Two Species of Symbiotic Bacteria Present in the Soybean Aphid (Hemiptera: Aphididae)

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ABSTRACT Aphids, which feed solely on plant phloem sap, have developed symbiotic associations with bacteria that provide them with the amino acids that are lacking in phloem. Three soybean aphid (*Aphis glycines* Mat samura) populations were screened for the presence of *Buchnera aphidicola* and three common species of secondary aphid symbionts (*Serratia symbiotica*, *Hamiltonella defensa*, and *Regiella insecticola*). Diagnostic polymerase chain reaction and subsequent DNA sequencing showed the presence of two species of symbiotic bacteria present in all three soybean aphid populations tested: *B. aphidicola* and *Arsenophonus* sp. Although *Buchnera* is commonly found in aphids, *Arsenophonus* is most commonly found in whiteflies (Hemiptera: Aleyrodidae), making the soybean aphid unique among aphids that have been tested for the presence of *Arsenophonus*.

KEY WORDS soybean aphid, bacterial symbiont, *Buchnera aphidicola*, *Arsenophonus*

Plant phloem sap is used as a sole food source by insects in the order Hemiptera (Douglas 2006). Aphids (Hemiptera: Aphidoidea) need essential amino acids for growth and fecundity but are only able to synthesize 9 of the 20 essential amino acids. The low ratio of essential:nonessential amino acids in phloem is insufficient to support the growth rate observed in aphids. To survive on an amino acid–deficient diet, nearly all aphids rely on an obligate symbiotic relationship with bacteria in the genus *Buchnera*, which synthesize essential amino acids that are lacking in phloem (Douglas 1998, Russell et al. 2003).

Buchnera are maternally transmitted γ-proteobacteria housed along the aphid digestive tract in specialized cells called bacteriocytes. Phylogenetic analysis of the relationship between aphids and *Buchnera* shows an initial infection followed by strict vertical transmission dating back 150–250 million years (Moran et al. 1993). The coevolutionary process between aphids and *Buchnera* has lead to the deletion of many of the genes necessary for *Buchnera* to survive outside of its host and to the amplification of genes required to synthesize amino acids lacking in the aphid diet (Gil et al. 2002).

Buchnera are considered to be the primary symbiont present in aphids, making up >90% of the symbiotic bacteria present in bacteriocytes (Haynes et al. 2003). In addition to *Buchnera*, many aphids harbor additional bacterial symbionts, known as secondary symbionts. Unlike *Buchnera*, secondary symbionts are not found in all aphid species and are thought to undergo horizontal as well as vertical transmission (Moran and Telang 1998, Gil et al. 2002, Russell et al. 2003). There are three main species of secondary symbionts found in aphids: *Serratia symbiotica* (R-type), *Hamiltonella defensa* (T-type), and *Regiella insecticola* (U-type) (Moran et al. 2005). In addition, *Rickettsia*, *Spiroplasma*, and *Wohlbachia* species have also been found in aphids (Gomez-Valero et al. 2004, Sakurai et al. 2005).

The soybean aphid, *Aphis glycines* Mat samura (Hemiptera: Aphididae), was first introduced to the United States in the summer of 2000, where it has since become an economically important pest of soybean, *Glycine max* L. Merr. (Hartman et al. 2001, Venette and Ragsdale 2004). In this paper, two species of symbiotic bacteria, *B. aphidicola* and *Arsenophonus* sp. (a genus closely related to *Hamiltonella*), present in the soybean aphid are described. Partial 16S rDNA sequences were obtained for both species of bacteria, and BlastN was used to determine the similarity between symbionts of the soybean aphid and other known aphid symbionts. This is the first report of the presence of bacterial symbionts in the soybean aphid.

