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Diversity of Bacteria Associated with Natural Aphid Populations

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The bacterial communities of aphids were investigated by terminal restriction fragment length polymorphism and denaturing gradient gel electrophoresis analysis of 16S rRNA gene fragments generated by PCR with general eubacterial primers. By both methods, the γ-proteobacterium *Buchnera* was detected in laboratory cultures of six parthenogenetic lines of the pea aphid *Acyrthosiphon pisum* and one line of the black bean aphid *Aphis fabae*, and one or more of four previously described bacterial taxa were also detected in all aphid lines except one of *A. pisum*. These latter bacteria, collectively known as secondary symbionts or accessory bacteria, comprised three taxa of γ-proteobacteria (R-type [PASS], T-type [PABS], and U-type [PAUS]) and a rickettsia (S-type [PAR]). Complementary analysis of aphids from natural populations of four aphid species (*A. pisum* [*n* = 74], *Amphorophora rubi* [*n* = 109], *Aphis sarothamni* [*n* = 42], and *Microlophium carnosum* [*n* = 101]) from a single geographical location revealed *Buchnera* and up to three taxa of accessory bacteria, but no other bacterial taxa, in each aphid. The prevalence of accessory bacterial taxa varied significantly among aphid species but not with the sampling month (between June and August 2000). These results indicate that the accessory bacterial taxa are distributed across multiple aphid species, although with variable prevalence, and that laboratory culture does not generally result in a shift in the bacterial community in aphids. Both the transmission patterns of the accessory bacteria between individual aphids and their impact on aphid fitness are suggested to influence the prevalence of accessory bacterial taxa in natural aphid populations.

Many symbioses between animals and microorganisms are traditionally described as binary associations, i.e., relationships between an animal and single taxon of symbiotic microorganism (16). However, it is becoming increasingly apparent that this perspective is an oversimplification because animals generally harbor multiple microbial taxa (24, 26). This shift in focus can be illustrated by the microbial symbiosis in aphids. Until recently, virtually all research on the microbial symbiosis in aphids has concerned the γ-proteobacterium assigned to the genus *Buchnera*, which accounts for >90% of the microbial cells in aphids (2, 15). *Buchnera* cells are restricted to specific aphid cells (known as bacteriocytes) in the insect body cavity, are obligately vertically transmitted, and are required by the insect for normal growth and reproduction (5, 15, 17). However, many aphids additionally harbor one to several other bacterial taxa, known as secondary symbionts or accessory bacteria, including taxa known informally as R-type (PASS), S-type (PAR), T-type (PABS) and U-type (PAUS). The accessory bacterial taxa generally have a broader tissue distribution in aphids than *Buchnera* does and can be transmitted among aphids both vertically and horizontally (6, 7, 8, 11, 17, 20, 34, 35). The pea aphid, *Acyrthosiphon pisum*, has also been reported to bear *Spiroplasma* (21), *Erwinia* (23), and *Staphylococcus* (3, 22) spp.

A key feature of the accessory bacterial taxa in aphids is that, unlike *Buchnera*, they are not generally universal in any one aphid species (8, 11, 12, 20, 34, 35). In principle, the distribution of accessory bacteria can be accounted for by the occasional horizontal acquisition and failure of vertical transmission, perhaps compounded by selection for or against insects containing the accessory bacteria. It has been suggested that the accessory bacteria may be transmitted horizontally by the oral route (i.e., feeding from plants contaminated with these bacteria) or by aborted parasitoid attack (i.e., attack by a parasitoid whose ovipositor has become contaminated by a previous attack of an aphid harboring the accessory bacterium) (13, 35). There is evidence that the accessory bacteria may have positive, negative, or no effect on aphid fitness, depending among the bacterial taxa and with environmental conditions (8, 12, 18, 30). However, a general limitation to these considerations is that much of the research to date on the interactions between aphids and accessory bacteria has been conducted on aphids in long-term laboratory culture. The bacterial diversity of some insects is altered by laboratory maintenance conditions (14, 27), and the possibility cannot be excluded that the microbial taxa of aphids are similarly changed or reduced by laboratory conditions.

