JOURNAL OF BACTERIOLOGY, May 1994, p. 3081–3084
0021-9193/94/$04.00+0

Vol. 176, No. 10

16S Ribosomal DNA Sequence Identities of β-Proteobacterial Endosymbionts in Three *Crithidia* Species

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Received 6 January 1994/Accepted 14 March 1994

The 16S ribosomal DNA sequences of endosymbionts from the trypanosomatid protozoa (*Crithidia* spp.) are most homologous to that of *Bordetella* spp. This finding extends the polyphyletic origin of endosymbionts for the first time to the β *Proteobacteria*. Biased base transitions and compensatory mutations of the symbionts' sequences that may contribute to their identity in the three *Crithidia* spp. are noted.

Obligate intracellular symbionts abound in nature, especially in protozoa and insects (9). How these symbionts evolve with their eukaryotic hosts is a question of particular interest that may bear on the origins of cell organelles, such as mitochondria and chloroplasts (7).

Endosymbionts have been found in the insect trypanosomatid protozoa *Crithidia oncopelti*, *C. deanei*, and *C. desouzai* (11). They naturally contain in each cell a single obligate intracellular symbiont as a cytoplasmic membrane-bound entity (13, 16, 23) which contains DNA and ribosomes typical of bacteria (2, 25). The ratio of one symbiont per protozoan is usually maintained apparently by their synchronous division. There is no lateral transmission of the symbionts in the protozoal populations. Symbionts cannot be grown outside of their hosts, and the hosts become nutritionally fastidious when rendered symbiont free (3, 5). The symbionts are thought to be heterogeneous (5), as they appear to supply their hosts with different nutrients, e.g., hemin, purines, vitamins, and/or amino acids.

The endosymbionts of proteobacterial origin so far examined from other sources are all in the α and γ subdivisions (15) and often coevolve with their eukaryotic hosts (14, 27). We report here that endosymbionts from three *Crithidia* spp. have identical small-subunit ribosomal DNA (rDNA) sequences that are most similar to that of *Bordetella bronchiseptica* in the β division of the *Proteobacteria*.

Cells. *C. oncopelti*, *C. deanei*, and *C. desouzai* were originally isolated from different insect species and grown in Difco brain heart infusion broth (11). Cloned cells of all species were rendered symbiont free by treatment with 800 μg of chloramphenicol per ml under conditions previously described (3, 4). The absence of endosymbionts in the symbiont-free lines was verified by microscopy (25) and by CsCl-Hoechst 33258 ultracentrifugation of the total DNA (4).

Cloning and sequencing of *Crithidia* symbiont small-subunit rDNA and the ITS region. Both strands of the 16S rRNA gene (1,532 bp) and the downstream internal transcribed spacer (ITS) region (583 bp) of the symbionts in question were completely sequenced, except for 8 bp at the 5′ end. We began this work by isolating the total DNA from symbiont-containing and symbiont-free strains of all three *Crithidia* spp. as previously described (4, 22). The eubacterial rRNA gene conserved sequences (10) were used as primers for PCR amplification of homologous fragments from the total DNA isolated under standard conditions for 30 cycles (4). As reported previously (4), PCR products of the expected size were obtained from symbiont DNAs and DNAs from symbiont-containing cells of the three *Crithidia* spp. but not from cognate symbiont-free strains. The amplified products were cloned into PCR cloning vector pGEM-T (Promega). The cloned fragments were used to screen genomic libraries prepared from all three species as follows: symbiont DNA was separated from *Crithidia* nuclear and mitochondrial DNAs by ultracentrifugation in a CsCl-Hoechst 33258 gradient (4), partially cut with *Pst*I, sized to about 5 kb by centrifugation in an NaCl gradient, and cloned in pBluescript (Stratagene) (22). Positive clones obtained from these libraries were each found to contain a 5-kb insert, as expected. Symbiont 16S rRNA genes and the ITS regions from these clones were sequenced by the dideoxyribonucleotide chain termination method (United States Biochemical Co. Sequenase version 2.0). Initial sequence analysis of these inserts indicated that they are identical, each containing part of the 16S rDNA (nucleotides [nt] 498 to 1532), the ITS region (nt 1533 to 2115), and the 23S rDNA plus its downstream 3′ flank (data not shown). Genomic DNAs from these inserts were used to sequence most of the 16S rRNA gene (nt 9 to 1532) and the ITS (nt 1533 to 2115), the first 492 bases being obtained from the PCR products.