Materials and Methods

Aphids and DNA Preparation. Two clones of *A. glycines* from Champaign and Brown Counties in Illinois were initially collected and maintained by Curt Hill (University of Illinois, Urbana, IL). The clones were established from single first-instar nymphs reared on a continuous supply of Williams 82 soybean

Trade and manufacturers' names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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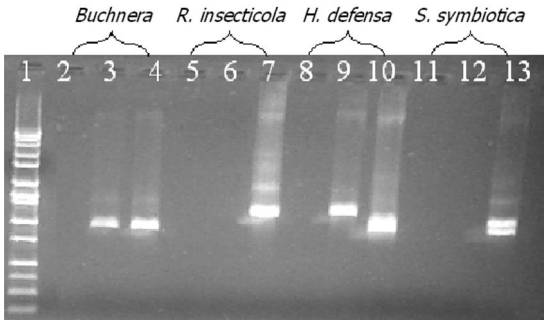

Fig. 1. Diagnostic PCR analysis of soybean aphid symbionts. Results for all three soybean aphid clones tested were the same. Data shown are from the Urbana, IL, clone. Lane 1 contains a 1-kb DNA ladder, lanes 2–4 contain products from BS16S and 16S4, lanes 5–7 contain products from R1279 F and 480R, lanes 8–10 contain products from T1279 F and 480R, and lanes 11–13 contain products from U1279 F and 480R. Lanes 2, 5, 8, and 11 contained no DNA (negative control), lanes 3, 6, 9, and 12 contained soybean aphid DNA, and lanes 4, 7, 10, and 13 contained DNA from *A. pisum* known to harbor the relative symbiont.

plants in plant growth chambers, with each population maintained in a separate growth chamber. The growth chambers were kept at 22°C, 70% RH, and continuous 300 μmol/m^2/s PAR radiation. In addition to the two Illinois soybean aphid clones, a soybean aphid population from Ohio (Kim et al. 2008) was established by placing a soybean leaf with multiple aphids into a pot containing four Williams 82 soybean plants in a growth chamber as previously described. To simplify data reporting, the Ohio soybean aphid population will be described as a clone in this paper.

Total aphid DNA was extracted using the CTAB/Na method as previously described (Keim and Shoemaker 1988). Each DNA sample was made from a random sample of 20 individual apterous adults from each aphid clone. Total aphid DNA samples from four *A. pisum* clones known to harbor *Buchnera*, *S. symbiotica*, *H. defensa*, and *R. insecticola* were obtained from Nancy Moran (University of Arizona) to use as positive controls.

Diagnostic 16S rDNA Amplification. To detect the presence of *Buchnera*, primers BS16S and 16S4 were used as previously described (Nakabachi and Ishikawa 1999). These primers were designed to specifically amplify *Buchnera* 16S rDNA, yielding a 984-bp product.

The presence of secondary symbionts was determined with a forward diagnostic primer (R1279, T1279, and U1279 for *S. symbiotica*, *H. defensa*, and *R. insecticola*, respectively) and a universal reverse primer (480R) as previously described (Russell et al. 2003). In secondary symbionts, the 16S rDNA and 23S rDNA genes were separated by an intergenic region of varying length (Fig. 1). The universal reverse primer binds to a region in the 23S rDNA gene that is conserved across all three secondary symbiont species. The diagnostic forward primers bind to species-specific regions in the 16S rDNA gene. The resulting fragment, spanning the 16S and 23S rDNA genes, along with the intergenic region, yields a product of characteristic length for each of the three secondary symbionts.

All PCR reactions were performed in 25-μl volumes with 1× polymerase chain reaction (PCR) buffer (Eppendorf/Qiagen, Qiagen Inc., Valencia, CA), 2 mM $Mg(OAc)_2$ (Eppendorf/Qiagen), 0.2 mM of each dNTP, 0.5 pmol/ml of each primer, 0.5 ng/ml genomic DNA, and 2 U of *Taq*DNA polymerase (Eppendorf/Qiagen). To prevent false-positive or -negative results, positive control reactions consisting of genomic DNA from *A. pisum* clones known to harbor the relative symbiont (obtained from Nancy Moran, University of Arizona) and negative controls consisting of water instead of DNA were used in all PCR reactions. The running conditions for all PCR reactions were 94°C for 3 min, followed by 30 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 1 min. PCR products were separated on an 0.8% agarose gel for 2 h at 125 V, stained with ethidium bromide, and visualized with UV light.

