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The association of western flower thrips, *Frankliniella occidentalis*, with near *Erwinia* species gut bacteria: transient or permanent?

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Chapter 2

ABSTRACT – Associations between insects and gut bacteria are ubiquitous. It is possible to make a distinction between permanent associations (called symbiosis) in which the same type of bacteria is present in more than one generation of the insect, and transient associations. Transient bacteria are ingested together with food but do not settle in the insect gut in such a way that they will be passed on to the next generation. In this study, we describe the permanent association between western flower thrips (*Frankliniella occidentalis*), a polyphagous insect species that is a major pest worldwide, and one type of gut bacteria. On basis of direct microscopical observations and counts of bacteria, it was found that thrips from the populations studied contained large numbers of bacteria in their hindgut. Bacteria were isolated from their host and grown on ten different agar media. The number of bacteria isolated on agar media equaled the number of direct counts. All isolates had the same colony morphology. On the basis of their 16S rDNA sequence these bacteria were identified as Enterobacteriaceae, closely related to *Escherichia coli*. Isolates tested with API 20E biochemical tests were *Erwinia* species. This was the only type of bacteria found in all thrips individuals on any of the ten different agar media. Universal primers, which would potentially pick up DNA from any bacterium present in the insect, were applied on crude DNA extracts from thrips with bacteria. We only found 16S rDNA sequences similar to those of the isolated thrips gut bacteria. The same type of bacteria was present in all life stages of the thrips and was found to persist in the thrips populations for at least two years (more than 50 generations).

KEY WORDS – Symbiosis, gut bacteria, *Frankliniella occidentalis*, *Enterobacter agglomerans*, *Erwinia herbicola*, RAPD-PCR, 16S rDNA

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Many microorganisms use insects as their host. These microbes colonise, grow, and reproduce inside the host and can be found in the host digestive system or reproductive tissue. They are called symbionts, because they reside inside their host for a prolonged period of time, and often they are reliably transmitted to successive host generations.

A few associations involving intracellular obligate symbiotic mutualists are well described (viz., aphids [Douglas, 1989; Moran and Baumann, 1994], weevils [Nardon, 1973] and tsetse flies [Aksoy, 1995]). These authors have found congruence between host and microorganism molecular phylogenies. This suggests that intracellular microorganisms can drive food plant adaptation of their host, resulting in speciation (Moran and Baumann, 1994). However, not in all intracellular permanent associations the host profits from the presence of the microorganisms, which is the case in the parasitic symbiotic interactions between arthropods and Rickettsiae or *Wolbachia* (Breeuwer *et al.*, 1992, Stouthamer *et al.*, 1993, Werren, 1995).

Symbiosis is not restricted to associations in which the microorganism has adopted an obligate intracellular lifestyle. It also applies to systems in which the hosts have to acquire their symbionts from external sources in every new generation (Boucher, 1982). These symbionts are called facultative because they are able to survive outside the host. Many bacteria that persist in the intestinal tract throughout the complete insect life cycle belong to this type of symbionts (Bignell, 1983). They may be located in the gut lumen or be attached to the gut wall. Such facultative associations have been reported in many different insect species, but only in a few examples, the host-guest interaction has been studied (Campbell, 1990). These associations may be parasitic or mutualistic. For example, termites and cockroaches are dependent upon facultative mutualistic bacteria in their hindgut, which take part in digestion (Breznak, 1982; Cruden and Markovetz, 1984). In a few other studies, mutualistic gut bacteria were shown to be an advantage to their host by providing nutrients or by degrading toxic compounds (Campbell, 1990, and references therein). Many of the microorganisms involved in such associations belong to the family Enterobacteriaceae. In addition to these facultative symbionts, transient microorganisms can be found in the gut. Transients are ingested during food uptake, but are removed with the faeces without settling in the gut and hence are not reliably transmitted to the next generation.

In this study, western flower thrips (*Frankliniella occidentalis* (Pergande) [Thysanoptera: Thripidae]) is used as model system to study the association between an insect species and gut bacteria. The western flower thrips is a small (2 mm long) polyphagous insect and has recently spread over the world from the Western part of the United States. Thrips are a major agricultural pest of both vegetables and ornamental crops. The few studies on thrips nutritional requirements suggest that thrips adults survive best on a mixed diet of leaves and pollen. However, it is able to feed and reproduce on leaves alone (Van Rijn *et al.*, 1995). The versatility of thrips regarding nutrition is still unexplained. The possible role of bacteria was hypothesised before (Mollema, 1995).

