Novel chlamydiae in whiteflies and scale insects: endosymbionts 'Candidatus Fritschea bemisiae' strain Falk and 'Candidatus Fritschea eriococci' strain Elm

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Bacteria called 'Fritschea' are endosymbionts of the plant-feeding whitefly *Bemisia tabaci* and scale insect *Eriococcus spurius*. In the gut of *B. tabaci*, these bacteria live within bacteriocyte cells that are transmitted directly from the parent to oocytes. Whiteflies cause serious economic damage to many agricultural crops; *B. tabaci* fecundity and host range are less than those of *Bemisia argentifolii*, possibly due to the presence of this endosymbiont. The *B. tabaci* endosymbiont has been characterized using electron microscopy and DNA analysis but has not been isolated or propagated outside of insects. The present study compared sequences for 11 endosymbiont genes to genomic data for chlamydial families *Parachlamydiaceae*, *Chlamydiaceae* and *Simkaniaceae* and to 16S rRNA gene signature sequences from 330 chlamydiae. We concluded that it was appropriate to propose 'Candidatus Fritschea bemisiae' strain Falk and 'Candidatus Fritschea eriococci' strain Elm as members of the family *Simkaniaceae* in the *Chlamydiales*.

The whitefly *Bemisia tabaci* biotype A contains several types of endosymbiont (Costa *et al.*, 1995). Two have been characterized, including rRNA gene sequence analyses (Clark *et al.*, 1992; Thao & Baumann, 2004a; Zchori-Fein & Brown, 2002). The third endosymbiont, when examined using electron microscopy (EM), has a biphasic appearance (Fig. 1). The electron-dense globular bodies of the biphasic endosymbiont are 0.2–0.25 µm wide and the less dense morphological forms are 0.7–0.8 µm wide and 0.7–2.5 µm long (Costa *et al.*, 1995). The chlamydial nature of the biphasic endosymbiont is suggested by PCR amplification of extracted total insect DNA using 23S rRNA gene signature sequence primers (Thao *et al.*, 2003; Everett *et al.*, 1999a). Analysing a 16.6 kb *XhoI*–*EcoRV* genome segment

from the organism, Thao *et al.* (2003) found that the full-length 16S rRNA and 23S rRNA gene sequences were 91 % identical to rRNA genes in *Simkania negevensis* Z^T, the type strain, species and genus of the *Simkaniaceae* (Table 1), and that, like *S. negevensis*, the 23S rRNA gene contained an intron (Everett *et al.*, 1999b). Although the endosymbiont was not isolated, Thao *et al.* (2003) proposed the names 'Fritschea bemisiae' for the endosymbiont and 'Fritschea eriococci' for a related endosymbiont.

A bacterial species must be culturable for valid publication of its name. The *B. tabaci* chlamydial endosymbiont, however, has only been found to live in bacteriocytes, which are transmitted vertically to oocytes (Costa *et al.*, 1996). In an effort to culture this endosymbiont, we used an *Acanthamoeba* co-cultivation method that we use routinely to isolate strains of the *Parachlamydiaceae*. This method has been shown to work for culture of *S. negevensis* (Kahane *et al.*, 2001). In the present study, co-cultivation of disrupted *Bemisia* with amoebae in trypticase soy yeast broth did not result in chlamydial growth in the broth or amoebae. Each time we thought the endosymbiont might be growing, it was lost from the amoeba culture, perhaps due to extremely slow

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The GenBank/EMBL/DDBJ accession numbers for genomic fragments containing the rRNA operons of 'Candidatus F. bemisiae' Falk and 'Candidatus F. eriococci' Elm are respectively AY140910 and AY140911.

An extended neighbour-joining tree is available as supplementary material in IJSEM Online.