Similarity of the *Crithidia* symbionts to *B. bronchiseptica* in rRNA gene sequence with notable biased base transitions and compensatory mutations. The sequences of the symbiont genes from all three *Crithidia* spp. are identical, with a G+C content of 52.7%. Comparison by using the Bestfit program of the Genetics Computer Group (University of Wisconsin, Madison) showed that the symbiont gene is 95.67% identical to that of *B. bronchiseptica* (GenBank accession no. X57026). Biased base transitions and compensatory mutations were noted in these genes. Of the 67 base substitutions, 42 in the *Crithidia* symbiont sequence are G-to-A (35%) and C-to-T (28%) transitions. Base transitions biased in these directions have been found previously in other symbiont genes (18). From the secondary structures of the *Crithidia* symbiont and *B. bronchiseptica* 16S rRNAs constructed on the basis of the *Escherichia coli* model (17) (data not shown), biased base transitions of the symbiont gene in relation to compensatory mutations (8) and its G · U intermediates (21) are evident. The stalk regions of the two sequences have 42 base substitutions, of which 24 in the symbiont sequence form 12 complementary pairs and all

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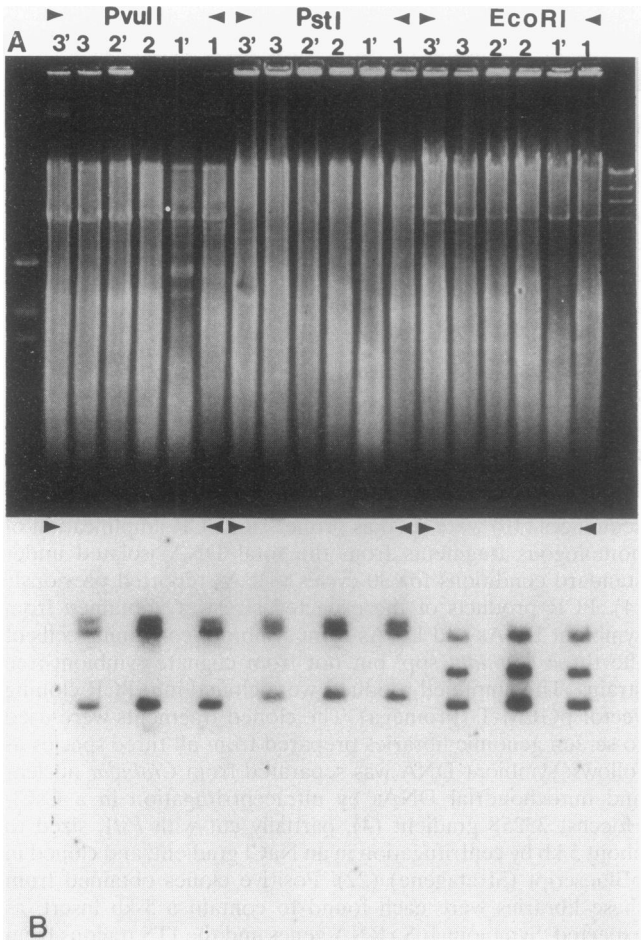

FIG. 1. Specificity and identity of the endosymbiont 16S rDNAs in three *Crithidia* spp. Total DNAs isolated from both symbiont-containing (lanes 1, 2, and 3) and symbiont-free (lanes 1′, 2′, and 3′) cells were digested with endonucleases (e.g., *Eco*RI, *Pst*I, and *Pvu*II), separated in gels, and probed with a plasmid containing the 492-bp fragment of the 16S rDNA gene. Panels: A, ethidium bromide-stained gel; B, Southern blot. Lanes: 1, 2, and 3, total DNAs from symbiont-containing cells of *C. oncopelti*, *C. deanei*, and *C. desouzai*, respectively; 1′, 2′, and 3′, total DNAs from the corresponding symbiont-free cells of the same three species.

except one conform to the expected biased base transitions. Further, single-base substitutions account for 11 changes from G · U intermediates in *B. bronchiseptica* to complementary pairs in the symbiont and two changes in the reverse direction.

The *Crithidia* symbiont sequence contains two putative tRNA genes, encoding $tRNA^{Ile}$ and $tRNA^{Ala}$, at nt 1663 to 1739 and 1771 to 1846 in the 16S-23S ITS region (data not shown). Similar tRNA genes have been found in the ITS region of rRNA operons of many other eubacteria, including *Bordetella* spp. (12). The symbiont tRNA genes contain the 14 universally conserved bases; unlike the primitive chloroplast tRNA genes (28), they have no introns in the anticodon region according to their secondary structures (data not shown).