Ullman (1989) observed large amounts of bacteria in the hindgut and Malpighian tubules of thrips. Recently, we were able to isolate these bacteria and grow them outside their host (De Vries *et al.*, 1995). Here, we report on the characterisation and phylogeny of the thrips gut bacteria and discuss the permanency of their association with thrips.

MATERIALS AND METHODS

Thrips populations

Two of the thrips cultures were collected from commercial greenhouse populations and reared at the Centre for Plant Breeding and Reproduction Research in Wageningen since 1988: a culture on cucumber (WCU) and a culture on chrysanthemum (WCH). The third population had been cultivated on chrysanthemum at the Institute for Biodiversity and Ecosystem Dynamics in Amsterdam (ACH) since 1993. This population was started from the WCH culture. The thrips were reared continuously on intact plants, with flowers, under controlled conditions (25 °C, 60% r.h., and 16 h light per day).

Isolation and cultivation of gut bacteria

Thrips samples were randomly collected from the mass rearing cultures to identify and quantify gut bacteria. Each sample included 20 to 30 individuals. Most samples contained only adults, but in four samples we specifically included larvae or eggs to check whether bacteria were present at all life stages of the thrips.

Bacteria were isolated from individual thrips. Thrips were first surface-sterilized by soaking them in 70% ethanol solution for 60 s and, subsequently, in 5% NaClO₃ for 60 s. After rinsing three times with sterile water, the thrips was homogenised with a glass rod in 35 µl TE buffer (10 mM Tris and 1 mM EDTA). All steps in the isolation procedure were done in a laminar flow hood to reduce the risk of contamination. Isolation of bacteria from the ACH thrips population was done in Amsterdam, from the WCH and WCU population in Wageningen. Individual thrips homogenate (35 µl) was streaked on enriched agar medium (agar from Hispanagar S.A.). Plates were incubated at 25 °C. After two days of incubation, bacterial number and morphology was scored, and cell morphology was determined with a microscope.

Bacteria isolation from thrips eggs

Eggs were obtained from 25 adult female thrips placed in a Murai cage (Murai and Ishii, 1982). One end of this cage was sealed with two layers of parafilm with water in between. The other end was closed with a fine mesh screen. Adult thrips were placed in these cages and pollen (Koppert Inc.) was supplied as a food source. The thrips adults can penetrate the parafilm layer with their mouthparts and ovipositor and deposit their eggs in the water enclosed by the parafilm. Eggs were collected from the water reservoir every 24 h. Egg samples were not sterilised before isolating bacteria. Homogenisation, transfer to bacterial medium and incubation of gut bacteria were done in the same way as with adults and larvae.

Bacterial media

Because the growth requirements of the thrips gut bacteria were unknown, thrips homogenates were plated on ten different bacterial media. Five samples of ten adult thrips were pooled, surface-sterilised and homogenised in 350 µl buffer, after which the homogenate was divided over ten different enriched agar media. The following media were

used: Nutrient broth, Brain Heart Infusion, MacConkey broth, Trypticase Soy broth (all from Sigma), Malt agar, Potato Dextrose agar, Corn Meal agar, Pepton-yeast extract, Trypton-yeast extract and Luria-Bertoni medium (all from Difco). We compiled this list of agar media from the various studies on associations of insects and facultative symbionts (Campbell, 1990, and references therein). This experiment was repeated three times. All media supported bacterial growth for all samples equally well, except for MacConkey broth and Corn Meal agar, which showed poor growth. All bacterial colonies were morphologically identical. For reasons of efficiency and economy, Luria-Bertoni (LB) medium was selected for further experiments.

Bacterial count

Gut bacteria that were not able to grow on any of the above mentioned media were detected by comparing the number of colony forming units on LB medium with the number of bacteria counted in thrips homogenate using a light microscope. The number of colony forming units was determined as follows. We made a tenfold dilution series of the thrips homogenate in isolation buffer and streaking each dilution separately on LB agar medium. Homogenates were diluted 10, 100 and 1000 times and only plates were counted with between 30 and 300 colonies. For direct counts of gut bacteria, individual thrips were homogenised in 5 µl TE isolation buffer. The number of bacteria was counted under a light microscope.

