CURRENT MICROBIOLOGY Vol. 41 (2000), pp. 300–304
DOI: 10.1007/s002840010138

Current Microbiology
An International Journal

Secondary Endosymbionts of Psyllids Have Been Acquired Multiple Times

MyLo L. Thao,[1] Marta A. Clark,[1] Linda Baumann,[1] Eric B. Brennan,[2] Nancy A. Moran,[3] Paul Baumann[1]

[1]Microbiology Section, University of California, Davis, CA 95616-8665, USA
[2]Plant Biology Section, University of California, Davis, CA 95616-8536, USA
[3]Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, USA

Received: 2 May 2000 / Accepted: 8 June 2000

Abstract. Previous studies have established that psyllids (Hemiptera, Psylloidea) contain primary endosymbionts, designated as *Carsonella ruddii*, which cospeciate with the psyllid host. This association appears to be the consequence of a single infection of a psyllid ancestor with a bacterium. Some psyllids may have additional secondary (S-) endosymbionts. We have cloned and sequenced the 16S–23S ribosomal RNA genes of seven representative psyllid S-endosymbionts. Comparison of the S-endosymbiont phylogenetic trees with those of *C. ruddii* indicates a lack of congruence, a finding consistent with multiple infections of psyllids with different precursors of the S-endosymbionts and/or possible horizontal transmission. Additional comparisons indicate that the S-endosymbionts are related to members of the *Enterobacteriaceae* as well as to several other endosymbionts and insect-associated bacteria.

Psyllids (Hemiptera, Psylloidea) are insects that utilize plant sap, a diet rich in carbohydrates but deficient in essential amino acids [3, 19]. Within the body cavity of psyllids is a structure called a bacteriome, which consists of a multinucleate syncytial region within which are uninucleate cells called bacteriocytes [3, 6]. The latter contain vesicles within which are polymorphic bacteria with a Gram-negative cell wall [7, 29, 31]. These bacteria appear to be present in all psyllids and are called the primary (P-) endosymbionts. Many psyllids, in addition, have morphologically different bacteria within the syncytial region, and these are called secondary (S-) endosymbionts [6, 7, 31]. Since the P-endosymbionts appears to be present in all psyllids, they must be able to fulfill all the requirements of the endosymbiotic association. By analogy with *Buchnera*, the extensively studied endosymbionts of aphids, it is probable that one of the functions of the P-endosymbionts is the synthesis of essential amino acids for the psyllid host [2, 3, 13]. The function (if any) of the S-endosymbionts is not known.

The phylogenetic affiliation of psyllid endosymbionts has recently been studied by molecular methods. With the sequence of 16S ribosomal DNA (rDNA), it has been established for four species of psyllids that the P-endosymbiont consists of a unique lineage within the gamma-3 subdivision of the *Proteobacteria* [14, 26]. Among its unusual properties is a low guanine + cytosine (G + C) content of the 16S rDNA. In contrast, the S-endosymbionts from three psyllid species appear to be related to the members of the *Enterobacteriaceae* [14, 26]. By using oligonucleotide probes to 16S ribosomal RNA and in situ hybridization, it has been shown that in the psyllid *Anomoneura mori* the P-endosymbiont is located in the bacteriocytes, while the S-endosymbiont is the syncytial region [14].

We have extended these studies by a phylogenetic analysis of rDNA from 32 species of psyllids [29]. Since 16S rDNA [about 1.5 kilobases (kb)] does not contain an adequate amount of information for the differentiation between closely related endosymbionts [3] we have used both 16S and 23S rDNA (about 4.0 kb). In all cases, following amplification of 16S-23S rDNA by the polymerase chain reaction (PCR), we detected bands of 4.0 kb which correspond to the P-endosymbiont. Phylogenetic analysis of the 16S-23S rDNA as well as a host gene indicated congruence between the two trees, a result consistent with a single infection of a psyllid ancestor with a bacterium and subsequent cospeciation of the

