA	532	Regnery et al. (1, 24)
		Rr190.602n	AGTGCAGCATTCGCTCCCCCT		

[a] Applies to each pair of primers.

from the American Type Culture Collection (Rockville, Md.): *R. conorii* 7 (Malish; ATCC VR-613), *R. rickettsii* R (Bitteroot; ATCC VR-891), and *R. akari* MK (Kaplan; ATCC VR-148). *R. parkeri* maculatum 20 and the *R. montana* tick strain were kindly supplied by D. H. Walker (University of Texas, Galveston). *R. australis* Phillips, *R. belli* 369L42-1, *R. slovaca* 13-B, and *R. rhipicephali* 3-7-6 were obtained from G. A. Dasch (Naval Medical Research Institute, Bethesda, Md.). Unnamed strain Mtu5 was isolated in Unité des Rickettsies (Marseille, France) (6).

All of the rickettsiae were cultivated in Vero cell monolayers maintained in a 5% CO_2 atmosphere at 35°C for *R. prowazekii* and *R. typhi* and at 32°C for spotted fever group rickettsiae. Dulbecco modified minimal essential medium (Eurobio, Les Ulis, France) supplemented with 10% fetal bovine serum (Eurobio) and 2% glutamine (Seromed, Berlin, Germany) was used for *R. prowazekii*, minimal essential medium (Eurobio) supplemented with 2% fetal bovine serum and 2% glutamine was used for *R. typhi*, and minimal essential medium supplemented with 4% fetal bovine serum and 1% glutamine was used for all other rickettsiae. Five to seven days after inoculation, the infected cells were Gimenez stained (14) and harvested.

The rickettsiae were purified by the combination of differential centrifugation, sonication, centrifugation through a 25% sucrose cushion, and ultracentrifugation in a 28 to 45% linear density gradient of Renografin (Radioselectan, Schering, France) as previously described (3). Purified rickettsiae were suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for DNA isolation.

DNA preparation. Rickettsial DNA was obtained by phenol and phenol-chlorophorm extraction as described elsewhere (20).

R. canada was not cultivated in this study. DNA from *R. canada* was obtained from an infected yolk sac suspension extracted with Chelex 100 resin (Bio-Rad, Hercules, Calif.) by boiling for 30 min as previously described (30).

PCR amplification and restriction analysis. PCR amplification was performed with the oligonucleotide primers (Eurogentec S.A., Seraing, Belgium) listed in Table 1. Reactions were performed with 100 μl as the final volume using 0.5 U of AmpliTaq DNA polymerase (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, N.J.) and appropriate reagents. A tube containing distilled water instead of DNA was included in all reactions as a negative control. PCR amplification was performed in a PREM III thermal cycler (Lep Scientific, Flobio, Courbevoie, France) under conditions optimized for each primer pair, as follows: 35 cycles of denaturation at 95°C for 20 s, annealing at 48°C for 30 s, and extension at 60°C for 2 min (for the CS gene fragment, *ompA* gene fragment, and *ompB* gene fragment) (11, 24); 32 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 70°C for 2 min (for the 534-bp DNA fragment of the 17-kDa-protein gene) (5); or 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 2 min, and extension at 70°C for 2 min (for the 434-bp DNA fragment of the 17-kDa-protein gene) (39).

Restriction profiles of amplified DNA fragments were compared following restriction endonuclease digestion using *Alu*I (Promega, Paris, France) and *Rsa*I (New England Biolabs, Beverly, Mass.) as previously described (11, 24).

DNA probe preparation and Southern hybridization. DNA fragments amplified from the CS gene of *R. prowazekii* and from the *ompB* gene of *R. rickettsii* were used as DNA probes. DNA was labeled with digoxigenin-conjugated 11-dUTP by using a random priming system according to the manufacturer's instructions (Nonradioactive DNA Labeling and Detection Kit; Boehringer Mannheim).

For Southern hybridization, electrophoresed DNA was depurinated, transferred to a Hybond-N membrane (Amersham International plc, Amersham, United Kingdom) in 20× SSC transfer buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) for 20 h, and baked at 80°C for 2 h. Hybridization was carried out at 68°C overnight. The hybrids were detected by enzyme-linked immunoassay with antidigoxigenin antibodies conjugated with alkaline phosphatase (Boehringer Mannheim) according to the manufacturer's instructions.