Morphological characterisation of bacteria

Bacterial types were classified on the basis of: (1) size, colour, clarity (opaque or clear) and shape (round or irregular) of the colony, (2) shape (rod or coccus), size and motility of the cells. All bacterial strains were labeled according to the thrips population from which they were isolated and the isolation date. For example, TAC.XII.93.8 represents the gut bacterial strain isolated in December 1993, followed by the serial colony number 8. TAC means Thrips Amsterdam Chrysanthemum, in other words the bacterial strain isolated from thrips out of the ACH population. TWK is the bacterial strain from the Cucumber thrips population in Wageningen (WCU), TWC are bacteria from the WCH thrips population.

To localise the bacteria inside the thrips body, bacteria were isolated from different body parts separately. The thrips were first externally sterilized. Then the gut was removed by fixing the insect head and pulling the abdomen. This was done under a binocular cleaned with 70% ethanol. Thrips were divided into two parts: abdomen and head/thorax. The gut was then separated from the rest of the abdomen. Each part was homogenised and streaked on LB medium separately.

Molecular characterisation of gut bacteria

We used two methods to isolate bacterial DNA: (1) isolation directly from thrips and (2) isolation from pure bacterial strains. Total DNA, of thrips and bacteria, was obtained directly from the thrips after surface sterilisation and rinsing with sterile distilled H₂O. The thrips were transferred to 0.5 ml microfuge tubes filled for 50% with Zirconium beads (0.1 and 0.5 µm diameter) in 200 µl DNA isolation buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl and

2.5 mM MgCl₂). Phenol (200 µl) was added and the tubes were shaken for 150 s in a bead beater (Antos Labtech). After centrifugation (10 min at 12,000 g), the water phase was transferred to a new tube. DNA was ethanol precipitated and re-dissolved in 20 µl water. DNA from pure bacterial strains was isolated in a similar procedure. A few colonies of each pure bacterial strain were transferred to tubes containing Zirconium beads and isolation buffer.

DNA sequence analysis

Fragments of 16S rDNA were amplified from bacterial DNA isolates with PCR. The PCR was performed in 50 µl total volume, including Taq polymerase (0.5 unit per reaction), PCR buffer, 0.2 mM nucleotides, primers (0.5 µM, Eurogentec) and 2 µl of DNA template. The primers for amplification of 16S rDNA were universal bacterial primers 27F and 1540R (Lane, 1991; Olsen and Woese, 1993). In a Hybaid Omnigene PCR machine the following amplification conditions were applied: 1 cycle of 95 °C (3 min), 55 °C (1 min), 72 °C (15 s); 28 cycles of 95 °C (1 min), 55 °C (30 s), 72 °C (15 s); 1 cycle of 72 °C (5 min). The complete 16S rDNA sequence was determined with the dideoxynucleotide chain termination method. We used primers that were designed according to conserved regions of the eubacterial gene 519R, 685R, 970R, 1100R, 1329R, 27F and 1540R (Lane, 1991). The primers were 5'-end labeled with ADP-³²P before sequencing. Fragments directly amplified from homogenised thrips were cloned before sequencing. The cloning procedure is described elsewhere (Breeuwer *et al.*, 1992). In this study, three bacterial clones from one particular thrips were sequenced (6.4, 6.9 and 6.12). The 16S rDNA PCR fragments from pure bacterial strains were sequenced without prior cloning. The Taq polymerase used for the sequence amplification reaction had proofreading ability (CircumVent, Biozym). Other reagents and cycle conditions were the same as described for 16S rDNA PCR.

Base pair sequences were recorded after gel electrophoresis and blotting. The sequences were manually aligned using *Escherichia coli* as a model. In the data matrix, thrips bacterial sequences were combined with several 16S rDNA sequences of representative bacterial species belonging to the gamma subdivision of the Proteobacteriaceae. These sequences were obtained from the Ribosomal Database Project of the University of Illinois, Urbana, Champaign. The data matrix was phylogenetically analysed with PAUP 3.1.1 (Swofford, 1991). Gaps were treated as missing data. A phylogram was created using the heuristic search option with 1000 fold bootstrapping. The 16S rDNA sequences of thrips bacteria presented in this article can be found in the GenBank database under accession numbers AF024607 till AF024613.

Biochemical characterisation

The gut bacterial strains were also characterised using an API 20E identification kit (Biomérieux). The biochemical reactions in this kit were incubated for two days at 25 °C. With the API 20E reference guide, we were able to identify the bacterial strains.