The core purpose of this study was to investigate the microbiota of aphids by two PCR-based methods, terminal restriction fragment length polymorphism (T-RFLP) and denaturing

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TABLE 1. Bacterial taxa present or absent in reference lines of *A. pisum*

A. pisum line	Presence of:				
	Buchnera	R-type bacteria	S-type bacteria	T-type bacteria	U-type bacteria
LMB95/28[a]	+	−	−	+	−
R[b,c]	+	−	+	−	−
IS[b,d]	+	+	−	−	−
FH[b,d]	+	−	−	−	+
MD[b,d]	+	−	−	−	−

[a] Aphid line in laboratory culture (20°C, 16 h of light and 8 h of darkness per day on *Vicia faba* plants).
[b] Aphid DNA.
[c] Provided by C. Montllor.
[d] Provided by T. Fukatsu.

gradient gel electrophoresis (DGGE) analysis of amplified 16S rRNA gene fragments, both of which have been used widely for the analysis of microbial communities. These approaches have the advantage over the methods previously used to study accessory bacteria in aphids (sequencing of clonal libraries, taxon-specific PCR assays) that they offer rapid and robust assays of bacterial taxa, whether or not they have been found previously in aphids (29, 32, 33). These methods are therefore well suited to studying the bacterial communities in natural aphid populations and particularly to investigating whether the bacterial community differed substantially between aphids from natural populations and in long-term laboratory culture.

MATERIALS AND METHODS

Aphids. Three classes of aphid material were used: DNA from five reference lines of *Acyrthosiphon pisum* with known bacterial community profiles as determined by 16S rRNA gene library sequencing, taxon-specific PCR, and in situ hybridization analysis (Table 1); two parthenogenetic lines of aphids, *A. pisum* JF98/24 and *Aphis fabae* HR91/3, in long-term culture on *Vicia faba* cv. The Sutton; and field samples of four species, collected from a damp meadow at Silwood Park, Berkshire, United Kingdom, between June and August 2000: *A. pisum* and *Aphis sarothamni* from *Cytisus scoparius*, *Amphorophora rubi* from *Rubus fruticosus*, and *Microlophium carnosum* from *Urtica dioica*. DNA was extracted from single aphids by using the DNeasy tissue kit (Qiagen) as specified by the manufacturer. Fresh material was used for the laboratory lines, and aphids from natural populations were transferred directly from the host plant to individual tubes of acetone (19) and stored at room temperature until DNA was extracted up to a year later.

T-RFLP analysis. 16S rRNA gene fragments were amplified from total aphid DNA by using the general bacterial primers Y2MOD (5′-ACT-YCT-ACG-GR A-GGC-AGC-AGT-RGG-3′) (*Escherichia coli* positions 338 to 361), modified from that described in reference 43 and labeled at the 5′ end with the phosphoramidite dye 6-FAM (MWG Biotech UK, Milton Keynes, United Kingdom), and 16SB1 (*E. coli* positions 1491 to 1512) (6), by 24 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min, but with the extension time increased to 2 min for the first and last cycles. The amplification reaction mixtures contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 100 μM each deoxynucleoside triphosphate (Promega), each primer at 0.2 μM, and 1 U of Platinum *Taq* polymerase (Gibco, Life Technologies) in 50-μl volumes. This followed preliminary experiments that (i), revealed that a greater diversity of 16S rRNA gene amplification products was obtained consistently with Platinum *Taq* polymerase than with other enzymes tested and (ii), optimized the PCR conditions by a Taguchi approach (10). A sample of the PCR product from each amplification was run on a 1.5% agarose gel, the remainder of the sample was purified with the Qiaquick PCR purification kit (Qiagen) as specified by the manufacturer, and subsamples (15 μl) were digested either with 3 U of *Alu*I and 2.5 U of *Bse*R1 (New England Biolabs) (37°C for 2 h) (AB digestion) or sequentially with 3 U of *Sma*I (25°C for 2 h) followed by 3 U of *Cla*I and 3 U of *Xba*I (37°C for 2 h) (Promega) (SCX digestion). A 2-μl volume of each restriction digest was mixed with 1 μl of GS500 size standard (Applied Biosystems) and run on a 4.5% polyacrylamide gel on an ABI 377 automated sequencer. The results were analyzed using Genescan version 2.02 (Applied Biosystems). Table 2 shows the predicted restriction sites for each of the five enzymes used, together with the 5′-terminal restriction fragments (5′-T-RFs), for bacteria previously reported in aphids and for *E. coli*, *Staphylococcus*, and *Wolbachia.* The restriction enzymes were selected from inspection of 5′-T-RF patterns for a variety of enzymes, initially in silico using the Ribosomal Database Project (RDP) database to construct T-RF outputs and subsequently in test experiments that checked the reproducibility of the method. With the restriction enzymes selected (but not some other apparently suitable enzyme combinations), the detected complement of bacteria did not differ among multiple amplifications from the same DNA templates or with 1:10 dilutions of the template, and the observed positions of the peaks in the electropherograms were ±1 bp from the predicted positions.