Sequencing. CS DNA fragments amplified by PCR using 7 pmol of each of the primers were sequenced. 5′-Biotin-labeled primers and 5′-fluorescein-labeled primers (Eurogentec) were derived from the same CS gene region and had the same nucleotide sequences as those used for the standard PCR.

5′-Biotin-labeled DNA fragments were affinity purified by 30 min of incubation at room temperature with streptavidin-coated magnetic beads (Dynabeads M-280; Biosys S.A., DYNAL, Oslo, Norway) according to the manufacturer's instructions. Immobilized DNA duplexes were denaturated in 0.1 M NaOH by 5 min of incubation at 37°C. The isolated single strands were then sequenced with 3 pmol of corresponding 5′-fluorescein-labeled primer by using the AutoRead sequencing kit (Pharmacia LKB, Uppsala, Sweden). The sequencing products were denaturated for 5 min at 85°C before loading onto the 0.35-mm 5.7% (wt/vol) polyacrylamide gel (Ready Mix Gel, A.L.F. grade; Pharmacia). The gel was run for 3 h at 45 W, 45 mA, and 1,500 V at a constant temperature of 45°C

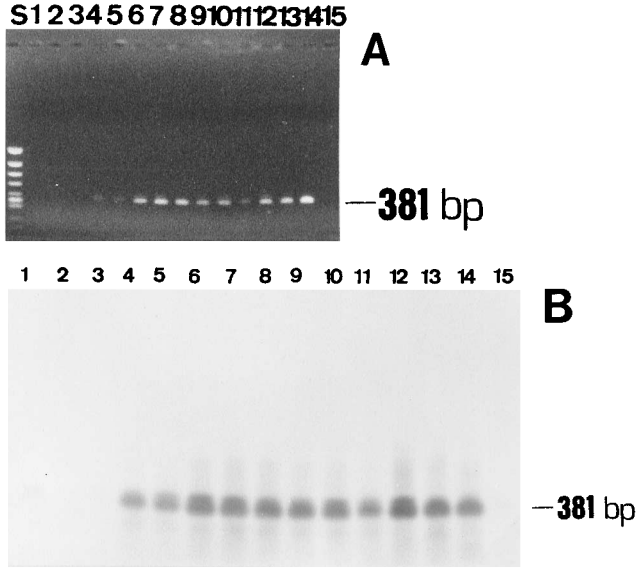

FIG. 1. Genotype identification of the AB bacterium with the RpCS.877p and RpCS.1258n primer pair. (A) Ethidium bromide-stained agarose gel of PCR-amplified DNA; (B) Southern blot hybridization with digoxigenin-labeled *R. prowazekii* DNA probe, prepared from the CS primer pair-amplified DNA fragment. Lanes 1 to 3, LB(−) strains; lanes 4 to 11, LB(+) strains; lanes 12, *R. prowazekii*; lanes 13, *R. typhi*; lanes 14, *R. canada*; lanes 15, negative control; lane S, molecular size markers.