RAPD identification

RAPD-PCR was used to fingerprint bacteria (Odinot *et al.*, 1995; Parent *et al.*, 1996). Bacterial DNA was isolated from single colonies of gut bacteria suspended in 5% Chelex

emulsion (Walsh *et al.*, 1991). Proteinase K was added (20 µg per DNA sample) and the samples were incubated for 2 h at 37 °C. After incubation, the samples were mixed and centrifuged, followed by incubation at 95 °C for 15 min to denature proteinase K. The RAPD reactions were performed in a 25 µl reaction volume, including 0.25 u Taq polymerase (HT Biotechnologies), PCR buffer and 0.1 mM nucleotides, 0.2 µM primer and 2 µl DNA template. RAPD reaction conditions were: one cycle of 3 min at 95 °C, 35 cycles of 1 min 95 °C, 15 s 36 °C and 30 s at 72 °C, and one cycle of 5 min at 72 °C. Six informative primers (A2, A3, A4, H5, C2 and F12; Operon Technologies Inc.) were selected in a primer test involving 25 different primers (data not shown). After electrophoresis on 1.5 % agarose gels at 4 V/cm, RAPD fragments were analysed with the IS1000 digital imaging system (Alfa Innotech). The bacteria were typified according to banding patterns. Fragment lengths were assessed with RFLPscan software (Scanalytics, CSPI) by aligning them with a 100 bp size marker ladder. To determine the optimal concentration of DNA template, a dilution series was made from the DNA isolates of a bacterial type strains. It was found that 10² to 10³ diluted DNA samples yielded the best results in terms of number and clarity of bands. In most experiments, *E. coli* and TAC.93.XII.8 were used as internal standards.

RESULTS

Isolation and morphological description of gut bacteria

Most adult thrips of the ACH population contained between 0.5 and 1.0 × 10⁵ internal bacteria (Table 2.1). Both estimation methods, direct counting with the microscope and dilution series plated on LB medium, gave similar results. The bacteria were primarily located in the gut of dissected thrips, they were absent from head and thorax. Most thrips (21 out of 23) had more than 10³ bacteria in their gut. Only two had more than 10² bacteria in any other part of the insect body. This is consistent with earlier microscopic observations by Ullman (1989). Bacteria were present in each life stage of the thrips (Table 2.2). The morphology of bacteria present in eggs and larvae was the same as in adult thrips.

Bacterial samples were taken regularly from our thrips cultures between 1993 and 1996 (Table 2.2). The colony morphology was the same during this period, which lasted for more than 50 generations of thrips. After 24 h of incubation at 25 °C, all colonies were white, clear, round (diameter 2 mm) and consisted of motile rod-like (1.0 to 2.0 µm) bacteria. This

TABLE 2.1 – Mean (± SE) number of bacteria per individual western flower thrips, determined by direct count of thrips homogenate (TC) and by dilution series of the homogenate, plated on LB agar medium (DS). n = number of individuals tested.

Sample date	Method	n	No. bacteria (× 10 ⁴)
April 1994	TC	8	9.3 ± 1.9
June 1996	TC	10	11 ± 2.9
May 1994	DS	10	5.0 ± 0.11
February 1996	DS	19	6.3 ± 0.35
March 1996	DS	18	10 ± 1.3

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TABLE 2.2 – The presence of gut bacteria in various thrips populations. AMC, Amsterdam chrysanthemum; WCH, Wageningen chrysanthemum; WCU, Wageningen cucumber. n = number of thrips included in the sample, %p = percentage of thrips with gut bacteria. In the last column the names of the type strains of bacteria are given, which were used throughout this study.

Population	Date	Life stage	n	%p	Type strain
AMC	Dec 93	adult	10	100	TAC.XII.93.8 TAC.XII.93.2
	Feb 94	larva	9	100	
	March 94	adult	20	75	TAC.III.94.10 TAC.III.94.1
	July 94	adult	40	55	TAC.VII.94.7
	Oct 94	egg	17	100	
	Nov 94	egg	10	50	
	June 95	adult	20	70	TAC.VI.95.5
	Jan 96	larva	26	81	
	Jan 96	adult	40	58	
	WCH	July 94	adult	15	53
April 95		adult	8	100	TWC.IV.95.5
June 95		adult	12	67	TWC.VI.95.3
July 96		adult	26	81	
WCU	July 94	adult	19	74	
	April 95	adult	8	88	TWK.IV.95.5
	June 95	adult	13	85	TWK.VI.95.3
	Jan 96	larva	25	88	
	Jan 96	adult	34	44	
	Nov 96	adult	10	60	

was the only bacterial type consistently present in thrips, and it always occurred in high numbers. It is called TAC hereafter. Occasionally, in less than 25% of the thrips in any of the samples, bacteria were found with a different morphology. These would be present in small numbers and always in addition to the TAC type. Probably, these are transient microbes.