Correspondence to: P. Baumann; *e-mail:* pabaumann@ucdavis.edu

P-endosymbiont and the host [29]. We have given the lineage consisting of the psyllid P-endosymbionts the designation *Carsonella ruddii* [29]. In the case of 22 species of psyllids, following amplification by PCR, we also detected additional single bands of about 4.3–4.7 kb which correspond to putative S-endosymbionts [29]. In the present study we have determined the nucleotide (nt) sequence of the 16S-23S rDNA of the S-endosymbionts of seven representative psyllid species. In addition, to gain a better understanding of the phylogenetic position of the S-endosymbionts, we have sequenced the DNA upstream of the 16S rDNA gene of one S-endosymbiont.

The results of past morphological studies as well as more recent phylogenetic analyses of insect endosymbionts suggest that the psyllid-*C. ruddi* association is an example of a common type of endosymbiotic association which is the result of a single infection and subsequent cospeciation of the host and the endosymbiont [3, 9, 11, 22, 23]. In a variety of insects, superimposed on this association may be another one involving S-endosymbionts. Both morphological and molecular studies suggest that the S-endosymbionts are diverse, and the associations may be the result of multiple infections or horizontal transmission [1, 3, 6, 15].

Materials and Methods

General methods. Most of the methods used have been described in detail by Thao et al. [29]. These include the isolation of whole psyllid DNA, amplification of endosymbiont 16S-23S rDNA by PCR, cloning into pBluescript (Stratagene, La Jolla, CA), and nt sequence determination. The oligonucleotide primers described in [29] were used for the PCR amplification of 16S-23S rDNA from seven representative psyllid species. The 4.3–4.7 kb bands, corresponding to the S-endosymbiont, were cloned and sequenced. In a previous study [29] we obtained the sequence of the 4.0-kb band corresponding to *C. ruddii* (P-endosymbiont). The abbreviations used for the S-endosymbionts as well as the GenBank accession numbers are given in Table 1.

Methods of phylogenetic analysis. In analyses involving 16S-23S rDNA, the intergenic space between 16S and 23S rDNA was removed and the resulting sequences aligned by using Pileup of the GCG package [16]. All analyses were heuristic parsimony searches with 1000 bootstrap replicates with PAUP 4b.1 [28]. Similar methods were used for analyses involving only 16S rDNA. The accession numbers of all the sequences used are given in Table 1.

The 9.6-kb DNA fragment of S-Gbr. Restriction enzyme and Southern blot analyses of DNA from Gbr, by using the probe shown in Fig. 4, indicated that upon digestion with *Xba*I and *Eco*RI, a prominent 9.3-kb band was present as well as a band of 5.6 kb of lesser intensity. The prominent band corresponded to *C. ruddii*, and the other band to the S-endosymbiont. Following digestion with *Xba*I and *Eco*RI, the Gbr DNA preparation was electrophoresed and the 5.6-kb band (Fig. 3) eluted and cloned into *Xba*I and *Eco*RI-digested λZAP (Stratagene) by the methods previously described [21]. This fragment was sequenced, and the 3′-region was found to be identical to the 16S rDNA of the 4.6-kb S-Gbr DNA fragment containing 16S and 23S rDNA, allowing assembly of a 9.6-kb sequence (Fig. 3).

Results and Discussion

Phylogenetic analyses. Fig. 1(a) presents a phylogenetic tree of the S-endosymbionts based on 16S-23S rDNA as well as other related bacteria within the gamma-3 subdivision of the *Proteobacteria* for which sequences of both 16S and 23S rDNA are available (Table 1). The S-endosymbionts form two major clusters (A, B) which are related to, but distinct from some of the well-studied members of the *Enterobacteriaceae* (cluster C). Cluster A is composed of two closely related strains, while the five organisms of cluster B are quite diverse.