DGGE analysis. A 16S rRNA gene fragment of ca. 200 bp was amplified with the general bacterial 16S rRNA gene primers P2 and P3 (*E. coli* positions 341 to 357 and 518 to 534, respectively) (32) from a template of total aphid DNA or in a nested PCR amplification using as template the products of a PCR amplification with primer pair 16SA1 and 16SB1 (*E. coli* positions 8 to 27 and 1491 to 1512, respectively) (6). All amplifications were performed in 50-μl volumes under the conditions given in reference 32. PCR products were checked by agarose gel electrophoresis (2% [wt/vol] agarose) and stained with ethidium bromide prior to DGGE analysis. DGGE was carried out as described previously (37, 41). PCR products (ca. 200 ng of each product) were separated using gradient polyacrylamide gels (6 to 12% [wt/vol] polyacrylamide) with a denaturing gradient between 40 and 60% (100% denaturing conditions are 7 M urea and 40% [vol/vol] formamide). Gels were poured with the aid of a 50-ml volume gradient mixer (Fisher Scientific, Loughborough, United Kingdom), and electrophoresis was done at 75 V for 16 h at 60°C. The polyacrylamide gels were visualized under UV following staining with ethidium bromide. All bands were excised, homogenized in 10 μl of sterile distilled water, and incubated at 4°C overnight. The sample (1 μl) was then reamplified by PCR (as above) and used as a template for a direct sequencing reaction with the BIG-Dye sequencing kit (Applied Biosystems) with the P3 primer. The partial 16S rRNA gene sequences obtained were analyzed for DNA sequence similarity using the BLASTN algorithm (1) to the search the online GenBank DNA database. All sequences were checked for chimeric artifacts by using the CHECK_CHIMERA program of RDP (28).

RESULTS

Evaluation of methods for bacterial community analysis. The T-RFLP electropherograms obtained from the PCR amplifications of the five reference lines of aphids are shown in Fig. 1A to J. They show the peaks conforming to the predicted T-RF of *Buchnera* and accessory bacterial taxa previously identified in each line (Tables 1 and 2). Peaks predicted for other bacterial taxa previously found in aphids (Table 2) were not detected in any electropherograms.

Additional peaks in some samples from all lines were detected at 163 and 172 bp for the AB digestion and 72, 80, and 88 bp for the SCX digestion (Fig. 1). Supplementary experiments (data not shown) revealed these peaks in digestions with different restriction enzymes and in undigested controls, suggesting that they were PCR artifacts, such as fluorescently labeled concatemers of primer-dimers and misprimed products. Aphid line IS also generated a peak at 313 bp (Fig. 1E), and some AB electropherograms of all lines included a broad peak at >500 bp. These peaks were interpreted as partial digestion products: 313 bp of R-type bacteria and >500 bp of all amplicons (*Buchnera* and R-, T-, and U- type bacteria have an *Alu*I restriction site at ca. 520 bp [Table 2]). Complete digestion could not be achieved by extending the incubation time because the manufacturer recommends that the enzyme *Bse*RI not be used in digestion reactions longer than 2 h. The partial digestion product of R-type bacteria at 313 bp in the AB T-RF could be used to identify R-type bacteria in samples that

TABLE 2. The restriction sites and restriction fragment lengths of the 16S rRNA gene amplicons

Bacterial taxon[a]	Cut site of restriction enzyme[b]:					T-RF length (bp)[c]	
	*Alu*I	*Bse*RI	*Cla*I	*Sma*I	*Xba*I	AB	SCX
Bacterial taxa previously described in aphids							
Buchnera (M27039) from *A. pisum*	395					57	
	862					524	
R-type (AF293617) from *A. pisum*	651	460				122	
	862					313	
						523	
S-type (U42084) from *A. pisum*	434			614	651	96	251
	832					497	288
T-type (AJ297720) from *A. pisum*	618	460			661	122	323
	862					280	
						524	
U-type (AF293618) from *A. pisum*	618		476			280	138
	862					523	
V-type (AY136158) from *Pemphigus populi*	443					105	
	455					117	
	618					280	
	847					508	
Ars-type (AY136168) from *Wahlgreniella nervata*	651			615		313	277
	862					524	
So-So type (AY136147) from *Eulachnus pallida*	395			626		57	288
	455					118	
	651					313	
	847					509	
	862					524	
Spiroplasma sp. (AB048263) from *A. pisum*	862					517	
Erwinia sp. (AJ233418) from *A. pisum*	862			615		524	277
				626			288
Bacterial taxa not previously described in aphids							
E. coli (AE000460)	651			615		313	277
	862					524	
Staphylococcus sciuri (Z26901)[d]		680				342	
Wolbachia sp. (AB038370)	395				651	57	288
	434					96	
	832					496	