There was no morphological variation among the bacteria isolated from thrips out of the two other populations, maintained on chrysanthemum and cucumber in Wageningen. Moreover, colonies from these two strains, labeled TWK and TWC respectively (Table 2.2), were morphologically indistinguishable from the TAC colonies. To further investigate the apparent permanent infection of thrips, bacteria were molecularly and biochemically characterised.

16S rDNA sequence analysis

The small subunit ribosomal DNA gene of four bacterial colonies, each from a different adult thrips, was amplified and sequenced. As expected, all PCR-fragments of 16S rDNA were approximately 1500 bp in size. Secondly, bacterial 16S rDNA was directly amplified

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from homogenised individual thrips with a specific PCR, and of these samples, three clones were sequenced. The overall similarity of the thrips bacterial sequences was very high, between 97 and 100%. From all sequences in the database, *E. coli* 16S rDNA was the closest with a similarity of 94%, so we decided to concentrate on Enterobacteriaceae in our phylogenetic analysis (Figure 2.1).

The phylogenetic tree shows that all sequences from the thrips bacteria belong to two sister clades, which are closely related to *E. coli* strains. The two clades were named type 1 and type 2. The base pair differences between type 1 and 2 were less than 2% or on average 35 positions in the stem region (based on *E. coli* 16S rDNA secondary structure). The split between type 1 and 2 was supported by a high bootstrap value (94%, Figure 2.1). A few differences were found between the sequences from pure bacterial strains, grown outside the thrips on artificial medium, and the other kind of sequence, directly obtained from the thrips body. Both clades, type 1 and type 2, contained each kind of sequence (Figure 2.1). There were nine base pair differences between TAC.XII.93.8, TAC.XII.93.2 and TAC.I.94.1 on the one hand and 6.4 and 6.9 on the other. There were six base pair differences between TAC.III.94.1 and 6.12.