Genomic identity and sequence specificity of the symbiont genes in the three *Crithidia* spp. Southern blot analysis provided further evidence for the symbiont origin and structural identity of the 16S rDNA among all three species (Fig. 1). The total DNAs isolated from the three symbiont-containing cells (lanes 1, 2, and 3) and the corresponding symbiont-free cells (lanes 1′, 2′, and 3′) were digested with different restriction enzymes (*Eco*RI, *Pst*I, and *Pvu*II) and probed with PCR-amplified fragments of the ITS region or the 16S rDNA, i.e., the first-492-bp fragment or a downstream fragment between nt 924 and 1540. Probes for Southern blot analysis (22) were labeled with [^{32}P]dCTP by using hexanucleotide random primers (USB), and hybridization was performed at 65°C overnight; this was followed by four washings of the blots under stringent conditions, i.e., 0.1% sodium dodecyl sulfate and 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). As expected, these probes hybridized with DNA from symbiont-containing cells (Fig. 1, lanes 1 to 3) but not with those from symbiont-free cells (Fig. 1, lanes 1′ to 3′). The identical patterns from symbiont-containing cells of the three *Crithidia* spp. (Fig. 1, lanes 1 to 3) suggest that the rRNA genes of their symbionts are identical not only in sequence but also in chromosomal organization. Perhaps divergence of the host protozoa into three species is too recent (11) for the evolution of their symbiont rRNA genes. The biased base transitions and compensatory mutations may also contribute to the sequence conservation observed among the three symbionts.

Phylogenetic affiliation of *Crithidia* symbionts with β *Proteobacteria*. Parsimony and distance analyses of the 16S rDNA sequences by PHYLIP (6) further confirmed that the *Crithidia* symbionts are *Proteobacteria* of the β division and that they are phylogenetically closest to *B. bronchiseptica* (Table 1 and Fig. 2). The 16S rRNA gene sequences of the *Crithidia* symbionts and 18 other purple bacteria were first aligned by the Genetics Computer Group Pileup program. Species selected for comparison included intracellular symbiotic and parasitic bacteria (the legend to Fig. 2 contains the GenBank accession numbers of their sequences). A total of 1,237 sites from these sequences were subjected to DNAdist analysis in accordance with Kimura's model (8). The distance matrix generated (Table 1) was then used to construct the tree by the neighbor-joining method, and the unrooted tree was drawn by Drawtree (6)

TABLE 1. Evolutionary distances and percent similarities of some β-division *Proteobacteria*[a]

Organism	*Eikenella* sp.	*Neisseria gonorrhoeae*	*Pseudomonas testosteroni*	*Rhodocyclus gelatinosus*	*Crithidia endosymbiont*	*B. bronchiseptica*	*A. faecalis*
Eikenella sp.		0.0601	0.1599	0.1443	0.1470	0.1250	0.1323
N. gonorrhoeae	0.9355		0.1697	0.1494	0.1354	0.1194	0.1411
Pseudomonas testosteroni	0.8585	0.8551		0.0934	0.1492	0.1276	0.1477
Rhodocyclus gelatinosus	0.8680	0.8659	0.9158		0.1127	0.0984	0.1197
Crithidia endosymbiont	0.8711	0.8803	0.8764	0.8988		0.0457	0.0743
B. bronchiseptica	0.8866	0.8933	0.8895	0.9140	0.9567		0.0530
A. faecalis	0.8812	0.8738	0.8740	0.8876	0.9309	0.9456	

[a] Evolutionary distances (above the diagonal) were calculated by the DNAdist program in PHYLIP by using Kimura's two-parameter model. Percent similarities (below the diagonal) are based on Bestfit (Genetics Computer Group) output.