[a] GenBank accession numbers are given in parentheses.
[b] The first and subsequent cut sites of each restriction site are indicated, using the nucleotide number (1 to 1542) of the 16S rRNA gene of *E. coli* (GenBank accession number AE000460 with the 16S rRNA gene at nucleotides 8271 to 9812).
[c] The top value in each entry refers to the 5′-T-RF, and the subsequent values refer to lengths arising from incomplete digestion.
[d] Sequence with close similarity to the sequence recovered from a clonal library of the PCR-generated 16S rRNA gene fragment from *A. pisum* (3).

additionally bore T-type bacteria. This overcame a limitation of the protocol, which in principle can discriminate R-type and T-type bacteria in single infections only, because both bacteria have a T-RF at 122 bp in the AB profile and T-type bacteria have a T-RF at 323 bp in the SCX digestion (Table 2). (The theoretical T-RF of *E. coli* is also at 313 bp, but *E. coli* and R-type bacteria can be discriminated in the SCX T-RF profile [Table 2]). The broad peak at >500 bp attributed to partial digestion products of various bacterial 16S rRNA gene fragments masked the predicted T-RF of *Spiroplasma*, and it was concluded that *Spiroplasma* could not be identified reliably by the T-RFLP protocol.

The T-RFLP electropherograms of the two laboratory lines *A. pisum* JF98/24 and *Aphis fabae* HR91/3 contained peaks attributable to *Buchnera* and U-type bacteria.

The DGGE profiles of the 200-bp 16S rRNA gene fragment amplified from the five reference lines and two laboratory lines of *A. pisum* yielded one to four bands (Fig. 2a). One band in each profile was identified by sequencing as an amplicon from *Buchnera* (Table 3); the *Buchnera* band in *Aphis fabae* HR91/3