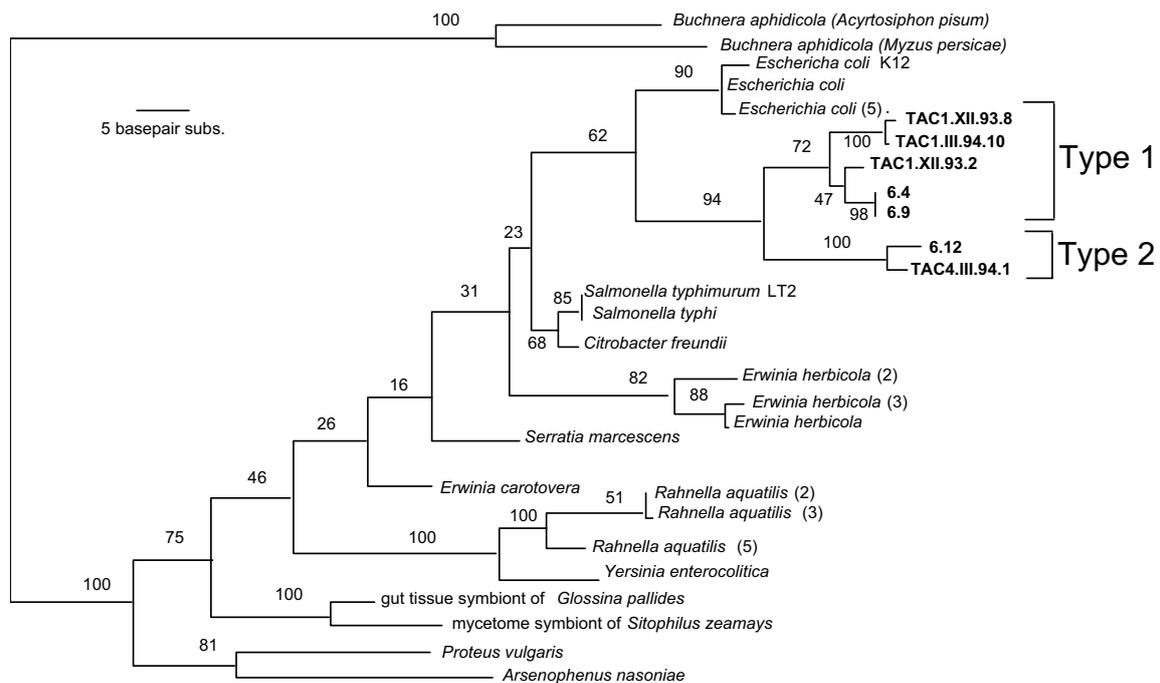


FIGURE 2.1 – Phylogenetic tree of Enterobacteriaceae, including thrips gut symbionts, on the basis of their 16S rDNA gene sequence using maximum parsimony approach. Thrips symbiont sequences are from isolated pure cultures of bacteria (Table 2.2) or were obtained from 16S rDNA fragments isolated directly from the thrips without pre-culturing of the bacteria outside the host (sequences indicated by 6.4, 6.9, and 6.12). Other sequences were selected from the Ribosomal Database Project on basis of their possible relation with thrips gut bacteria. The phylogram represents the maximal parsimonious tree after bootstrapping 1000 times. The total length of this tree is 769. The consistency index is 0.58. *Buchnera aphidicola*, the primary symbiont of aphids and closely related to Enterobacteriaceae, is used as outgroup to root the tree. Numbers on the branches represent the bootstrap values.

Biochemical description of bacteria with API 20E

Biochemical tests of different strains of isolated gut bacteria showed that all strains shared the same characteristics. Positive in beta-galactosidase, acetoin production, glucose utilisation (ut), mannitol ut, rhamnose ut, sucrose ut, amygdalin ut and arabinose ut. Negative in arginine dehydrolase (dh), lysine dh, ornithine dh, citrate ut, H₂S production, urease, tryptophane deaminase, indole production, gelatinase, inositol ut, sorbitol ut and cytochrome oxidase. Biochemical identification using the API reference guide indicated that our strains were *Erwinia* species, possibly *Enterobacter agglomerans*. The accuracy of this identification could be questioned because the identification 'Erwinia species' using API 20E systems is given to a large number of different API 20E biochemical patterns (Mergaert *et al.*, 1984). On the other hand, our results showed that 19 out of 20 tested biochemical properties of symbiotic bacterial strains of western flower thrips were shared with one subphenon of Mergaerts' studies, i.e. phenon F2. There is one difference between the biochemical characteristics of this phenon and thrips bacterial strains: the acid production from inositol in the latter.

Bacterial type strains TWC and TWK, obtained from the Wageningen thrips populations included in this study, were biochemically identical to TAC bacteria from the Amsterdam thrips. This uniformity was confirmed by the RAPD data. The two clades found in 16S rDNA sequence analysis, type 1 and type 2 (Figure 2.1), could not be distinguished in the biochemical tests.

RAPD identification of gut bacterial strains

In total 56 RAPD markers from ten different primers were scored for presence/absence in thrips bacteria and *E. coli* strains. First, the results of RAPD fingerprinting were compared with the 16S rDNA sequence results of the same strains. The two data sets were compatible: the clade 1 and clade 2 strains found in the sequence analysis could also be discriminated with RAPD fingerprinting. The two clades differed in 41 markers and were similar in only fifteen. According to the RAPD markers, thrips gut bacteria were completely different from *E. coli*. This validates the use of RAPD fingerprints for clade identification.

Secondly, we tested whether bacteria with identical morphology from one thrips were the same. This was confirmed in three different experiments where five individual bacterial colonies from a single thrips had (nearly) identical banding patterns, the frequency of shared bands was 0.95 to 1.0 (Table 2.3). Thirdly, bacterial variation among thrips from one population was studied. We compared bacteria from two different thrips pair-wise. The compilation of all these comparisons showed that the frequency of shared bands ranged between 0.65 and 0.9 (Table 2.3). Fourthly, the variation between thrips from different generations was tested. To test this, we compared the bacterial isolates from the type strains of each population. The variation among generations was not larger than between thrips bacteria from the same generation but different populations. Both cases showed a frequency of shared bands between 0.5 and 0.75 (Table 2.3). It was found that the type 2 bacteria, described according to the 16S rDNA sequence, only occurred in a small number of thrips samples.

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TABLE 2.3 – Variation between thrips gut bacteria assessed with RAPD-PCR. Results of several comparisons are given: between bacteria of one thrips, between bacteria of several thrips of one generation or population and between bacteria from thrips of different populations and generations. For each of the four comparisons a summation is presented of pair-wise comparisons of thrips gut bacterial strains. Variation is expressed as the mean and the range of the frequency (freq.) of shared (homologous) bands. N_{rep} = number of repetitions of the experiment, N_{thrips} = number of thrips tested per replicate, N_{bact} = number of randomly picked bacterial colonies per thrips included in one replicate, N_{mark} = number of markers scored.