Diagnostic PCR testing was done one time per month for 8 consecutive mo for the two Illinois aphid clones. The Ohio soybean aphid clone was subjected to diagnostic PCR testing three times.

Sequencing and Phylogenetic Analysis of Symbiont 16S rDNA. Samples that yielded a distinct band in the diagnostic screening tests were purified using a QIAquick PCR purification kit (Qiagen) and sequenced directly. Sequencing reactions were performed at the University of Illinois W.M. Keck Center for Comparative and Functional Genomics. BlastN was used to compare the sequences of symbiont 16S rDNA from *A. glycines* to other known aphid symbionts in GenBank. Samples were considered positive for a specific symbiont only if the diagnostic PCR reaction yielded a band of the expected length and if the sequenced 16S rDNA was at least 95% similar to entries for the relevant symbiont using BlastN. Samples were considered to be negative for a specific symbiont if they did not amplify with the diagnostic primers, yielded a PCR product of the incorrect length, or if the partial 16S rDNA sequence was <95% similar to other previously described symbionts.

CLUSTAL W (Thompson et al. 1994) was used to align the experimental sequences with the sequences obtained from the BlastN search. Bacterial species and accession numbers used in this study are as follows: *Arsenophonus* sp. isolated from *Dialeurodes hongkongensis* (AY264667.1), *Glycaspis brimblecombei* (AF263561.1), *Pentastiridius* sp. (DQ834351.1), and *Triatoma rubrofasciata* (DQ508185.1); *B. aphidicola* isolated from *Acyrthosiphon pisum* (AB033776.1), *Aphis fabae fabae* (AY518294.1), *Myzus persicae* (AY849937.1), *Pterocomma populeum* (AJ296747.1), *Rhopalosiphum maidis* (M63247.1), *Rhopalosiphum padi* (M63246.1), *Schizaphis graminum* (L18927.1), and *Uroleucon sonchi* (M63250.1); *E. coli* (V00331); and *Hamiltonella defensa* isolated from *A. pisum* (AY296733.1).

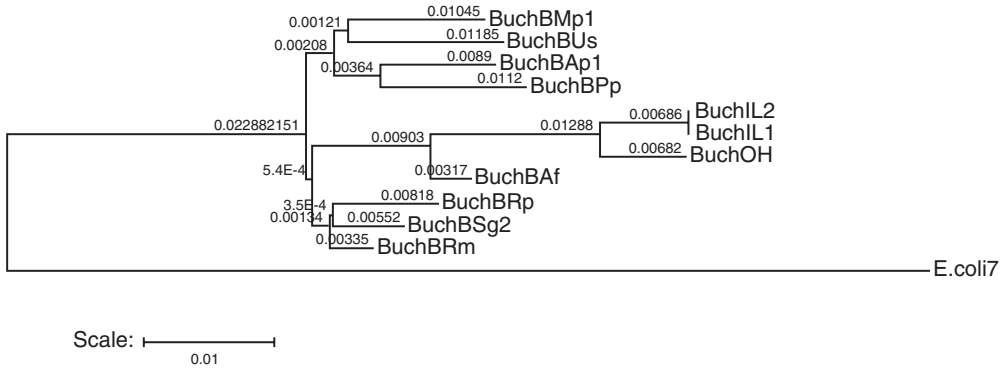

Fig. 2. Neighbor-joining phylogenetic tree for *B. aphidicola* found in three clones of the soybean aphid (BuchIL1, BuchIL2, and BuchOH). Strains and accession numbers used are as follows: *B. aphidicola* isolated from *Acyrthosiphon pisum* (BuchAp1, AB033776.1), *Aphis fabae fabae* (BuchAf, AY518294.1), *Myzus persicae* (BuchMp1, AY849937.1), *Pterocomma populeum* (BuchPp, AJ296747.1), *Rhopalosiphum maidis* (BuchRm, M63247.1), *Rhopalosiphum padi* (BuchRp, M63246.1), *Schizaphis graminum* (BuchSg2, L18927.1), *Uroleucon sonchi* (BuchUs, M63250.1), and *E. coli* (V00331).