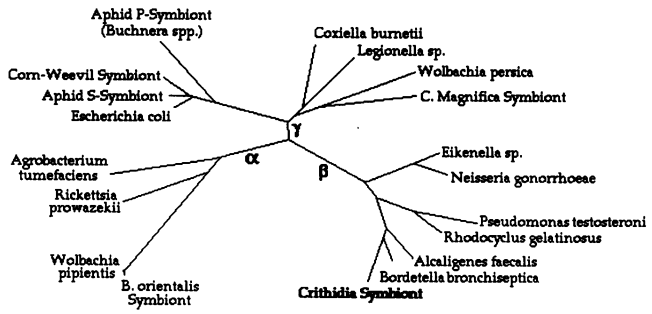

FIG. 2. Phylogenetic position of the *Crithidia* endosymbionts. This phylogenetic tree was constructed by using the PHYLIP programs (6). The 16S rRNA gene sequences of these symbionts and 18 other purple bacteria were aligned and subjected to DNAdist analysis in accordance with Kimura's model. The distance matrix generated (Table 1) was then used to construct the tree as described in the text. The GenBank nucleotide sequence accession numbers for the sequences of the organisms named (clockwise from *Coxiella burnetti*) are as follows: M21291, X60080, M21292, M99446, L06165, X07714, M11224, M60682, M22508, X57026, L29303, M85266, X61768, M21789, M11223, J01695, M27040, M85270, and M27039.

(Fig. 2). Analysis of the same sequences or 490 informative sites by DNApars, DNAcomp, or DNApenny produced trees with identical topologies. The results obtained are completely consistent with the prokaryote small-subunit rDNA tree generated by the Ribosomal Database Project (19). In addition, DNApars analysis of 500 bootstrapped replicas resulted in a >97.9% level of confidence for the branches including the *Crithidia* symbionts, *B. bronchiseptica*, and *Alcaligenes faecalis* (Fig. 2).

The *Crithidia* symbionts are the sole endosymbionts reported so far in the β division (26). The insect endosymbionts from aphids (14) and tsetse flies (20) fall into the γ division, whereas those from weevils (1), *Paramecium caudatum* (24), and other sources, like *Wolbachia* spp. (15), as well as intracellular pathogens, e.g., legionellae and rickettsiae, are all in either the α or the γ division (Fig. 2). The β division does include extracellular animal pathogens, such as *Bordetella*, *Neisseria*, and *Eikenella* spp. The grouping of *Crithidia* endosymbionts with these extracellular pathogens suggests that further studies may find intracellular parasites and even cell organelles in the β division.

The difference of *Crithidia* symbionts from all other noncultivable endosymbionts in phylogenetic affiliation is of interest. The evolutionary significance of this finding awaits further study of similar symbionts from additional species of trypanosomatid protozoa. The finding of identical symbionts in three presumably different *Crithidia* spp. is unexpected. The implication of this finding in symbiont-host coevolution cannot be fully assessed until further analysis of the host gene sequences.

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number of the *Crithidia* symbiont sequence determined in this study is L29303.

We thank Wanderley de Souza and M. J. Soares for providing the *Crithidia* spp., Joe Felsenstein for help with PHYLIP, and Tony Dean, Michael Fennewald, John Keller, and Michael Votkin for suggestions.