Fig. 1. ‘*Candidatus Fritschea bemisiae*’ within its *B. tabaci* host cells. Reproduced with permission of Springer-Verlag KG from Costa *et al.* (1995). C2, Reticulate bodies (replicating chlamydiae); f, filamentous structures within reticulate bodies; gb, elementary bodies (electron dense, compact chlamydiae); P, primary pleomorphic endosymbiont (Thao & Baumann, 2004a). Bar, 1 µm.

growth. We have not been able to confirm growth by fluorescent *in situ* hybridization or by sequencing and have not tried to culture the endosymbiont by other means. The

category *Candidatus* accommodates more than two dozen bacterial taxa not yet isolated or cultivated *in vitro*. For these reasons, we propose that ‘*Fritschea*’ and similar endosymbionts belong to a new genus designated ‘*Candidatus Fritschea*’.

The cloned and sequenced 16·6 kb genomic fragment from the endosymbiont contained 10 predicted open reading frames (ORFs) and an rRNA operon. To characterize the relationship of the endosymbiont to chlamydiae, we compared these sequences to the genome sequences of four species of the *Chlamydiaceae* and strain UWE25 from the *Parachlamydiaceae* (Horn *et al.*, 2004; Kalman *et al.*, 1999; Read *et al.*, 2000, 2003; Stephens *et al.*, 1998). In the genomes from the *Chlamydiaceae*, three of the endosymbiont ORFs were absent from all four sequenced species: glycosylhydrolase, transposase and GTP-binding protein (Table 2). Six ORFs were present in the species from the *Chlamydiaceae*, but were more similar to UWE25 (*Parachlamydiaceae*) ORFs (Table 2). None was located in the vicinity of an rRNA operon, but the tenth ORF was an endonuclease in the 23S rRNA gene intron. The endosymbiont 16S and 23S rRNA gene sequences were more than 80 % identical to chlamydial rRNA genes (Table 1).

Nine of the endosymbiont ORFs were present in strain UWE25 (*Parachlamydiaceae*) (Table 2). The six ORFs that were clustered downstream of the endosymbiont rRNA genes were also clustered in UWE25 (Locus Tags pc0231–39; Table 2), although not in the vicinity of an rRNA operon. Two of the endosymbiont genes upstream of the rRNA genes, *yaeL* and a putative transposase, were also in close proximity in UWE25. These transposase ORFs were located in large predicted, complementary hairpins (not shown). The endosymbiont transposase was related to an IS982 element in *Photorehabdus luminescens*, a broad-spectrum insect

Table 1. Full-length rRNA gene sequence identity between the *B. tabaci* endosymbiont ‘*Candidatus Fritschea bemisiae*’ and selected chlamydial sequences

Sequences were identified by BLAST and aligned using CLUSTAL. Gaps were not deleted, but the introns in the *S. negevensis* and ‘*Candidatus F. bemisiae*’ 23S rRNA genes were deleted. GenBank accession numbers are in parentheses. ND, Not determined.

Species/strain	Sequence identity (%) with ‘ <i>Ca. F. bemisiae</i> ’	
	16S rRNA gene	23S rRNA gene
Endosymbiont of <i>B. tabaci</i> biotype Jatropa	99·8 (AF400484)	ND
‘ <i>Candidatus Fritschea eriococci</i> ’	97 (AY140911)	97 (AY140911)
<i>Simkania negevensis</i>	91 (U68460)	91 (U68460)
‘ <i>Candidatus Rhabdochlamydia porcellionis</i> ’	87 (AY223862)	ND
<i>Parachlamydia acanthamoebae</i> Bn ₉	87 (Y07556)	87 (Y07555)
<i>Neochlamydia hartmannellae</i>	86 (AF177275)	ND
<i>Waddlia chondrophila</i>	86 (AF042496)	85 (AF042496)
UWE25 (<i>Parachlamydiaceae</i>)	85 (BX908798)	86 (BX908798)
<i>Chlamydomydia psittaci</i> 6BC	85 (U68447)	84 (U68447)
<i>Chlamydia trachomatis</i> L2/434/BU	85 (U68443)	83 (U68443)

Table 2. 'Candidatus Fritschea bemisiae' and other chlamydial genomes: protein similarities and conservation of gene order

GenBank accession numbers and Locus Tags are provided. Values in parentheses indicate percentage identity/similarity/gaps. Similarity is based on the possible codons for each amino acid and scored positive if amino acid comparison suggests no more than one nucleotide difference. None, Absent from the genome.