Phylip version 3.6 (Felsenstein 1989) was used to create distance matrix calculations using the Kimura two-parameter method. These calculations were used to create neighbor-joining trees for the endosymbiotic bacteria detected in the soybean aphid, with *E. coli* used as an outgroup for each tree.

Results

Diagnostic PCR and Sequencing of Symbiont 16S rDNA. Amplification of *Buchnera* 16S rDNA with the specific diagnostic primers BS16S and 16S4 yielded a product of ≈985 bp in the three aphid clones (Fig. 1). Subsequent purification and sequencing of the PCR products (submitted to GenBank) confirmed the presence of *Buchnera* in the three soybean aphid clones. The partial *Buchnera* 16S rDNA sequences of the soybean aphid clones tested were at least 98% similar to each other, and BlastN searches confirmed that the obtained sequences were at least 95% similar to *Buchnera* 16S rDNA sequences from other aphid species. The neighbor-joining phylogenetic tree showed that the *Buchnera* strains found in the soybean aphid are most closely related to *Buchnera* found in *A. fabae fabae* (Fig. 2).

Amplification of *S. symbiotica* secondary symbiont 16S rDNA with the diagnostic forward primer R1279 F and the universal reverse primer 480R did not yield a product in the soybean aphid populations tested (Fig. 1), indicating the absence of *S. symbiotica.* Diagnostic PCR tests for *R. insecticola* using the primers U1279 F and 480R did not yield a product in any of the aphid clones tested (Fig. 1), indicating the absence of *R. insecticola.*

PCR tests using the diagnostic primers T1279 F and 480R yielded a product of ≈1,000 bp for the three soybean aphid clones (Fig. 1). This product was longer than the PCR product obtained in the positive control reaction using *A. pisum* genomic DNA known to contain *H. defensa*, indicating that the bacterium detected was not *H. defensa.* Subsequent sequencing and BlastN results further confirmed the absence of *H. defensa* in the soybean aphid clones, because BlastN searches with the partial 16S rDNA sequences obtained from the diagnostic PCR reactions were <95% similar to known *H. defensa* 16S rDNA sequences. Instead, BlastN searches indicated that a different γ-proteobacterium, *Arsenophonus*, was present in the soybean aphid clones tested. Phylogenetic analysis placed the secondary symbiont found in the soybean aphid closest to *Arsenophonus* found in *Glycaspis brimblecombei* (Fig. 3). Sequencing results were submitted to GenBank.

The two Illinois soybean aphid clones were tested for the presence of *Buchnera*, *S. symbiotica*, *H. defensa*, and *R. insecticola* monthly over the course of 8 mo. The species of bacteria present in each aphid clone did not change throughout the testing period, providing evidence that the 16S rDNA sequences obtained were not the result of bacterial contamination, as could be the case if a clone was found to gain a new symbiont during the test period. Likewise, the Ohio soybean aphid clone was tested for the presence of symbiotic bacteria three times. Results for all three tests were the same.

Discussion

Many aphid species are known to harbor multiple bacterial symbionts, with most studies focusing on the symbiosis between aphids, *Buchnera*, and the secondary symbionts *S. symbiotica*, *H. defensa*, and *R. insecticola.* In addition to the aforementioned species, *Wohlbachia*, *Spiroplasma*, *Erwinia*, and *Staphylococcus* spp. have been reported in *C. cedri* and *A. pisum* (Haynes et al. 2003, Gomez-Valero et al. 2004). These additional species of bacteria are considered to be facultative, because they are found in insect orders other than Hemiptera and in some instances decrease host fitness. In total, secondary symbionts of 10 separate bacterial groups have been discovered in aphids, and it is possible that there are additional undiscov-