Figure 1(b) is a phylogenetic tree based only on 16S rDNA. The analysis included, in addition to the S-endosymbionts of psyllids, other related endosymbionts and insect-associated bacteria as well as several other bacteria within the gamma-3 subdivision of *Proteobacteria*. Clusters A, B, and C are maintained in both the 16S and the 16S-23S analyses. Cluster A contains closely related organisms and includes species of the bacterial genus *Arsenophonus* (Fig. 1b). *A. triatominarum* is an endosymbiont of the triatomine bug that has not been cultured [20]; *A. nasoniae* causes a change in the sex ratio of a parasitic wasp and has been cultured on laboratory media [17]. A possible relationship between *Arsenophonus* and *Proteus vulgaris* (Fig. 1b) has been previously noted [17, 20]. Cluster B, which is composed of rather diverse organisms, contains several previously studied P- and S-endosymbionts of insects [12, 18] (Fig. 1b). These include *Sodalis glossinidius*, the S-endosymbiont of tsetse, which has been cultured on laboratory media [12]. Also in this cluster and related to S-Cmy is the S-endosymbiont of the psyllid *A. mori*, which has been localized to the syncytial space by in situ hybridization [14]. This cluster also contains the S-endosymbiont of a mealybug [15], as well as the P-endosymbionts of carpenter ants (*Blochmannia*) [24, 25] and a weevil [18]. Both clusters A and B are distinct from the S-endosymbionts of a species of whitefly and a species of aphid [10, 30].

Although Clusters A and B are strongly supported by phylogenetic analyses of both 16S-23S combined (Fig. 1a) and 16S rDNA alone (Fig. 1b), the relationships between these two clusters and other bacteria within the *Enterobacteriaceae* are not well resolved on the basis of 16S rDNA (Fig. 1b). The association with insect hosts appears to have arisen separately in ancestors of Clusters A and B, since these appear to be independently derived from bacteria not associated with insects.

Comparisons of the phylogeny of S- and P-endosymbionts. Figure 2 compares the phylogenetic trees obtained from an analysis of 16S-23S rDNA from the S-endosymbionts and P-endosymbionts (*C. ruddii*) of

Table 1. List of sequences used in this study and their source

Abbreviations[a]	Psyllid species[b]	Bacterial designation (source of psyllids)	GenBank 16S-23S	GenBank 16S	GenBank 23S	Reference
S-Ain	*Aphalaroida inermis*	S-endosymbiont (Arizona, USA)	AF263556			This study
S-Bco	*Bactericera cockerelli*	S-endosymbiont (Arizona, USA)	AF263557			This study
S-Boc	*Blastopsylla occidentalis*	S-endosymbiont (Arizona, USA)	AF263558			This study
S-Cmy	*Cacopsylla myrthi*	S-endosymbiont (Malta)	AF263559			This study
S-Csc	*Calophya schini*	S-endosymbiont (California, USA)	AF263560			This study
S-Gbr	*Glycaspis brimblecombei*	S-endosymbiont (California, USA)	AF263561			This study
S-Hte	*Heteropsylla texana*	S-endosymbiont (Arizona, USA)	AF263562			This study
	Anomoneura mori	S-endosymbiont (Japan)		AB013087		[14]
	Trioza magnoliae	S-endosymbiont (South Carolina, USA)		AF077607		[26]
		Arsenophonus nasoniae		M90801		[17]
		Arsenophonus triatominarum		U91786		[20]
		Blochmannia sp.		X92550 X92552		[24, 25]
		Sodalis glossinidius (S-endosymbiont)		M99060		[12]
		Endosymbiont of *Sitophilus oryzae*		AF005235		[18]
		S-endosymbiont *Acyrthosiphon pisum*		M27040		[30]
		S-endosymbiont *Antonina crawii*		AB030020		[15]
		S-endosymbiont of *Bemisia argentifolii*		Z11926		[11]
		Acetobacter intermedius		Y14694	Y14680	
		Aeromonas hydrophila		AF099022	X67943	
		Citrobacter freundii		AJ233408	U77928	
		Escherichia coli	AE000474			
		Erwina carotovora		Z96091		
		Klebsiella pneumoniae		AJ233420	X87284	
		Proteus vulgaris		X07652		
		Ruminobacter amylophilus		AB004908		
		Salmonella enterica		X80681	U77919	
		Serratia entomophila		AJ233427		
		Yersinia enterocolytica		Z47828	U77925	