Simkaniaceae	Parachlamydiaceae		Chlamydiaceae		
'Candidatus F. bemisiae' (AY140910)	UWE25 (BX908798)	Chlamydia trachomatis (AE001273)	Chlamydia muridarum (AE002160)	Chlamydia caviae (AE015925)	Chlamydia pneumoniae (AE001363)
YaeL (metalloprotease)	pc0261 (41/69/3)	CT072 (32/54/6)	TC0344 (32/54/6)	CCA00442 (32/55/6)	CPn0344 (34/55–56/5)
OrfA (glycosylhydrolase)	pc1825 (25/47/9)	None	None	None	None
OrfB (transposase)	pc0265 (58/76/3)	None	None	None	None
EndA (23S rRNA intron endonuclease)*	None	None	None	None	None
ArgS (arginyl-tRNA synthetase)	pc0231 (56/82/1)	CT454 (49/65/3)	TC0739 (49/65/2)	CCA00172 (49/67/2)	CPn0570 (47/64/3)
OrfC (conserved hypothetical protein)	pc0233 (57/84/1)	CT538 (45/70/0)	TC0825 (45/69/0)	CCA00083 (47/69/0)	CPn0658 (45/68/0)
YjeE (ATPase or kinase)	pc0234 (57/84/1)	CT537 (35/53/10)	TC0824 (35/53/5)	CCA00084 (32/49/10)	CPn0657 (33/57/16)
OrfD (GTP-binding protein)	pc0235 (43/71/6)	None	None	None	None
DnaQ (DNA polymerase III, epsilon chain)†	pc0236 (56/84/0)	CT536 (51/68/0)	TC0823 (52/70/1)	CCA00085 (52/69/1)	CPn0655 (52/69/0)
Pgk (phosphoglycerate kinase)	pc0239 (52/81/2)	CT693 (46/64/2)	TC0065 (45/63/2)	CCA00061 (46/64/2)	CPn0679 (47/63/1)

*Highest similarity to *S. negevensis* (55/70/6).

†DNA polymerase III, epsilon chain (gene duplicates that are 34–37% identical are also present in all four chlamydial genomes).

pathogen and gammaproteobacterial symbiont of nematodes (Duchaud *et al.*, 2003). Between *yaeL* and the transposase gene in the endosymbiont was a glycosylhydrolase gene. UWE25 had three different ORFs between *yaeL* and the transposase (pc0262–64); a glycosylhydrolase ORF (pc1825) was located 1562 ORFs away on the UWE25 chromosome. Only the intron ORF of the endosymbiont was absent from UWE25, which did not have a 23S rRNA gene intron. The ORF, however, did share 55% amino acid sequence identity with the endonuclease in the 23S rRNA group I intron of *S. negevensis* Z^T. Both introns were located in the I-*CpaI* restriction site (position 1910, by *Escherichia coli* numbering; data not shown).