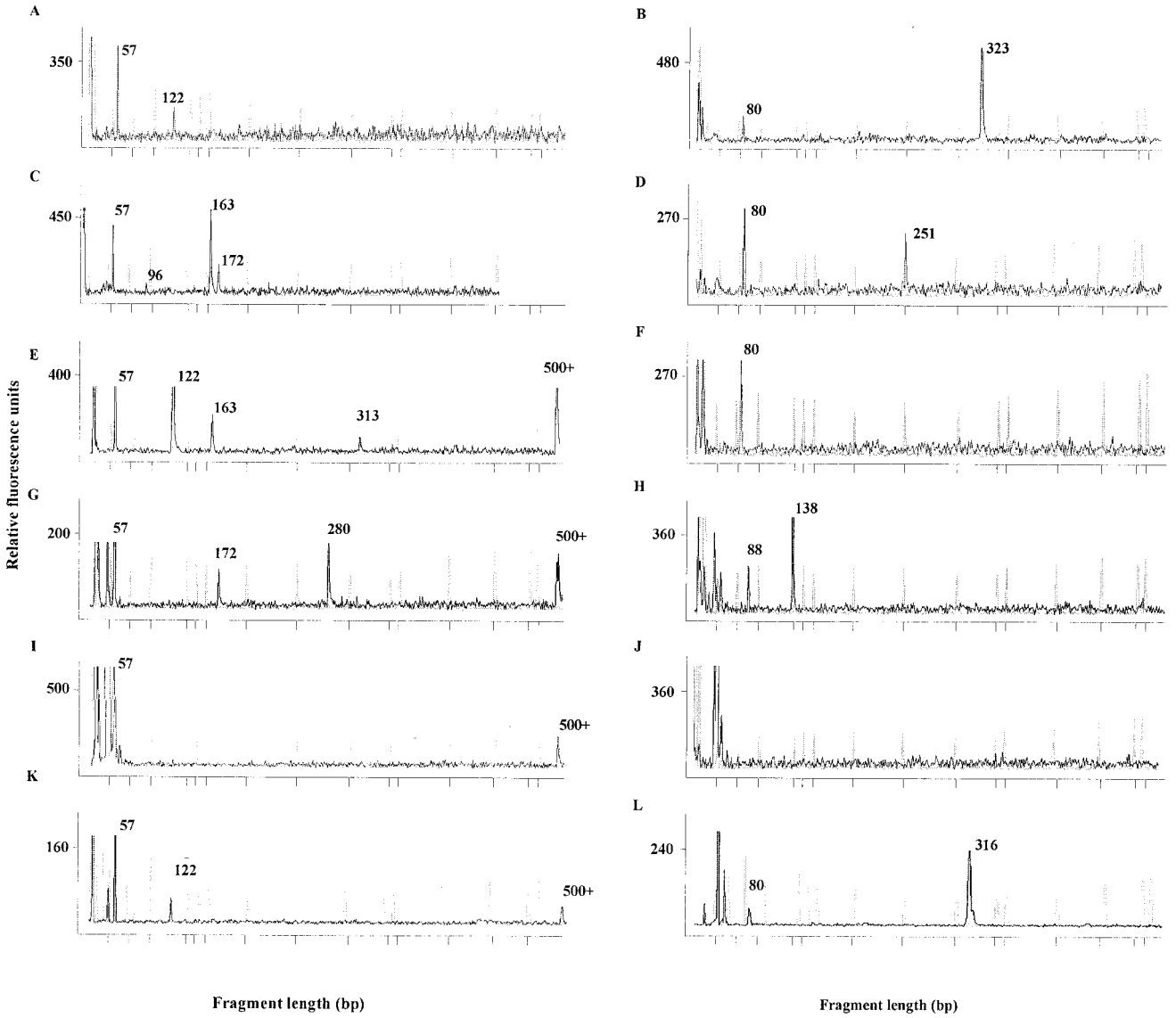

FIG. 1. Electropherograms of the T-RFs produced by *Alu*I-*Bse*RI (AB) digestion (A, C, E, G, I, and K) and *Sma*I-*Cla*I-*Xba*I (SCX) digestion (B, D, F, H, J, and L) of PCR amplicons from 16S rRNA genes from the reference lines of the pea aphid *A. pisum* LMB95/28 (A and B), R (C and D), IS (E and F), FH (G and H), and MD (I and J) and aphid 1330 from the natural population of *A. pisum* (K and L). The T-RFs are shown in black, and the internal standards (50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bp) are shown in gray.

was markedly less mobile within the DGGE gel than in the *A. pisum* lines, indicating that the sequence of the amplicons differed between *Aphis fabae* and *A. pisum.* The accessory bacteria identified in each aphid line by DGGE matched the results from the T-RFLP analysis precisely. However, two bands in the profile for line IS were assigned to R-type bacteria and two bands in line R were assigned to S-type bacteria, indicative of microdiversity in both of these accessory bacterial taxa detectable by DGGE but not by T-RFLP (Fig. 2a; Table 3). Many of the reactions also yielded a band with slightly greater mobility than the *Buchnera* fragment (e.g., the bands labeled "artifact" for lines LMB95/28, IS, JF98/24, and HR91/3 in Fig. 2a). The sequences of the band at this position varied between reactions and, when checked for chimeras, were found to include *Buchnera* sequence and a region(s) of low homology to other γ-proteobacteria sequences, whose identity varied among samples. When these sequences were included in phylogenetic analyses, they disrupted the tree topology by forming an unsupported outgroup clade (<50%) to the *Buchnera* sequences (data not shown). This type of tree disruption is characteristic of chimeric artifacts (25).

Bacterial community in aphids from natural populations. The T-RFLP electropherograms of all 324 aphids scored included a peak identical to the predicted T-RF of *Buchnera*, and many also had peaks that could be assigned to the R-, S-, T-, and U-type accessory bacteria. In addition, some *A. pisum* individuals (but none of the other three aphid species) that generated a 122-bp peak in the AB T-RF profile (Fig. 1K), as expected for R-type and T-type bacteria, had a previously undescribed peak at 316 bp after SCX digestion (Fig. 1L).