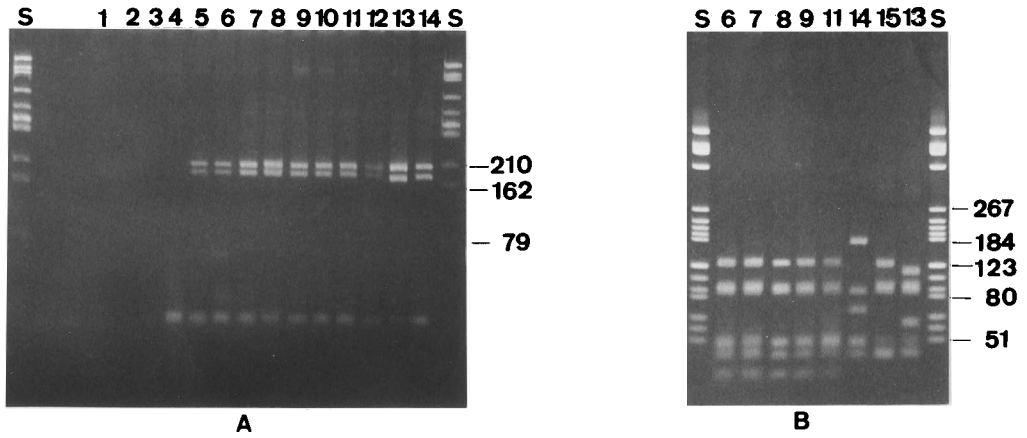

FIG. 2. Ethidium bromide-stained polyacrylamide gel electrophoretograms of restriction endonuclease *Rsa*I-digested (A) and *Alu*I-digested (B) DNA PCR amplified with the RpCS.877p and RpCS.1258n primer pair. Lane 1, negative control; lanes 2 to 4, LB(−) strains; lanes 5 to 12, LB(+) strains; lanes 13, *R. prowazekii*; lanes 14, *R. typhi*; lane 15, *R. canada*; lanes S, molecular size markers (sizes are indicated on the side in base pairs).

in 1× Tris-borate-EDTA buffer with an Automate Laser Fluorescent DNA Sequencer (Pharmacia LKB).

Numerical analysis. The CS gene fragment sequences were aligned to the sequence of bp 877-to-1258 fragment of the *R. prowazekii* E CS gene (GenBank accession number M17149) by using the multisequence alignment program CLUSTAL, which is part of the BISANCE software package (10). Relationships were inferred with version 3.42 of the PHYLIP (J. Felsenstein, Seattle, Wash.) software package. A dendrogram was constructed by using DNABOOT bootstrap confidence intervals on DNA parsimony. The program was repeated in 100 replicates to estimate grouping of studied strains.

Nucleotide sequence accession numbers. The CS gene fragment nucleotide sequences for the AB bacterium, *R. prowazekii* Breinl, *R. typhi* Wilmington, *R. canada* 2678, and *R. conorii* Malish will be available in the GenBank database under accession numbers U20242, U20244, U20245, U20241, and U20243, respectively. The GenBank accession number for the *gltA* gene of *Coxiella burnetii* is M36338, and that for the *gltA* gene of *Escherichia coli* is J01619.

RESULTS

PCR-RFLP analysis with the genus-specific CS oligonucleotide primer pair. All LB(+) strain samples were amplified with CS primer pairs, whereas no product was amplified from the LB(−) strains (Fig. 1A). The amplified product of 381 bp was similar to amplicons of *R. prowazekii*, *R. typhi*, and *R. canada*. Specificity of PCR amplification was confirmed by Southern hybridization with DNA probes derived from the *R. prowazekii* Breinl CS amplicon (Fig. 1B). The pattern of polymorphism of the PCR-amplified fragment of LB(+) strains was similar to *R. prowazekii* and *R. typhi* patterns after digestion with *Rsa*I restriction endonuclease (Fig. 2). After digestion with *Alu*I, migration profiles of restricted products of LB(+) strains were different from those of *R. prowazekii*, *R. typhi*, and *R. canada* (Fig. 2).

PCR-RFLP analysis with the primer pairs derived from the 17-kDa-protein gene. Two sets of primer pairs derived from the 17-kDa-protein gene were used. Oligonucleotide primers Rr17.61p and Rr17.492n are designed from the conserved region of the 17-kDa common rickettsia protein. They yielded amplicons from six of the eight LB(+) strains (Fig. 3A). The 434-bp amplicons of the test samples were similar in size to those of *R. prowazekii*, *R. typhi*, and *R. canada*. The *Rsa*I restriction profile of the LB(+) amplicon was similar to that of *R. typhi* but differed from those of *R. prowazekii*, *R. canada*, and *R. belli* (Fig. 3B).

Another primer pair, Rr17.A25p and Rr17.C508n, used for the amplification of the entire 17-kDa-antigen gene in several spotted fever group rickettsiae gave negative results when LB(+) samples were tested.

PCR-RFLP analysis with the group- and species-specific *ompB* and *ompA* gene-derived primers. All LB(+) strain samples but not LB(−) samples yielded amplicons with the RrBG.5-23 and RrBG.6-22 primers. The positive PCR amplification result was confirmed by an intensive signal of all LB(+) samples in Southern hybridization with a DNA probe derived from the *R. rickettsii* amplicon (Fig. 4). In addition, two of three LB(−) strains were detected in the hybridization reaction.