[a] Abbreviations used in text.
[b] Host plant of psyllids given in [29].

psyllids. Previously it has been established that the phylogeny of *C. ruddii* is congruent with that of the psyllid host, a finding consistent with vertical evolution of the host and the P-endosymbiont [29]. In contrast to this result, the tree obtained for the S-endosymbionts differs greatly from that obtained for *C. ruddii*. For example, *C. ruddii* Gbr and Boc are closely related but have S-endosymbionts from the A and B clusters, respectively. Similarly, many conflicts are found between the phylogenies of *C. ruddii* and S-endosymbionts of the B cluster. These results suggest multiple infections of psyllids with ancestors of the S-endosymbionts and/or horizontal transfer. The geographic source of six of the psyllid species included in this study is similar (Table 1), and it is possible that S-endosymbionts are occasionally transferred between psyllid species with overlapping geographic ranges.

Recently it has been shown that the 16S rDNA of *S. glossinidius* (S-endosymbiont) from different species of tsetse is virtually identical, indicating multiple infections or horizontal transmission of the same organism [1]. Although it would appear that the association between the S-endosymbiont and the host is stable, there has been one documented case in which the S-endosymbiont was lost from an aphid [8].

Genetic characterization of a 9.6-kb S-Gbr DNA fragment containing 16S-23S rDNA. A genetic map of the 9.6-kb DNA fragment from S-Gbr is presented in Fig. 3. The order of the genes is identical to that of the region upstream of *E. coli rrnG* operon [5]. The G + C content of the region upstream of 16S-23S rDNA of S-Gbr is 40.1 moles%, similar to the G + C content of *P. vulgaris*, to which S-Gbr may be related (Fig. 1a). The amino acid