Experiment	N_{rep}	N_{thrips}	N_{bact}	N_{mark}	Populations	Mean frequency of shared bands (range)
Within one thrips	2	10	5	15	ACH, WCH	0.97 (0.92–1.00)
Between thrips of one population and from one sample date	4	10	1	20	All	0.78 (0.71–0.85)
Between thrips of one population and from different generations	3	5	1	20	All	0.68 (0.60–0.80)
Between thrips from different populations and one sample date	5	5	1	20	ACH–WCH	0.60 (0.43–0.70)
					WCH–WCU	0.62 (0.54–0.70)
					ACH–WCU	0.57 (0.40–0.70)

DISCUSSION

Each isolation experiment showed that western flower thrips possess large amounts of morphologically identical bacteria, on average 0.5×10^5 , which are primarily located in the thrips hindgut. This confirms earlier electron microscopic studies (Dallai *et al.*, 1991, and Ullman *et al.*, 1989). The numbers of bacteria growing on enriched agar medium (LB or most other media that we tested) were comparable to the numbers of bacteria in direct counts of the thrips homogenate (Table 2.1). This strongly suggests that our bacterial isolation and cultivation method is an accurate representation of the bacterial flora in thrips.

It is possible that bacterial species remain undetected. For example, certain bacteria may have completely lost the capacity to grow outside the insect body tissue, such as Rickettsiae and *Wolbachia* (Breeuwer *et al.*, 1992; Moran and Baumann, 1994; Stouthamer *et al.*, 1993; Werren *et al.*, 1995) and endosymbionts in mycetomes (Aksoy *et al.*, 1995; Douglas, 1989; Nardon, 1973). In addition, some bacterial species may not be able to grow on plates. Alternatively, rare species may remain undetected due to the presence of more abundant species. Low abundance does not necessarily imply that these bacteria are functionally unimportant. In other insect-bacteria systems it was shown that the number of symbionts is not always indicative for the importance of the association (Potrikus and Breznak, 1977).

We used PCR based techniques to look for non-culturable bacterial species by amplifying the bacterial ribosomal DNA directly from thrips using universal primers for 16S rDNA of prokaryotes (Lane, 1991). In theory, these primers should be able to pick up all bacterial species present in the thrips. With these primers, bacterial DNA was picked up that had the same sequence as was found in pure cultures of thrips gut bacteria. We specifically tested

for *Wolbachia* with specific *ftsZ* primers (Holden, 1993). However, no *Wolbachia* were detected. On the bases of all results obtained with DNA sequencing it is clear that the TAC bacterium is indeed the only one consistently present and abundant in thrips.

There are several reasons to argue against the possibility that the thrips-bacteria association was an accidental finding, a result of a flaw in our experimental design of the isolation. In other words, that we were culturing transient microbes. The possibility that this infection was an incident may be ruled out by studying insects from various populations. All three populations were reared under different conditions in separate greenhouses (Amsterdam and Wageningen). The isolation of thrips bacteria was done by three individuals and in two different laboratories, to reduce the risk of a bias due to a certain laboratory practice. Also, bacteria were isolated at different moments in time, spanning more than 50 thrips generations.

A striking fact is the absence of bacteria in some of the thrips individuals. This occurs mostly in adult thrips, all second instar larvae have gut bacteria (Table 2.2). At this moment, we have no idea why this difference exists. It is possible that bacteria have different effect in adults than in larvae. The relation between number of bacteria, larval growth, and thrips oviposition needs to be clarified in future research.

Plant as source of microbes

The option that the plant was the source of one particular type of transient bacteria was excluded by rearing thrips on different host plants, chrysanthemum and cucumber, from different growers. In all the samples, the typical thrips gut bacteria were found in large numbers, regardless of host plant. In a few isolates, more than one morphological type of bacterium was present, in addition to these bacteria. They were mostly *Pseudomonas* species, determined on the basis of biochemical characteristics (E. de Vries, unpublished data). These may have been transient microbes, ingested by the thrips during feeding on plant tissue. A variety of bacteria can indeed be found on the leaf surface of cucumber and chrysanthemum (E. de Vries and G. Jacobs, unpublished data).