REFERENCES

1. **Campbell, B. C., T. S. Bragg, and C. Turner.** 1992. Phylogeny of symbiotic bacteria of four weevil species (*Coleoptera*: *Curculionidae*) based on analysis of 16S ribosomal DNA. Insect Biochem. Mol. Biol. **22**:415–421.
2. **Chang, K.-P.** 1974. Ultrastructure of symbiotic bacteria in normal and antibiotic-treated *Blastocrithidia culicis* and *Crithidia oncopelti*. J. Protozool. **21**:699–707.
3. **Chang, K.-P., and W. Trager.** 1974. Nutritional significance of symbiotic bacteria in two species of hemoflagellates. Science **183**:531–532.
4. **Du, Y., and K.-P. Chang.** 1993. PCR amplification and sequence analysis of pre-ribosomal RNA genes from *Crithidia* endosymbionts, p. 499–504. *In* S. Sato, M. Ishida, and H. Ishikawa (ed.), Endocytobiology V. Tübingen University Press, Tübingen, Germany.
5. **Dutra de Menezes, M. C. N., and I. Roitman.** 1991. Nutritional requirements of *Blastocrithidia culicis*, a trypanosomatid with an endosymbiont. J. Protozool. **38**:122–123.
6. **Felsenstein, J.** 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle.
7. **Gray, M. W., and W. F. Doolittle.** 1982. Has the endosymbiont hypothesis been proven? Microbiol. Rev. **46**:1–42.
8. **Kimura, M.** 1991. Recent development of the neutral theory viewed from the Wrightian of theoretical population genetics. Proc. Natl. Acad. Sci. USA **88**:5969–5973.
9. **Krieg, N. R., and J. G. Holt (ed.).** 1984. Bergey's Manual of Systematic Bacteriology, vol. 1, p. 795–833. The Williams & Wilkins Co., Baltimore.
10. **McLaughlin, G. L., D. K. Howe, D. R. Biggs, A. R. Smith, P. Ludwinski, B. C. Fox, D. N. Tripathy, C. E. Frasch, J. D. Wenger, and R. B. Carey.** 1993. Amplification of rDNA loci to detect and type *Neisseria meningitidis* and other eubacteria. Mol. Cell. Probes **7**:7–17.
11. **Motta, M. C. M., A. M. C. Cava, P. M. F. Silva, J. E. Fiorini, M. J. Soares, and W. de Souza.** 1991. Morphological and biochemical characterization of the trypanosomatids *Crithidia desouzai* and *Herpetomonas anglusteri*. Can. J. Zool. **69**:571–577.
12. **Muller, M., and A. Hildebrandt.** 1993. Nucleotide sequences of the 23S rRNA genes from *Bordetella pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. avium* and their implications for phylogenetic analysis. Nucleic Acids Res. **21**:3320.
13. **Mundim, M. H., I. Roitman, M. A. Hermans, and E. W. Kitajima.** 1974. Simple nutrition of *Crithidia deanei*, a reduviid trypanosomatid with an endosymbiont. J. Protozool. **21**:518–521.
14. **Munson, M. A., P. Baumann, M. A. Clark, L. Baumann, N. A. Moran, D. J. Voegtlin, and B. C. Campbell.** 1991. Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. J. Bacteriol. **173**:6321–6324.
15. **Neefs, J., Y. Van de Peer, P. De Rijk, S. Chapelle, and R. De Wachter.** 1993. Compilation of small ribosomal subunit RNA structures. Nucleic Acids Res. **21**:3025–3049.
16. **Newton, B. A., and R. W. Horne.** 1957. Intracellular structures in *Strigomonas oncopelti*. I. Cytoplasmic structures containing ribonucleoprotein. Exp. Cell Res. **13**:563–574.
17. **Noller, H. F.** 1984. Structure of ribosomal RNA. Annu. Rev. Biochem. **53**:119–162.
18. **Ohtaka, C., and H. Ishikawa.** 1993. Accumulation of adenine and thymine in a groE-homologous operon of an intracellular symbiont. J. Mol. Evol. **36**:121–126.
19. **Olsen, G. J., R. Overbeek, N. Larsen, T. L. Marsh, M. J. McCaughey, M. A. Maciukenas, W.-M. Kuan, T. J. Macke, Y. Xing, and C. R. Woese.** 1992. The ribosomal database project. Nucleic Acids Res. **20**(Suppl.):2199–2200.
20. **O'Neill, S. L., R. H. Gooding, and S. Aksoy.** 1993. Phylogenetically distinct symbiotic microorganisms reside in *Glossina* midgut and ovary tissues. Med. Vet. Entomol. **7**:377–383.
21. **Rousset, F., M. Pelandakis, and M. Solignac.** 1991. Evolution of compensatory substitutions through G·U intermediate state in *Drosophila* rRNA. Proc. Natl. Acad. Sci. USA **88**:10032–10036.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Soares, M. J., M. C. M. Motta, and W. de Souza.** 1989. Bacterium-like endosymbiont and virus-like particles in the trypanosomatid *Crithidia desouzai*. Microbios Lett. **41**:137–141.
24. **Springer, N., W. Ludwig, R. Amann, H. J. Schmidt, H.-D. Gortz, and K.-H. Schleifer.** 1993. Occurrence of fragmented 16S rRNA in

an obligate bacterial endosymbiont of *Paramecium caudatum*. Proc. Natl. Acad. Sci. USA **90:**9892–9895.
25. **Tuan, R. S., and K.-P. Chang.** 1975. Isolation of intracellular symbiotes by immune lysis of flagellate protozoa and characterization of their DNA. J. Cell Biol. **65:**309–323.
26. **Woese, C.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
27. **Wren, H. N., J. I. Johnson, and D. G. Cochran.** 1989. Evolutionary inferences from a comparison of cockroach nuclear DNA and DNA from their fat-body and egg endosymbionts. Evolution **43:**276–281.
28. **Xu, M. Q., S. D. Kathe, H. Goodrich-Blair, S. A. Nierzwicki-Bauer, and D. A. Shub.** 1990. Bacterial origin of a chloroplast intron: conserved self-splicing group I introns in cyanobacteria. Science **250:**1566–1570.