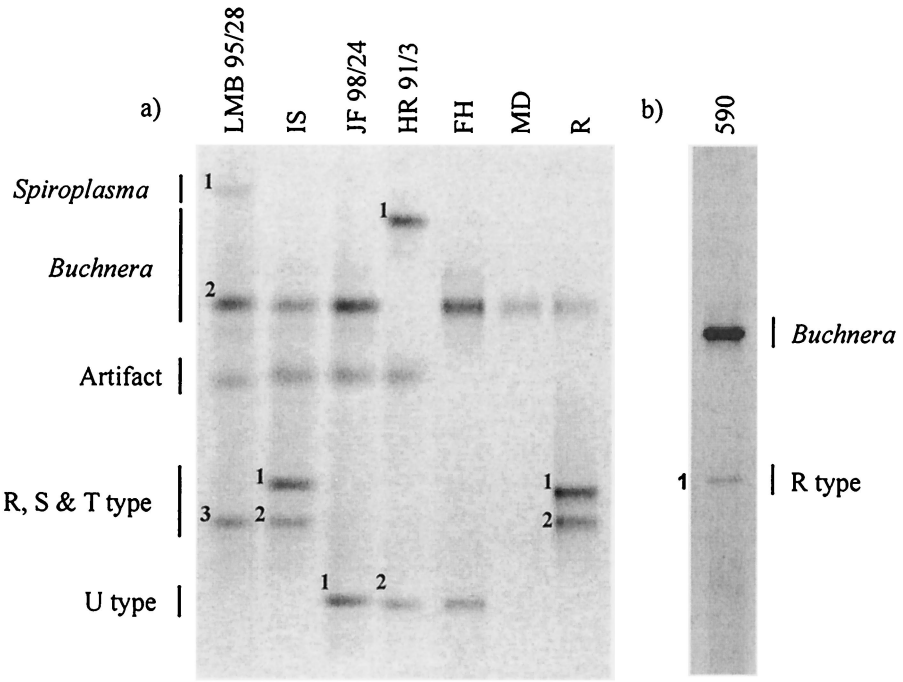

FIG. 2. DGGE profiles of 16S rRNA gene fragments amplified by PCR using general primers (P2 and P3) from whole-aphid DNA templates. (a) Aphid reference lines and lines in long-term laboratory culture; (b) Bacterial community in one *A. pisum* aphid with a novel T-RF profile (sample 590, collected from *Cytisus scoparius*). Numbers in the lanes relate to bands excised from the gel for sequencing (Table 3).

DGGE analysis of these aphids yielded two bands. One band had the mobility characteristic of the *Buchnera* product, and the other band could be assigned to R-type bacteria on the basis of its sequence (Fig. 2; Table 3). In other words, the SCX T-RF of 316 bp could be attributed to a variant of R-type bacteria with a *Xba*I restriction site at 316 bp downstream of the 5′ end of the amplicon. Among the 72 individuals of *A. pisum* tested, 38 bore R-type bacteria with the standard T-RF profile and 46 bore the "variant" R-type bacteria with a *Xba*I restriction site at 316 bp.

TABLE 3. Similarities of sequences of 16S rRNA gene fragments derived from DGGE bands to reference database sequences

DGGE band[a]	Closest match (GenBank accession no.)	Alignment length (bp)	% Similarity	E-value[b]
LMB95/28				
Band 1	*Spiroplasma* sp. (AJ132412), Firmucutes	136	88.5	6.1E-54
Band 2	*Buchnera* sp. (AP001118), γ-proteobacteria	110	99.4	8.7E-54
Band 3	T-type (AF293626), γ-proteobacteria	154	99.4	8.4E-79
IS				
Band 1	R-type (AF293617), γ-proteobacteria	124	99.2	3.7E-60
Band 2	R-type (AB033779), γ-proteobacteria	140	99.3	9E-69
JF98/24				
Band 1	U-type (AF293627), γ-proteobacteria	140	99.2	9E-69
HR91/3				
Band 1	*Buchnera* sp. (AP001118), γ-proteobacteria	161	100	2.5E-73
Band 2	U-type (AF293627), γ-proteobacteria	124	99.3	1.5E-61
R				
Band 1	*Rickettsia* sp. (U12458), α-proteobacteria	132	100	1.7E-66
Band 2	*Rickettsia* sp. (U12458), α-proteobacteria	131	98.5	3E-63
590				
Band 1	R-type (AY136140), γ-proteobacteria	135	99.3	7.3E-68

[a] The band number refers to the band excised from the DGGE gel shown in Fig. 2.
[b] Expectation value, i.e., the number of different alignments with scores equivalent to or better than the raw alignment score expected to occur in a database by chance.