Chlamydiae are increasingly being identified and classified on the basis of rRNA gene sequence, but as yet there are few well-characterized chlamydial genera. Current standards rely on percentage rRNA gene sequence identity to facilitate orderly discovery, comparison and eventual classification of novel species (Everett *et al.*, 1999a). *Parachlamydiaceae* and *Chlamydiaceae*, for example, are separate families because they have < 90% 16S rRNA gene sequence identity. In fact, we now know that their 85% 16S rRNA gene sequence identities correlate with great genomic, pathogenic and evolutionary differences (Horn *et al.*, 2004). Despite clear standards for the taxonomic placement of novel chlamydiae, errors are sometimes made. For example, the 16S rRNA gene sequence of 'Candidatus Rhabdochlamydia' is 91% identical to the environmental chlamydiae lineage (ECL) VI of chlamydiae and only 84% identical to *S. negevensis* (Kostanjšek *et al.*, 2004). Nonetheless, solely based on a neighbour-joining analysis of 16S rRNA gene sequences, 'Candidatus Rhabdochlamydia' was placed in the family *Simkaniaceae* (Kostanjšek *et al.*, 2004). Phylogenetic instability increases with the addition of novel, deeply branching 16S rRNA gene sequences, and branch points lose bootstrap and treepuzzle support. Phylogenetic analysis alone does not resolve chlamydial relationships.

To delineate the relationship between chlamydiae and the *B. tabaci* biphasic endosymbiont, two 16S rRNA gene sequence datasets were aligned. The first contained only full-length or nearly full-length sequences; it was analysed for percentage identity. The result was consistent with previous analyses of chlamydiae and in Fig. 2 provided a backbone for the analysis of the second dataset. The second dataset comprised 331 chlamydia-like 16S rRNA gene signature sequences. Bootstrap analysis of the aligned sequences identified the most strongly supported similarity clusters. The signature sequences correspond to nucleotide positions 40–337 (*E. coli* numbering) and contain the highest proportion of differences in the chlamydial 16S rRNA gene (Everett *et al.*, 1999a) (Fig. 2 and Supplementary Fig. S1 available in IJSEM Online). A reliably aligned 202-base segment of this dataset contained 43 identical positions. Bootstrap analysis identified six reliably clustered families and 92 lineages containing only one or two sequences (Fig. 2). Of all of them, the endosymbiont signature sequence was most

Fig. 2. Chlamydial 16S rRNA gene sequence clusters. This figure summarizes the diversity of 331 chlamydial lineages using two levels of 16S rRNA gene sequence similarity clustering: (i) percentage identity of full-length or nearly full-length sequences (vertical bars) and (ii) signature sequence clusters having >50% bootstrap support (branches and numbers). Clusters are labelled with the genus and with ECL group names (Horn & Wagner, 2001). The nine species of the *Chlamydiaceae* represent the 177 available sequences. Supplementary Fig. S1 shows a neighbour-joining tree of the complete selection of sequences (Chua, 2003; Chua *et al.*, 2005; Corsaro *et al.*, 2001, 2002; Devereaux *et al.*, 2003; Fritsche *et al.*, 2000; Greub & Raoult, 2002; Henning *et al.*, 2002; Horn & Wagner, 2001; Kaplan & Kitts, 2004; Labrenz & Banfield, 2004; von Bomhard *et al.*, 2003; Meijer *et al.*, 2000; Ossewaarde & Meijer, 1999). Bar, 0.05 substitutions per site.

similar to the *S. negevensis* signature sequence. The next closest were CRG26 (from human tissue) and CRG34 (from fertilizer) (Meijer & Ossewaarde, 2002). Bootstrapping placed *S. negevensis* with '*Candidatus Fritschea*', but not with any other sequences. Clusters that were identified had varying traits, including pathogenicity, endosymbiosis, human infectivity and host specificity (see Supplementary Fig. S1). Obtained from many continents, these groups showed no evidence of geographical specificity.

In screening DNA extracts from additional insects, a second chlamydia-like sequence was obtained from the scale insect *Eriococcus spurius* (Sternorrhyncha, Eriococcidae; Thao *et al.*, 2003). The *E. spurius* 16S rRNA gene sequence (presumably from another endosymbiont) was 97 % identical to the *B. tabaci* endosymbiont sequence (Table 1). The full-length 16S rRNA gene sequence of the endosymbiont was also nearly identical to a DNA sequence obtained from *B. tabaci* biotype Jatropha. *E. spurius* did not have a 23S rRNA gene intron.