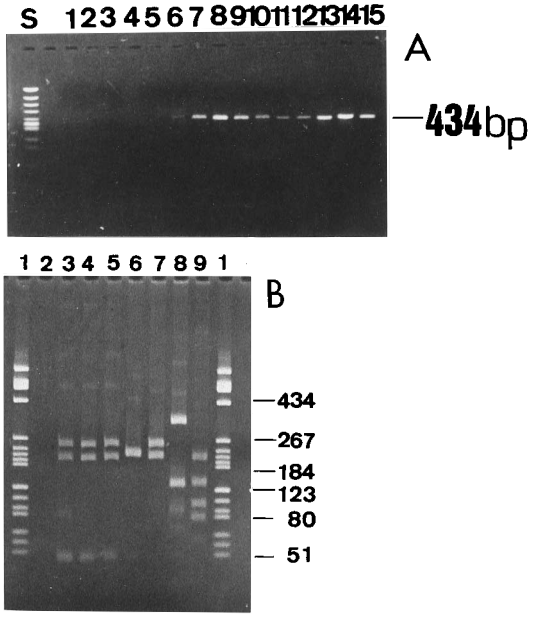

FIG. 3. PCR amplification with the 17-kDa-protein gene-derived primer pair. (A) Ethidium bromide-stained agarose gel of PCR-amplified DNA fragments. Lane 1, negative control; lanes 2 to 4, LB(−) strains; lanes 5 to 12, LB(+) strains; lane 13, *R. prowazekii*; lane 14, *R. typhi*; lane 15, *R. canada*; lane S, molecular size markers. (B) Polyacrylamide gel electrophoretogram of *Rsa*I-digested amplicons. Lanes 1, molecular size markers (sizes are indicated in base pairs); lane 2, negative control; lanes 3 to 5, LB(+) strains; lane 6, *R. prowazekii*; lane 7, *R. typhi*; lane 8, *R. canada*; lane 9, *R. belli.*

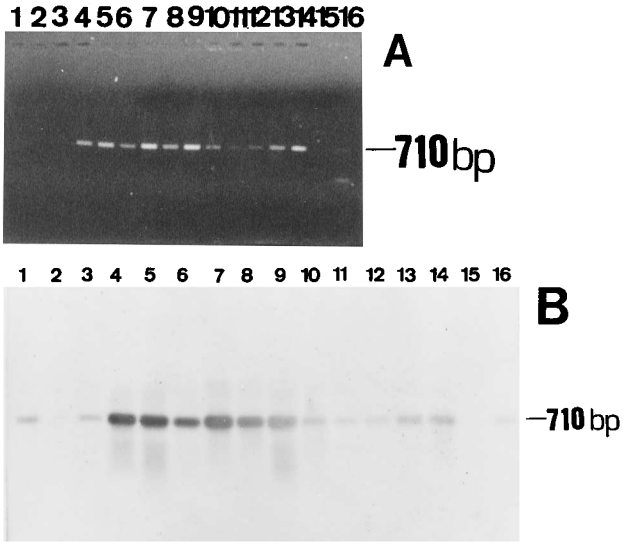

FIG. 4. Genotype identification of the AB bacterium with the RrBG.5-23 and RrBG.6-22 primer pair derived from the *ompB* gene. (A) Ethidium bromide-stained agarose gel of PCR-amplified DNA fragments; (B) Southern blot hybridization with the digoxigenin-labeled *R. rickettsii* DNA probe amplified by PCR. Lanes 1 to 3, LB(−) strains; lanes 4 to 11, LB(+) strains; lanes 12, *R. prowazekii*; lanes 13, *R. canada*; lanes 14, *R. conorii*; lanes 15, *R. belli*; lanes 16, *R. typhi.*

The 710-bp amplicon of LB(+) strain samples resulting from use of the RrBG.5-23 and RrBG.6-22 primer pair was similar in size to the amplicon of *R. prowazekii*, *R. conorii*, and *R. canada* obtained with the same primers. *Rsa*I restriction profiles of RrBG.5-23 and RrBG.6-22 primer pair amplicons of LB(+) samples differed from the restriction patterns of *R. prowazekii*, *R. canada*, *R. rickettsii*, *R. sibirica*, *R. slovaca*, *R. conorii*, strain Mtu5, *R. montana*, *R. rhipicephali*, *R. parkeri*, *R. akari*, and *R. australis* used in our study (Fig. 5). *R. typhi* and *R. belli* DNAs were not amplified with this pair of primers.