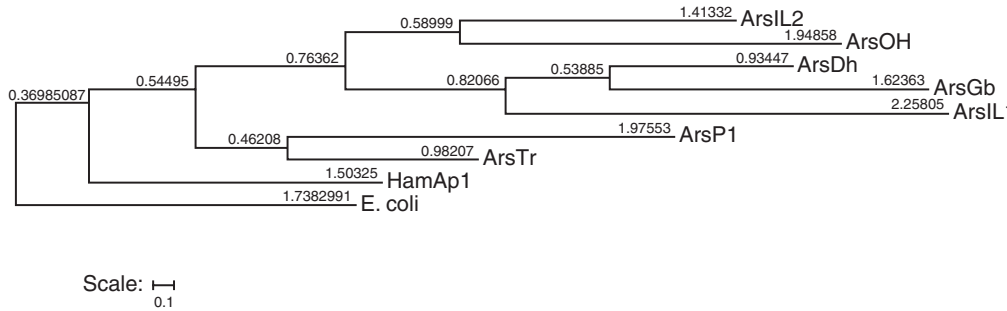

Fig. 3. Neighbor-joining phylogenetic tree for *Arsenophonus* sp. found in three clones of the soybean aphid (ArsIL1, ArsIL2, and ArsOH). Strains and accession numbers used are as follows: *Arsenophonus* sp. isolated from *Dialeurodes hongkongensis* (ArsDH, AY264667.1), *Glycaspis brimblecombei* (ArsGp, AF263561.1), *Pentastiridius* sp. (ArsP1, DQ834351.1), and *Triatoma rubrofasciata* (ArsTr, DQ508185.1); *E. coli* (V00331) and *Hamiltonella defensa* isolated from *A. pisum* (HamAp1, AY296733.1).

ered associations between aphids and bacterial symbionts (Russell et al. 2003).

This study determined that two species of symbiotic bacteria are present in soybean aphids: *Buchnera* and *Arsenophonus.* Evidence presented in this study strongly suggests that the partial 16S rDNA sequences obtained from soybean aphid total genomic DNA are from symbiotic bacteria and not from bacterial contamination. The three soybean aphid populations tested were reared in isolated growth chambers over many generations, and the two bacteria species were found to be consistently present in the aphid clones throughout the study. Aphid clones did not acquire or lose symbionts, as would be expected if the bacteria species reported were contaminants. In addition, diagnostic PCR reactions showed very strong bands for both *Buchnera* and *Arsenophonus.* If either of these bacteria was a contaminant instead of a symbiont, it would not likely be present in very large quantities and therefore would not amplify strongly in diagnostic testing. Finally, the reported partial 16S rDNA sequences obtained from the soybean aphid clones tested were similar (>95%) to other known symbionts. The 16S rDNA sequences of bacteria are very highly conserved, and it is therefore unlikely that the reported sequences do not correspond to *Buchnera* or *Arsenophonus.*

The Hemipteran subfamily Aphidinae, which includes aphids, is composed of multiple tribes. The soybean aphid is a member of the Aphidini tribe, which contains a number of important agricultural pests. The partial *Buchnera* 16S rDNA sequences obtained from soybean aphids in this study were closest to the *Buchnera* 16S rDNA sequences in *A. fabae fabae, R. maidis,* and *S. graminum,* all of which belong to the Aphidini tribe. Soybean aphid 16S rDNA *Buchnera* sequences were less consistent with the *Buchnera* species present in the largest aphid tribe, Macrosiphini. This tribe includes *A. pisum* and *Uroleucon* species, which has been the subject of most of the work on aphid symbionts. *Buchnera* are known to have an ancient relationship with aphids (Moran et al. 1993), during which they have been transmitted vertically through their aphid hosts. It therefore seems logical that the *Buchnera* in soybean aphids would be most closely related to *Buchnera* in other members of the Aphidini tribe.