This study brings together evidence for a chlamydial endosymbiont in the whitefly *B. tabaci* (Sternorrhyncha, Aleyrodidae): it lives inside cells, is biphasic, has > 80 % full-length rRNA gene sequence identity with species of the *Chlamydiales*, clusters with 91 % rRNA gene sequence identity in the family *Simkaniaceae* and has nine ORFs most closely related to members of the *Parachlamydiaceae*, compared with *Chlamydiaceae* or any other available lineage. Chlamydiae are often pathogenic, whereas primary endosymbionts typically benefit their hosts. Indeed, three observations indicate that the biphasic endosymbiont of *B. tabaci* may be pathogenic: (i) the 18S rRNA gene sequences of *B. tabaci* and *B. argentifolii* are indistinguishable, indicating that these whiteflies are closely related; (ii) EM and PCR show that *B. argentifolii* lacks the chlamydial endosymbiont of *B. tabaci* (Costa *et al.*, 1995; Thao *et al.*, 2003); and (iii) *B. argentifolii* causes serious disorders not caused by *B. tabaci* and is more vigorous with respect to growth and progeny production (Costa *et al.*, 1995). Thus, Costa *et al.* (1995) proposed that the lower fecundity and narrower host range of *B. tabaci* may be due to the chlamydial endosymbiont. The prevalence of chlamydiae in insects is not known. Thao *et al.* (2003) studied a strain of *B. tabaci* that is maintained by Bryce Falk at the University of California, Davis. Zchori-Fein & Brown (2002) examined 20 *B. tabaci* strains and placed a chlamydiae-like 'jatropha' sequence from *B. tabaci* in GenBank, but *B. tabaci* 'jatropha' was not described in their publication. Although *B. tabaci* is less pathogenic than *B. argentifolii*, many agricultural crops are severely infested with *B. tabaci*, including watermelons, pumpkins, squash, tomatoes, cotton, soybeans and peanuts, as are numerous weeds (R. Sprenkel and S. Olson, unpublished; http://n-fl-bugs.ifas.ufl.edu/more/Insect_week/2-18.htm). Due to its status as a pest, the availability of *B. tabaci* for research is restricted.

Symbionts in general belong to many bacterial phyla, including the *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and

Chlamydiae, and the *Archaea*. Endosymbiont phyla in insects are similarly diverse (Wernegreen, 2002). The primary and secondary non-chlamydial endosymbionts in *B. tabaci* collections are gammaproteobacteria (Clark *et al.*, 1992; Thao & Baumann, 2004a, b; Zchori-Fein & Brown, 2002). The primary gammaproteobacterial endosymbionts have been designated '*Candidatus Portiera aleyrodidarum*' (Thao & Baumann, 2004a). They are obligate and group with *Zymobacter* in the *Halomonadaceae*. The secondary endosymbionts group with *Enterobacteriaceae*, are not obligate and are thought to have been recently acquired (Clark *et al.*, 1992; Thao & Baumann, 2004b; Zchori-Fein & Brown, 2002). Chlamydial endosymbionts and intracellular bacteria live in many host lineages: mammals, marsupials, birds, reptiles, amphibians, amoebae, the crustacean *Porcellio scaber* and now insects (for up-to-date reviews see <http://www.chlamydiae.com>). Crustaceans and insects are sister taxa (Roehrdanz *et al.*, 2002). This suggests that an ancestor of both might have acquired chlamydiae long ago, but there is also evidence that chlamydiae may have played an ancestral role in the evolution of plants (Greub & Raoult, 2003; Horn *et al.*, 2004). The strongest support for this is that 11 % of the ORFs in the UWE25 genome (*Parachlamydiaceae*) are most closely related to sequences from chloroplasts, cyanobacteria and plants (Horn *et al.*, 2004); 4 % of the ORFs in the genomes from the *Chlamydiaceae* are closely related to sequences from plants (Brinkman *et al.*, 2002). In addition, the 23S rRNA gene group I introns of the endosymbiont and *S. negevensis* are most closely related to chloroplast rRNA gene introns (Everett *et al.*, 1999b). Chlamydiae have not yet been found in plants, but *B. tabaci* and *E. spurius* feed on plant phloem, so it may be that plant genes were acquired by chlamydiae via horizontal transfer through insect feeding activity.