TABLE 4. Number of bacterial taxa per aphid in natural populations

No. of bacterial taxa	No. of aphids			
	Microlophium carnosum	*Amphorophora rubi*	*A. pisum*	*Aphis sarothamni*
1[a]	1	103	4	18
2	27	5	60	17
3	70	1	7	7
4	3	0	1	0
Total no. of aphids	101	109	72	42

[a] The sole taxon detectable in these aphids was *Buchnera*.

The number of different bacterial taxa detected in the natural populations of four aphid species is summarized in Table 4. No individual aphid harbored more than three taxa of accessory bacteria, and the number of bacterial taxa per aphid varied significantly among the aphid species (Kruskall-Wallis test: $H = 226.73$, 3 degrees of freedom [df], $P < 0.001$), from 94% of *Amphorophora rubi*, which harbored no accessory bacteria, to 69% of *Microlophium carnosum* with two accessory bacterial taxa. The distribution of different accessory bacterial taxa is shown in Table 5. T-type was the only accessory bacterium detected in at least one individual of all four aphid species, harbored by 57% of *Aphis sarothamni* individuals and <10% of the other aphid species. Between 93 and 94% of both *Microlophium carnosum* and *A. pisum* had R-type. The U-type was rarely detected as the sole accessory bacterium: of the 84 aphids bearing U-type bacteria, 82 (all but two *Microlophium carnosum* individuals) also had R-type or T-type bacteria. The S-type was rare, detected in just 3 (<1%) of the 324 aphids scored. The frequency of accessory bacteria in *A. pisum* and *Aphis sarothamni*, the two aphid species with the same host plant, *Cytisus scoparius*, was significantly different ($\chi^2 = 79.01$, 2 df, $P < 0.001$; S-type bacteria excluded from analysis).

The data sets for all aphid species except *Amphorophora rubi* were suitable for analysis of the variation in the frequency of accessory bacteria with month of collection. For each aphid species, seasonal variation was not statistically significant (Table 6).

None of the T-RFLP electropherograms of the 324 aphids from natural populations yielded the expected T-RFs for *Erwinia* or *Staphylococcus* sp., bacteria previously found in aphids in laboratory culture (3, 22, 23).

TABLE 5. Frequency of accessory bacterial taxa in aphids in natural populations

Bacterial taxon	Frequency (%) of occurrence in[a]:			
	Microlophium carnosum	*Amphorophora rubi*	*A. pisum*	*Aphis sarothamni*
R-type	95	3	67	0
S-type	0	1	2	0
T-type	9	3	3	24
U-type	72	0	5	7

[a] Number of aphids of each species as shown in Table 4.

TABLE 6. Variation in frequency of accessory bacterial taxa with month of collection

Aphid species[a]	χ^2	df	*P*
A. pisum (June to August)	0.744	2	>0.05
Aphis sarothamni (June and July)	0.189	1	>0.05
Microlophium carnosum (June to August)	2.571	2	>0.05

[a] S-type bacteria excluded from all tests, and U-type and R-type bacteria excluded from the test for *A. pisum* and *Aphis sarothamni*, respectively, because of low frequencies.

DISCUSSION

The concordance between the bacterial taxa identified by DGGE and T-RFLP was excellent for the reference lines (aphids with previously described accessory bacterial complements), laboratory lines, and aphids from natural populations. All aphids were confirmed to have a low apparent diversity of bacteria, with no more than four taxa (*Buchnera* and three taxa of accessory bacteria) in any individual aphid (Table 4). The possibility should not be excluded, however, that aphids may harbor additional bacterial taxa that yield poor or no 16S rRNA gene PCR amplification products with the methods used as a result of PCR primer bias or low template abundance (38, 40).

This study additionally identified sequence microdiversity from analysis of the length of the 5′-TF in the T-RFLP electropherograms of R-type bacteria in *A. pisum* (Fig. 1K and L) from natural populations and from the different DGGE migration patterns for both R-type and S-type bacteria in the reference lines of *A. pisum* (Fig. 2; Table 3). Such heterogeneity could limit the applicability of the currently used diagnostic PCR assays, which generally have been designed on the basis of a few uniform sequences. For example, the R-type sequences obtained from amplicons used in DGGE analysis included sequence variation within a widely used taxon-specific primer, PASSF (7, 8, 30), but the impact of this variation on the reliability of the R-type-specific assay remains to be established. Variation in both 16S rRNA gene sequence between bacterial cells (36) and between multiple gene copies within single bacterial cells, as occurs in some but not all bacterial taxa (4, 9, 31), may contribute to this observed heterogeneity. The importance of multiple gene copies could be investigated by fluorescent in situ hybridization studies using probes which target the variable sites. This microheterogeneity, together with the similar mobility of the amplicons of different accessory bacteria in DGGE (Fig. 2), requires that DGGE bands be analyzed further by sequencing or Southern blotting for bacterial taxa to be identified, and this can limit the usefulness of DGGE as a rapid screen for bacterial communities in aphids.