The diagnostic primers used in this study to detect *H. defensa* (Russell et al. 2003) yielded a PCR product that, when sequenced, corresponded to *Arsenophonus* instead of *H. defensa. Arsenophonus* is a γ-proteobacterium known to establish secondary symbiotic relationships with whiteflies, psyllids, and aphids in the tribe Aphidini (Thao and Baumann 2004, Dale et al. 2006). Blood-feeding Diptera from Streblidae (bat flies) and Hippoboscidae (louse flies), which rely on symbiotic bacteria to supplement an amino acid–deficient diet, also harbor *Arsenophonus* (Trowbridge et al. 2006). Unlike the primary symbionts of aphids, *Arsenophonus* has been cultured in insect cell lines (Dale et al. 2006).

The16S rDNA and 23S rDNA genes of *Arsenophonus* and *H. defensa* are separated by an intergenic spacer. Primers used to detect *H. defensa* in this study included a universal reverse primer, which binds to a region of the 23S rDNA, and a diagnostic forward primer, which binds to a region in the 16S rDNA. The resulting PCR fragment, spanning the intergenic spacer, yields a product of ≈900 bp. This PCR product length is considered to be diagnostic for *H. defensa,* and previous aphid symbiont studies have discarded PCR products that were not of the correct length (Sandstrom et al. 2001, Russell et al. 2003). When soybean aphid populations were tested for the presence of *H. defensa,* the resulting PCR product was ≈1,000 bp long, indicating that the bacterial 16S rDNA being amplified was not that of *H. defensa,* because the 16S rDNA and intergenic spacer of this species are highly conserved. Sequencing of the PCR product instead indicated the presence of a different insect symbiont: *Arsenophonus.*

Aphid symbiosis with bacteria from the genus *Arsenophonus* has not been widely reported in the past. Tsuchida et al. (2002) tested 858 aphids from 43 Japanese locations and did not find *Arsenophonus. Aphis spiraecola, Wahlgreniella nervata,* and *Myzocallis* spp. are known to contain *Arsenophonus* (Russell et al. 2003, Dale et al. 2006). Previous restriction analysis

studies have detected the presence of aphid secondary symbionts of unknown identification, and it is possible that the unknown bacteria detected in these studies could be *Arsenophonus* (Sandstrom et al. 2001, Russell et al. 2003). In these cases, diagnostic PCR fragments that yielded incorrect product lengths were discarded without sequencing the product. Because of the genetic similarity between *Arsenophonus* and *H. defensa*, it is possible that a diagnostic PCR reaction meant to detect *H. defensa* would also amplify the 16S rDNA gene of *Arsenophonus.* The intergenic spacer of these two species is different in length, so the aphid would be determined to be negative for the presence *H. defensa*, and the PCR product would not be sequenced. This study suggests that *Arsenophonus* may be present in more aphids than previously thought and that current diagnostic PCR screening methods for aphid symbionts may be discarding essential data.

The soybean aphid is an economically important pest of soybeans, and the discovery of two types of symbiotic bacteria, *Buchnera* and *Arsenophonus*, in this aphid species could aid in the development of future control strategies. Because this is the first report of symbionts in the soybean aphid, little is known about their potential role in the insect's biology. *Arsenophonus* has been cultured outside of its host in insect cells lines, allowing recombinant DNA techniques to manipulate its genome (Dale et al. 2006). Secondary symbionts are known to carry genes that protect their aphid host from heat stress or parasitism, so the potential ability to manipulate the *Arsenophonus* symbiont of the soybean aphid in the laboratory could lead to innovative future control strategies. In addition, recently discovered soybean aphid resistance genes to the soybean aphid (Hill et al. 2006) and aphid biotypes that overcome this resistance (Kim et al. 2008) may be caused by some interaction with aphid symbionts. The role of symbionts in the soybean aphid has not been determined, but it has been shown that enteric bacteria are required for *Bacillus thuringeinsis* insecticidal activity (Broderick et al. 2006). More research on the soybean aphid–symbiont interactions may show more about how symbionts influence aphid behavior.

Acknowledgments

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