PCR amplification of LB samples was negative with the RrBG.1-21 and RrBG.2-20, RrBG.3-19 and RrBG.4-18, and Rr190.70p and Rr190.602n primer pairs.

CS gene fragment sequencing. The RpCS.877p and RpCS.1258n primer pair-amplified DNAs of four LB(+) strains (numbers 6, 7, 8, and 9) were sequenced and compared with the known sequence of *R. prowazekii* E and with the sequences of *R. prowazekii* Breinl, *R. typhi* Wilmington, *R. canada* 2678, and *R. conorii* 7 obtained in our study (Fig. 6A).

LB(+) strains had identical sequences which were easily differentiated from those of other rickettsiae, especially in the region corresponding to bp 1078 to 1110 of the *R. prowazekii* gene. In 348 nucleotides aligned, AB bacteria were found to have 33 differences with *R. prowazekii*. Of these, 24 are conservative differences, but the nine substitutions at nucleotide positions 957, 1027, 1037 and 1038, 1079, 1199 and 1100, 1110, and 1137 lead to changes of seven amino acids in the putative CS structure. Two leucines, two asparagines, one valine, one serine, and one proline in *R. prowazekii* were changed for three isoleucines, one asparagine, one leucine, one lysine, and one glycine in AB bacterium CS (Fig. 6B). Comparison with *R. typhi* and *R. canada* revealed 31 and 30 nucleotide substitutions, respectively, and eight and six amino acid changes (Fig. 6). The sequenced fragment of the AB bacterium reveals close homology (93.7%) to *R. conorii*, though 22 nucleotide substitutions which gave five amino acid differences between their putative CS structures were found (Table 2 and Fig. 6).

A numerical analysis based on nucleotide sequence data was performed (Fig. 7). This method separated *R. prowazekii* and *R. typhi*, which form a closely related group divergent from other compared strains. *R. conorii* and the AB bacterium were clustered in a monophyletic group and separated from *R. canada*, which had an intermediate position between typhus and spotted fever group rickettsiae.

DISCUSSION

The genus *Rickettsia* is composed of strict intracellular bacteria which have as vectors insects, ticks, or mites and can cause severe or mild disease accompanied by fever in mammals (36). Sequence analysis of the DNA encoding 16S rRNA showed that these bacteria are closely related among themselves (29, 34) and include bacteria involved in sex ratio bias in LBs (38).