Two principal conclusions can be drawn from this study of the bacterial community in aphids. First, the similarity of bacterial taxa identified in the aphid samples from long-term laboratory cultures and natural populations indicates that laboratory culture, which is known to affect the microbiota of some insects (see introduction), does not generally result in a substantial shift or reduction in the microbial community in aphids. In general terms, this conclusion confirms the relevance of laboratory-based studies of the aphid-microbe interactions (see, e.g., references 8 and 13). Exceptionally, however,

Erwinia and *Staphylococcus* spp. have been found previously in laboratory cultures of *A. pisum* (3, 22, 23) but were not detected in the aphids (including six *A. pisum* lines from long-term laboratory culture) studied here. Further research is required to establish whether this discrepancy reflects a susceptibility of aphids in laboratory culture to occasional colonization by bacteria not representative of natural populations or limitations of the molecular assays, perhaps linked to poor template availability or PCR bias (38, 40). The results of the T-RFLP analysis are, however, consistent with the report (42) that *Wolbachia* is apparently absent from aphids.

The second conclusion is that the four taxa of accessory bacteria initially identified in *A. pisum* (6, 7, 11, 35) occur in multiple aphid species but that none of these taxa was universally present, at least for the natural populations of the four aphid species investigated here. This conclusion confirms and extends published data sets obtained for one to several individuals of multiple aphid species by using taxon-specific PCR assays (34, 35), although the accessory bacteria referred to as V, So-So and Ars types in reference 34 were not detected in any aphids studied here. Horizontal transmission between aphid species is expected to contribute to the distribution of accessory bacteria because the phylogeny of accessory bacteria in different aphid species is not congruent with the phylogeny of their aphid partners (34, 35). Therefore, aphids generally can be considered a "habitat" for the accessory bacteria. These bacterial taxa have not, to date, been found under the free-living conditions, raising the possibility that aphids, and possibly other insects (11, 34), are essential for the long-term persistence of these bacteria.

Immediately arising from these considerations are questions about the processes underlying the observed interspecific variation in frequency of aphids bearing the various accessory bacteria. In addition to stochastic effects, contributory factors may include differences in the capacity of different accessory bacterial taxa to colonize and persist in the various aphid species and effects of the bacteria on aphid fitness. Three comparisons suggest that the impact of these factors may vary with bacterial taxon and environmental circumstance. First, experimental manipulations indicate that *A. kondoi* is incompatible with R-type bacteria from *A. pisum* (8) but that *Aphis fabae* is fully compatible with T-type bacteria from *A. pisum* (13). Second, a study of the significance of temperature as a determinant of the incidence of accessory bacteria has linked the elevated incidence of aphids bearing R-type bacteria in natural populations experiencing high temperatures in California to enhanced tolerance of high temperature in *A. pisum* harboring this bacterial taxon in the laboratory (30). However, no significant between-month variation in the frequency of accessory bacteria in British aphid populations was found either in the aphid species examined in this study (Table 6) or for T-type bacteria in *A. pisum* (12). This difference may reflect the more equable climate in the United Kingdom than in California. Finally, the host plant has also been implicated as an important correlate of the accessory bacterial complement in *A. pisum* (8). For example, individuals of *A. pisum* affiliated with *Trifolium* (clover) species tend to harbor U-type bacteria (39). However, the significantly different bacterial complements of *A. pisum* and *Aphis sarothamni* on *Cytisus scoparius* (Table 5) indicate that this link between plant and accessory bacterial complement does not necessarily extend to members of different aphid species.

In summary, the application of community-based molecular methods in this study to investigate the microbiota of aphids has provided the basis to explore the ecology of accessory bacterial taxa. The two chief issues to resolve are the habitat range of accessory bacteria, including the significance of aphids to their persistence, and the factors determining the incidence of accessory bacteria in aphids, predicted to include both transmission patterns and the impact of accessory bacteria on aphid fitness. The ecology of the various accessory bacterial taxa is not thought to be uniform; studies of these bacterial taxa offer the opportunity to explore the range of strategies available to nonpathogenic microorganisms associated with animals.

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