We propose that the chlamydia-like endosymbionts of *B. tabaci* (Sternorrhyncha, Aleyrodidae) and *E. spurius* (Sternorrhyncha, Eriococcidae) should be given *Candidatus* status (Murray & Schleifer, 1994). The 16S rRNA gene sequences of these endosymbionts are ≥ 95 % identical to each other and have ≥ 90 % identity to the 16S rRNA gene sequence of *S. negevensis* Z^T, so they belong to a separate genus in the family *Simkaniaceae* (Everett *et al.*, 1999a). They should be specifically detectable with the labelled 16S rRNA-targeted oligonucleotide probe 5'-TTCTCTTT-CCGCAAGGCC-3' (showing at least four mismatches with all other 16S rRNA sequences currently available). PCR using primers U23F (5'-GATGCCTTGGCATTGATAGGCGATGAAGGA-3') and 23S1GR (5'-TGGCTCATCATGCAAAAGGCA-3') amplifies '*Candidatus Fritschea*' endosymbionts but not gammaproteobacteria (Everett *et al.*, 1999a; Thao *et al.*, 2003).

Description of '*Candidatus Fritschea bemisiae*'

'*Candidatus Fritschea bemisiae*' (Frit'sche.a. N.L. fem. n. *Fritschea* after Thomas R. Fritsche, American physician and parasitologist, whose studies initiated a major change in our perception of the natural history and diversity of the

Chlamydiae. be.mi'si.ae. N.L. gen. fem. n. *bemisiae* of *Bemisia*, the genus of whitefly insects in which it was first discovered).

The host range currently includes the whitefly *Bemisia tabaci* (Sternorrhyncha, Aleyrodidae). 'Candidatus Fritschea bemisiae' can be detected and identified using EM, PCR amplification and DNA sequence analysis. EM detection of 'Ca. F. bemisiae' in the bacteriocytes of *B. tabaci* shows intracellular growth, small rod-like reticulate bodies and electron-dense elementary bodies (0.2–0.8 µm). There is evidence for a single ribosomal operon in 'Ca. F. bemisiae'. The G + C content is 40 mol% in a 16.6 kb 'Ca. F. bemisiae' genome fragment containing the rRNA operon (GenBank accession no. AY140910). Gene organization in this fragment is most similar to gene organization in strain UWE25 from the *Parachlamydiaceae*, the only genome sequenced to date from an organism in the order *Chlamydiales* not belonging to the *Chlamydiaceae*. The rRNA operon does not include a tRNA. Like *S. negevensis*, 'Ca. F. bemisiae' has a group I intron in the 23S rRNA gene (position 1910, *E. coli* numbering). Strain Falk is the reference strain of the species 'Ca. F. bemisiae' and was reared in the laboratory of B. W. Falk at UC Davis, CA, USA.

Description of 'Candidatus Fritschea eriococci'

'Candidatus Fritschea eriococci' (e.ri.o.coc'ci. N.L. gen. masc. n. *eriococci* of *Eriococcus*, the genus of scale insects in which it was first discovered).

The host range currently includes the scale insect *Eriococcus spurius* (Sternorrhyncha, Eriococcidae). 'Candidatus Fritschea eriococci' is currently known only by PCR and sequencing of DNA extracted from *Eriococcus* collected from an elm tree in Davis, CA, USA. The rRNA operon does not include a tRNA (GenBank accession no. AY140911); unlike 'Ca. F. bemisiae' and *S. negevensis*, 'Ca. F. eriococci' strain Elm does not have a 23S rRNA gene group I intron. Strain Elm is the reference strain of the species 'Ca. F. eriococci'.

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