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Identification And Location Of Symbionts Associated With Potato Psyllid (*Bactericera cockerelli*) Lifestages

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ABSTRACT The potato psyllid (*Bactericera cockerelli*, Sulc) is an invasive pest of solenaceous plants including potatoes (*Solanum tuberosum* L.)and tomatoes (*Solanum lycopersicum* L.). The insect transmits the phytopathogen *Candidatus* Liberibacter solanacearum, which has been identified as the causal agent of Zebra Chip in potatoes. The microbiome of the potato psyllid provides knowledge of the insect's bacterial makeup which enables researchers to develop targeted biological control strategies. In this study, the microbes associated with four *B. cockerelli* life stages were evaluated by 16S bTEFAP pyrosequencing. The sequences were compared with a 16S-rDNA database derived from NCBI's GenBank. Some bacteria identified are initial discoveries. Species of *Wolbachia*, *Rhizobium*, *Gordonia*, *Mycobacterium*, *Xanthomonas* and others were also detected and an assessment of the microbiome associated with *B. cockerelli* was established.

KEY WORDS pyrosequencing, psyllids, microbiome, symbionts, massively parallel bacterial tag-encoded FLX-Titanium amplicon pyrosequencing

Psyllids are jumping plant lice (Order: Hemiptera) and approximately forty species (19 genera) are known to be of economic importance. These insects are pests of a wide array of plants including pistachio (*Agonoscena targionii*, Lichtenstein); avocado (*Trioza aguacate*, Hollis & Martin); pear (*Cacopsylla pyricola*, Förster), berry (*Trioza tripunctata*, Fitch); and many ornamentals (Percy 2000). Potato psyllids (*Bactericera cockerelli*) are phloem feeding pests of solanaceous plants, such as potatoes, tomatoes, peppers, and have become a major limiting factor in the production of potatoes in both Central and North America (CNAS 2006). Historically, potato psyllid infestations were transient and sporadic (Eyer and Crawford 1933) but in recent years have become much more persistent with year-long and multiyear infestations becoming the norm.

Zebra Chip, an emerging disease of the potato (*Solanum tuberosum* L.) (Abad et al. 2009), originally was identified in 1994 in fields around Saltillo, Mexico where the disease was called "papa manchada" or stained potato. Above ground symptoms include aerial tubers and yellowing and curling of leaves. Alternating light and dark bands can be seen in freshly cut tubers and result from starches being converted to soluble sugars in the medullary rays (Gao et al. 2009). Frying caramelizes the bands and gives the chips a characteristic unpalatable taste. These symptoms reduce the value of potato crops and result in losses in the millions of dollars (Secor and Rivera-Varas 2004, CNAS 2006, Hernandez-Garcia et al. 2006, Salas-Marina et al. 2006); in some cases entire fields have been abandoned (Flores et al. 2004).

The U.S. potato industry has suffered from a severe loss in plant longevity and crop quality because of Zebra Chip. *B. cockerelli* have long been associated with the disease (Goolsby et al. 2007, Munyaneza et al. 2007), which was thought to have been caused by a bacteria, virus, or toxigenic effects of the insect's saliva. A recent transmission electron microscopy and 16S rDNA sequencing study by Liefting et al. (2009) has now implicated *Candidatus* Liberibacter solanacearum as the putative cause of Zebra Chip. As the potato psyllid has spread throughout Mexico, the southwestern United States, and northward, insect related crop losses have followed and are likely to continue.

To counter a diet largely deficient in amino acids (Sandström and Moran 1999), phloem-feeding insects have adapted by the incorporation of symbiotic bacteria. In exchange for permanent residence in specialized bundles of cells called bacteriomes (Baumann et al. 1995) bacteria like *Candidatus* Carsonella ruddii (Thao et al. 2001) supplement missing nutrients. Sequencing analysis of *Candidatus* Carsonella ruddii conducted by Nakabachi et al. (2006) identified genes related to amino acid biosynthesis, whereas other genes considered essential for life were missing. *Wolbachia*, a well known insect endosymbiont and potent influencer of sex ratios, has been used as a genetic marker to differentiate between potato psyllids from different locations (Liu et al. 2006). It is probable that more symbionts are exploited by the

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potato psyllid to perform various metabolic activities and an in depth knowledge of these interactions could expand current strategies and methods of psyllid pest management.