Our purpose in this work was to apply the PCR-RFLP tool

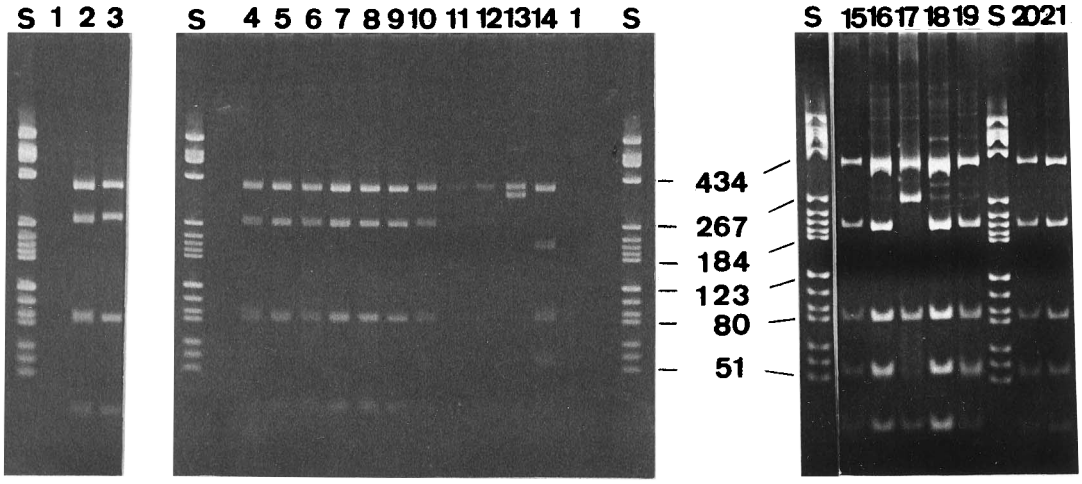

FIG. 5. Ethidium bromide-stained polyacrylamide gel electrophoretogram of restriction endonuclease *Rsa*I-digested DNA PCR amplified by using the RrBG.5-23 and RrBG.6-22 primer pair. Lane 1, negative control; lane 2, *R. akari*; lane 3, *R. australis*; lanes 4 to 11, LB(+) strains; lane 12, *R. prowazekii*; lane 13, *R. canada*; lane 14, *R. conorii*; lane 15, strain Mtu5; lane 16, *R. montana*; lane 17, *R. rhipicephali*; lane 18, *R. parkeri*; lane 19, *R. rickettsii*; lane 20, *R. slovaca*; lane 21, *R. sibirica*; lanes S, molecular size markers (sizes are indicated in base pairs).