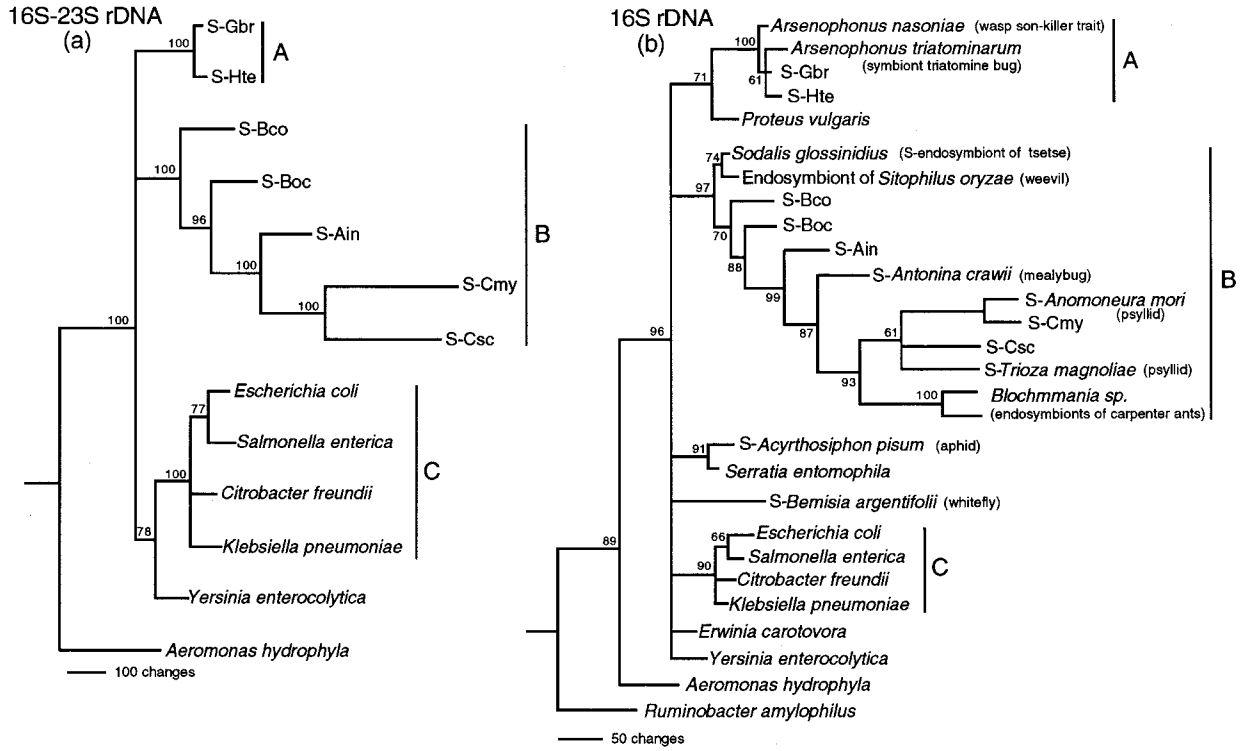

Fig. 1. Phylogenetic trees resulting from parsimony analyses of (a) 16S-23S rDNA of psyllid S-endosymbionts and related members of the *Enterobacteriaceae*; (b) 16S rDNA of psyllid S-endosymbionts, other related endosymbionts, insect-associated bacteria and other related organisms. The abbreviation or name, preceded by S-, designates S-endosymbionts. Letters next to vertical lines indicate major clusters. Numbers above or below nodes are bootstrap percentages from 1000 replicates. *Acetobacter intermedius* is the outgroup.

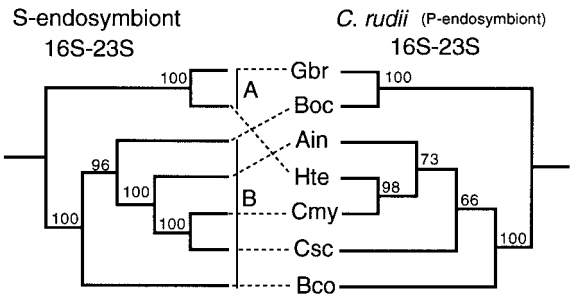

Fig. 2. Comparisons of the cladograms resulting from parsimony analyses of the 16S-23S rDNA sequence of psyllid S- and P-endosymbionts. Dashed lines indicate associations. Letters next to vertical lines indicate major clusters observed in Fig. 1a. Numbers above nodes are bootstrap percentages from 1000 replicates. Data for *C. ruddi* from [29].

Fig. 3. Genetic map of a 9.6-kb DNA fragment of S-Gbr. Thick lines, structural genes; striped thin line, region used for probe.

sequence identity of S-Gbr RluD (23S rRNA pseudouridine synthase), YfiH (no known function), and ClpB (protease) to the *E. coli* proteins is 78.3%, 54.7%, and 83.0%, respectively. Figure 4 presents the intergenic sequence between *cpIB* and 16S rDNA. Conserved sequences could be identified which correspond to the −35-10 region of the rRNA promoter as well as *BoxA* and *BoxC*. The function of *BoxC* is not known; *BoxA* is involved in antitermination [4, 27]. In the intergenic region between 16S and 23S rDNA is a sequence identical to *E. coli* tRNA-glu (Fig. 3). These results are in agreement with the results of the phylogenetic analysis (Fig. 1) in that the gene order of S-Gbr and the moles % G + C content are consistent with it being a member of the *Enterobacteriaceae*.

ACKNOWLEDGMENTS

This material is based on work supported by National Science Foundation Awards DEB-9817795 (P. Baumann), DEB-9815413 (N. A. Moran), and DEB-9978518 (N. A. Moran, P. Baumann) and the University of California Experiment Station (P. Baumann).