Identity of thrips gut bacteria

Comparison of 16S ribosomal DNA gene sequences showed that all thrips gut bacterial strains are closely related and belong to the Enterobacteriaceae. Two types were found, and of those types, type 1 prevailed. Biochemical identification of thrips gut bacteria indicated that these belong to the genus *Erwinia* and may be related to *Enterobacter agglomerans* (first described by Ewing and Fife, 1972). The different strains classified as *E. agglomerans* are genetically and phenotypically diverse and are not a monophyletic group in the genus *Erwinia* (Mergaert *et al.*, 1984, 1993). Recently, the species was renamed *Pantoea agglomerans* (Mergaert *et al.*, 1993). The names *E. agglomerans* and *E. herbicola* are used interchangeably. *Erwinia herbicola* strains are routinely isolated from roots and leaves of a number of different plants, and they are an important plant pathogen (Mergaert *et al.*, 1984). Molecular phylogeny of thrips gut bacteria clearly showed that they are unrelated to *E. herbicola* strains sequenced by other researchers (Figure 2.1). Biochemical similarity may be the result from convergent evolution of biochemical characteristics of bacterial species that live in a similar environment. Thus, we should be careful to attach a species name to bacterial clones on the basis of biochemical API-test alone.

Currently, the best way to resolve the above taxonomic issue is using molecular phylogenetic methods (Olsen and Woese, 1993). Unfortunately, molecular sequence data are not available for most of the type strains, and phylogenetic relationships in this genus are unresolved. There is variation among strains of thrips gut bacteria to the same extent as in other bacterial species. In *E. coli*, *Rahnella aquatilis* and *E. herbicola* 16S rDNA sequence variation among various strains was also found (Figure 2.1). Moreover, our phylogenetic analysis suggests that even the whole genus *Erwinia* is not monophyletic because *E. carotovera* and *E. herbicola* are unrelated.

Related gut symbionts

In other insect species, symbionts were identified biochemically with API 20E systems as *E. agglomerans*. This species has been reported in orthopteran (Dillon and Charnley, 1995; Mead *et al.*, 1988), termite (Potrikus and Breznak, 1977), coleopteran (Bridges, 1981), homopteran (Harada *et al.*, 1996), lepidopteran (Thibout *et al.*, 1995), and dipteran species (Cole *et al.*, 1990; Daser and Brandl, 1992; McCollem *et al.*, 1994). Some of these authors assume that *E. agglomerans* represents a laboratory borne infection or transient microbe (Lloyd *et al.*, 1986; Thibout *et al.*, 1995). Others describe them as facultative symbionts (Harada *et al.*, 1996; McCollem *et al.*, 1994). However, in many of these studies only data from a few insect individuals from one particular population are presented. Therefore, we cannot determine whether in these cases the bacteria are symbionts, *i.e.* whether the association lasts for more than one generation. In addition, there are the above-mentioned drawbacks of biochemical identification. In contrast to their studies, we have determined that the symbiont is the dominant bacterial species in thrips, because it is present in all thrips populations for the entire study period, regardless of host plant and culture conditions.

At this point, the role of gut bacteria on thrips life history is unclear. There seems to be ample opportunity for thrips to become invaded by other microbes that occur particularly on the plant leaves that thrips feed upon. Indeed, a more diverse composition of insect gut microflora is typically found (Campbell, 1990; Daser and Brandl, 1992; Thibout *et al.*, 1995; Ulrich *et al.*, 1981). The fact that thrips are only infected with this type of gut bacteria suggests that the association may be under control of one or both partners. In order to understand the evolutionary dynamics of thrips and gut bacteria, we need to understand the mechanisms of colonisation and establishment but also what the costs and benefits of the symbiosis are for both participants.

There are several not mutually exclusive explanations for the persistence of this symbiotic association. One possibility is that thrips are infected with different microbes, but that the establishment of these bacteria in the gut is somehow prevented by the thrips. The gut bacterium may be able to resist microbial defense systems of the thrips. Alternatively, the thrips gut bacterium may be capable of eliminating or preventing settlement of other bacteria, for example via the production of bacteriocins (Dykes, 1995). According to this scenario, the gut bacterium is expected to impose a cost on the thrips host. On the other hand, the thrips host may facilitate colonisation and transmission of thrips bacteria. This is possible when the gut symbiont is beneficial. The gut symbiont *E. agglomerans* produces toxins that eliminate entomopathogenic fungi in the desert locust, *Schistocerca gregaria* (Dillon and Charnley, 1995), plays a role in food digestion for the carrot fly, *Psila rosae* (Cole *et al.*, 1990), and fixates nitrogen in termites and bark beetles (Bridges, 1981; Potrikus and

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Breznak, 1977). Now that we have established that there is a permanent association between thrips and *Erwinia* species bacteria, we can start studying facultative symbiotic interactions.

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