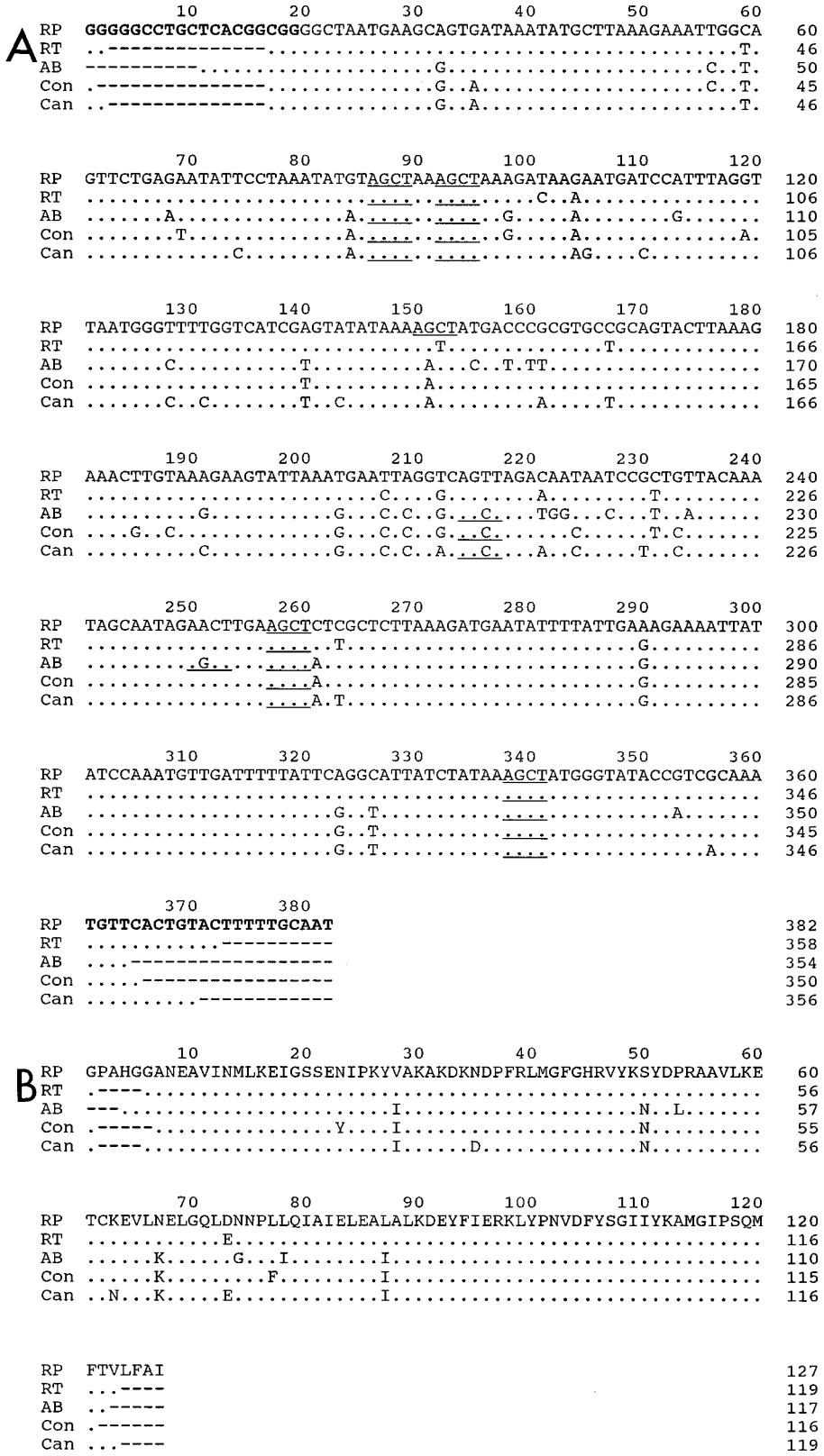

FIG. 6. Nucleotide sequence of (A) and predicted amino acid sequence from (B) the CS gene fragment amplified by PCR. The primer sequence is indicated by boldface letters. *Alu*I restriction sites are underlined. Homology is indicated by dots. Abbreviations: RP, *R. prowazekii*; RT, *R. typhi*; AB, AB bacterium; Con, *R. conorii*; Can, *R. canada.*

TABLE 2. Estimated nucleotide matching between the compared CS gene fragments

Bacterium	% Nucleotide matching with:			
	R. typhi	AB bacterium	*R. conorii*	*R. canada*
R. prowazekii	96.8	90.5	92.8	91.1
R. typhi		91.1	93.1	92.2
AB bacterium			93.7	91.4
R. conorii				94.0

used for rickettsiae to identify the AB bacterium. These data extend the available genotype characterization of the AB bacterium on the basis of the sequence of the 16S rRNA gene and the conserved region of the gene encoding the 17-kDa protein common to the spotted fever and typhus fever rickettsia groups (38). According to this phylogenetic analysis, the 16S rRNA gene sequence of the AB bacterium is more similar to that of spotted fever group rickettsiae, whereas the sequence of the gene encoding part of the 17-kDa protein clustered with those of *R. prowazekii* and *R. typhi.*

CS, the main metabolic enzyme of rickettsial bacteria, is highly conserved among the different species of this genus (35). At the same time, its nucleotide sequence has a variable region between bp 877 and 1258 which allows recognition of different isolates, as shown by PCR-RFLP analysis (11, 24). In our study sequence-based analysis of PCR-obtained fragments of four LB(+) strains (numbers 6, 7, 8, and 9) revealed considerable similarity of the AB bacterium to *R. prowazekii*, *R. typhi*, *R. canada*, and *R. conorii*. The most variable part in the CS gene amplicon sequence among the AB bacterium and the above-listed rickettsiae was between bp 1078 and 1110. Numerical analysis based on the CS gene sequence showed the close relatedness of the AB bacterium to the bacteria of the genus *Rickettsia*. AB bacteria were grouped with spotted fever group rickettsiae, represented in our study by *R. conorii*, and both of them were separated from typhus group rickettsiae. On the basis of comparing sequences within the evolutionary lineage rate of nucleotide substitutions, the DNABOOT method applied to CS sequence data indicates the phylogenetic position

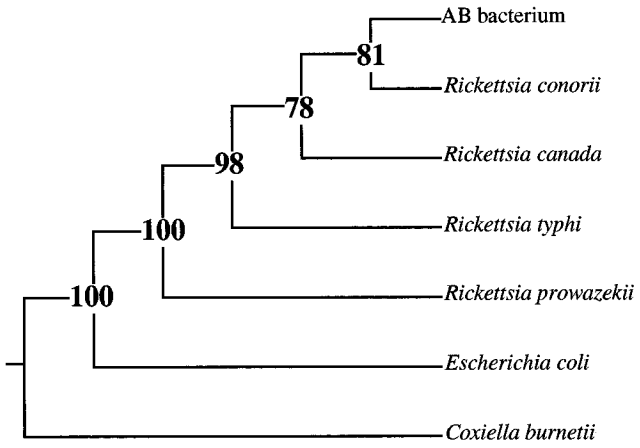

FIG. 7. Phylogenetic relationship dendrogram of the AB bacterium and typhus and spotted fever group rickettsiae established from the nucleotide sequences of the CS gene fragment. The tree was constructed by using DNABOOT bootstrap confidence intervals with the DNA parsimony method and the PHYLIP software package. The numbers at the forks indicate the number of times the group consisting of the species to the right of that fork occurred among the 100 bootstrap replicates.

of the AB bacterium, which is in good agreement with that determined by the 16S rRNA gene sequence analysis (29, 38).