Literature Cited

1. Aksoy S, Chen X, Hypsa V (1997) Phylogeny and potential transmission routes of midgut-associated endosymbionts of tsetse. Insect Mol Biol 6: 183–190

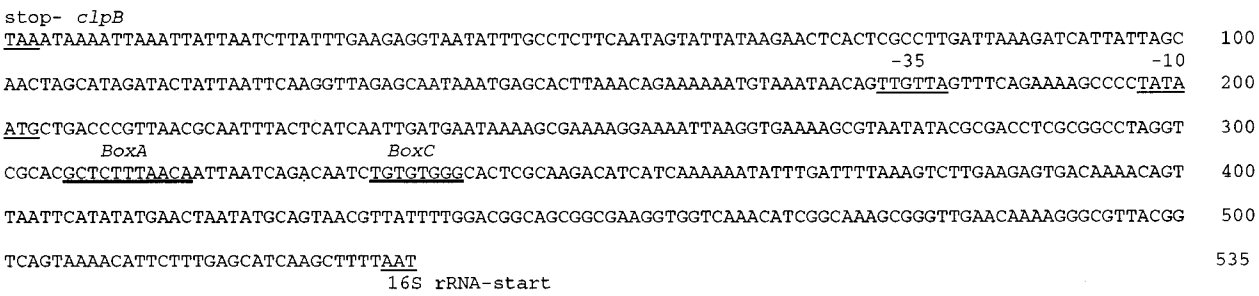

Fig. 4. Nt sequence of the intergenic region between *clpB* and 16S rDNA of S-Gbr, indicating some of the conserved sequences.