It is necessary to emphasize that available data of sequence-based analysis of the 16S rRNA gene, the gene encoding part of the 17-kDa protein, and the CS gene are limited and these data for all members in the genus *Rickettsia* are necessary in order to carry out a complete phylogenetic analysis and make definitive conclusions about the phylogenetic position of each rickettsial isolate. The use of DNA sequence data for bacterial strain differentiation has a major advantage, in that sequences generated in different laboratories can easily be compared by using a computer database (5, 27, 28, 38).

Using genotype PCR-RFLP analysis, we determined that eight LB(+) strains showing mortality of male embryos were infected with rickettsiae since the rickettsial genus-specific CS gene RpCS.877p and RpCS.1258n primer pair and group-specific *ompB* gene RrBG.5-23 and RrBG.6-22 primer pair gave amplicons that were confirmed by Southern hybridization with *R. prowazekii*- and *R. rickettsii*-specific DNA probes. Positive hybridization of LB(−) strains with the *R. rickettsii* DNA probe probably related to different stages of beetle infection which are not expressed phenotypically. Their PCR-amplified products were not seen in agarose gel because of the different sensitivities of UV detection and Southern hybridization. Six of eight LB(+) strains were specifically amplified with the Rr17.61p and Rr17.492n primer pair from the gene encoding the 17-kDa common protein of spotted fever and typhus group rickettsiae. They had the 434-bp PCR-generated fragment similar to that described in early studies of the AB bacterium (38). PCR amplification of LB(+) strains was negative with the group-specific primers derived from other regions of the *ompB* gene and from the *ompA* gene. The lack of these sequences indicates that the AB bacterium differs from most of the spotted fever group rickettsiae.

Restriction polymorphism patterns of RpCS.877p and RrCS.1258n pair- and RrBG.5-23 and RrBG.6-22 pair-obtained amplicons appeared to be identical in all LB(+) strains and were unique in comparison with those of known rickettsiae of the spotted fever and typhus groups (11, 24). Only one similarity with known rickettsiae was found. The restriction profile of the Rr17.61p and Rr17.492n pair-obtained amplicon of the AB bacterium was similar to that of *R. typhi*. But the *R. typhi* restriction *Alu*I polymorphism pattern with the CS gene-derived primer pair was different from that of the AB bacterium. The *ompB* gene-derived pair primers did not give an amplicon when used with *R. typhi*, in contrast to the AB bacterium and another rickettsia used in our study. Thus, the RFLP pattern of the AB bacterium appeared to be different from those of all known rickettsiae.

Sex ratio bias from the normal sex ratio to female predominance is a well-known phenomenon among the members of the animal kingdom, especially arthropods. Cytoplasmically inherited genes affect feminization and are associated with bacteria that are transmitted only by females. These bacteria have often been described as rickettsia-like agents, and some of them have been classified in the genus *Wolbachia*, which is closely related to the *Rickettsia* group of bacteria (22, 27, 28).

The cytoplasmically maternally inherited AB bacterium expressing the male-killing trait in *A. bipunctata* is the second member of the genus *Rickettsia* associated with a female bias in sex ratio. To date, only *R. tsutsugamushi* is known as a causative agent of sex ratio distortion to female bias in the scrub typhus arthropod vectors, the *Leptotrombidium* mites: *L. fletcheri*, *L. arenicola*, and *L. deliense* (25, 26). In *Leptotrombidium pallidum*, *R. tsutsugamushi* is vertically transmitted only from parents to the progeny without a sex ratio affect (33). Typical

rickettsia species are not known to cause sex bias of arthropod vectors, but transovarial transmission has been observed for *R. typhi* (12) and the ELB agent in fleas (4) and for spotted fever group rickettsiae in ticks (9).

Identification of the AB bacterium affecting feminization in *A. bipunctata* as a rickettsia expands our knowledge about the arthropod vectors of rickettsiae and rickettsia distribution in nature and also raises a question about the width of the distribution of this rickettsia and rickettsiae with similar abilities. It may be supposed that *A. bipunctata* is not a single Coccinellidae species carrying rickettsiae. The presence of male-killing maternally inherited agents sensitive to tetracycline has been shown in two species of Japanese Coccinellidae: *Menochilius sexmaculants* and *Harmonia axyridis* (see the references in reference 17). The latter is distributed widely in the Altaj region of Russia (43). In earlier studies rickettsia-like organisms in a wide range of insects were described (40).

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