2. Baumann P, Moran NA, Baumann L (1997) The evolution and genetics of aphid endosymbionts. Bioscience 47: 12–20
3. Baumann P, Moran MA, Baumann L (2000) Bacteriocyte-associated endosymbionts of insects. In Dworkin M (ed) The prokaryotes [Online.] New York: Springer, http://link.springer.de/link/service/books/10125
4. Berg KL, Squires CS, Squires CL (1989) Ribosomal RNA operon antitermination. Function of leader and spacer region boxB-boxA sequences and their conservation in diverse micro-organisms. J Mol Biol 209: 345–358
5. Berlyn MKB (1998) Linkage map of *Escherichia coli* K-12, edition 10: the traditional map. Microbiol Mol Biol Rev 62: 814–984
6. Buchner P (1965) Endosymbiosis of animals and plant microorganisms. New York: Interscience, pp 207–432
7. Chang KP, Musgrave AJ (1969) Histochemistry and ultrastructure of the mycetome and its "symbiotes" in the pear psylla, *Psylla pyricola* Foerster (Homoptera). Tissue Cell 1: 597–606
8. Chen DQ, Purcell AH (1997) Occurrence and transmission of facultative endosymbionts in aphids. Curr Microbiol 34: 220–225
9. Chen X, Li S, Aksoy S (1999) Concordant evolution of a symbiont with its insect species: molecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont *Wigglesworthia glossinidia*. J Mol Evol 48: 49–58
10. Clark MA, Baumann L, Munson MA, Baumann P, Campbell BC, Duffus JE, Osborne LS, Moran NA (1992) The eubacterial endosymbionts of whiteflies (Homoptera: Aleyrodoidea) constitute a lineage distinct from the endosymbionts of aphids and mealybugs. Curr Microbiol 25: 119–123
11. Clark MA, Moran NA, Baumann P, Wernegreen JJ (2000) Cospeciation between bacterial endosymbionts (*Buchnera*) and a recent radiation of aphids (*Uroleucon*) and pitfalls of testing for phylogenetic congruence. Evolution 54:517–525
12. Dale C, Maudlin I (1999) *Sodalis* gen. nov. and *Sodalis glossinidius* sp. nov., a microaerophilic secondary endosymbiont of tsetse fly *Glossina morsitans morsitans*. Int J Syst Bacteriol 49: 267–275
13. Douglas AE (1998) Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. Annu Rev Entomol 43: 17–37
14. Fukatsu T, Nikoh N (1998) Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). Appl Env Microbiol 64: 3599–3606
15. Fukatsu T, Nikoh N (2000) Endosymbiotic microbiota of the bamboo pseudococcid *Antonina crawii* (Insecta, Homoptera). Appl Env Microbiol 66: 643–650
16. Genetics Computer Group (1997) Program manual for the Wisconsin package, Version 9.1. Madison, WI: Genetics Computer Group
17. Gherna RL, Werren JH, Weisburg W, Cote R, Woese CR, Mandelco L, Brenner DJ (1991) *Arsenophonus nasoniae* gen. nov., sp. nov., the causative agent of son-killer trait in the parasitic wasp *Nasonia vitripennis*. Int J Syst Bacteriol 41: 563–565
18. Heddi A, Charles H, Khatchadourian C, Bonnot G, Nardon P (1998) Molecular characterization of the principal symbiotic bacteria of the weevil *Sitophilus oryzae*: a peculiar G + C content of an endosymbiotic DNA. J Mol Evol 47: 52–61
19. Hodkinson ID (1974) The biology of the Psylloidea (Homoptera): a review. Bull Entomol Res 64: 325–339
20. Hypsa V, Dale C (1997) In vitro culture and phylogenetic analysis of "*Candidatus Arsenophonus triatominarum*," an intracellular bacterium from the triatomine bug, *Triatoma infestans*. Int J Syst Bacteriol 47: 1140–1144
21. Lai CY, Baumann P, Baumann P (1994) Amplification of *trpEG*: adaptation of *Buchnera aphidicola* to an endosymbiotic association with aphids. Proc Natl Acad Sci USA 91: 3819–3823
22. Moran NA, Baumann P (2000) Bacterial endosymbionts of animals. Curr Opin Microbiol 3:270–275
23. Moran NA, Telang A (1998) Bacteriocyte-associated symbionts of insects: a variety of insect groups harbor ancient prokaryotic endosymbionts. Bioscience 48: 295–304
24. Sauer C, Stackebrandt E, Gadau J, Hölldobler B, Gross R (2000) Systematic relationships and their cospeciation of bacterial endosymbionts and their carpenter ant host species. Int J Syst Evol Microbiol, in press
25. Schröder D, Deppisch H, Obermayer M, Krohne G, Stackebrandt E, Hölldobler B, Goebel W, Gross R (1996) Intracellular endosymbiotic bacteria of *Camponotus* species (carpenter ants): systematics, evolution and ultrastructural characterization. Mol Microbiol 21: 479–489
26. Spaulding AW, von Dohlen CD (1998) Phylogenetic characterization and molecular evolution of bacterial endosymbionts in psyllids (Hemiptera: Sternorrhyncha). Mol Biol Evol 15: 1506–1513
27. Squires CL, Greenblatt J, Li J, Condon C, Squires CL (1993) Ribosomal RNA antitermination *in vitro*: requirement for Nus factors and one or more unidentified cellular components. Proc Natl Acad Sci USA 90: 970–974
28. Swofford DL (1999) PAUP: Phylogenetic analysis using parsimony, Version 4.0. Sunderland, MA: Sinauer
29. Thao ML, Moran NA, Abbot P, Brennan EB, Burckhardt DH, Baumann P (2000) Cospeciation of psyllids and their prokaryotic primary endosymbionts. Appl Env Microbiol 66:2898–2905
30. Unterman BM, Baumann P, McLean DL (1989) Pea aphid symbiont relationships established by analysis of 16S rRNAs. J Bacteriol 171: 2970–2974
31. Waku Y, Endo Y (1987) Ultrastructure and life cycle of the symbionts in the Homopteran insect, *Anomoneura mori* Schwartz (Psyllidae). Appl Entomol Zool 22: 630–637