The identification of large varieties of bacterial symbionts is impractical when using culture- or biochemical reaction-based methods because many types of reagents are required for characterization. Cultures are rarely able to accurately represent the true richness in environmental samples (Rondon et al. 2000) because the growth of some organisms can limit others rendering them undetectable. Biochemical classification methods are also less reliable than polymerase chain reaction (PCR) or sequencing because their results are sometimes variable and preconceived notions defining a taxon can be misleading (Francino et al. 2006), especially for microbes that are not well characterized.

In this study, bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) (Dowd et al. 2008a,b,c; Wolcott et al. 2009a,b; Bailey et al. 2010) was used to survey the bacterial constituents of four lifestages of *B. cockerelli.* Pyrosequencing using the bTEFAP methodology is a more representative, faster, and more cost-effective way to survey microbiomes.

Materials and Methods

Insect Colonies. Potato psyllids initially were provided by Drs. Tong-Xian Liu and Xiangbing Yang (Texas AgriLife Research) and were used to set up a colony maintained on potato plants (*Solanum tuberosum* L.) of various ages grown from tubers at 25°C, 40% humidity and a photoperiod of 12:12 (L:D) h. Adult, fifth-instar, and early-instar (first-fourth) psyllids were collected from the leaves by attaching a 1,000-μl filter pipette tip to the end of a 25-ml serological pipette connected to a vacuum line. These were placed in 1.5-ml microcentrifuge tubes and stored at −80°C. Potato psyllid eggs were removed by hand using fine point tweezers under a dissecting scope.

Sample Preparation. All psyllid lifestages were collected and the psyllids that were surface sterilized (Table 1) were prepared by three washes with 70% ethanol, one wash with 10% bleach, and then three washes with Nanopure water. *B. cockerelli* then were resuspended in sterile 1X phosphate buffered saline. Psyllid sugars, bead-like waxy excrement, were collected from the adaxial surface of the leaf and also suspended in 1X PBS buffer. A sample also was taken from the faucet used to water the plants to check for water borne contaminants and all the samples were shipped to the Research and Testing Lab in Lubbock, Texas for pyrosequencing.

Although sequence analysis, specifically Sanger sequencing, is more reliable and faster, its limitation comes from its inability to produce de novo sequence information from complex mixtures of DNA. Newer generations DNA sequencing technology continually are increasing in capability while cost continues to drop. Massively parallel pyrosequencing is based on sequencing by synthesis and relies on the detection of the pyrophosphate ion (PPi; $P_2O_7^{4-}$), which is released as DNA polymerase incorporates the nucleotides present into the growing chain. Enzymatic processes convert $P_2O_7^{4-}$ into ATP and then to a light signal—the strength of which is proportional to the number of nucleotides consecutively incorporated (Ronaghi 2001).

Table 1. Samples Collected for the 16S Microbiome Study

Potato psyllid samples		
Sample	Life stage	Note
1	Adult	Not surface sterilized
2	Adult	Surface sterilized
3	Fifth instar	Not surface sterilized
4	Fifth instar	Surface sterilized
5	Early instars[a]	Not surface sterilized
6	Early instars	Surface sterilized
7	Eggs	Not surface sterilized
8	Eggs	Surface sterilized
9	Psyllid sugars	Excrement from leaf surface
10	Water	Used to water the plants

The surface sterilized samples were prepared with 3 washes in 70% EtOH, 1 wash in 10% bleach, then 3 washes in nanopure water. Unsterilized samples were immediately submerged in sterile PBS buffer.

[a] Here, "early instars" means all instars except the fifth.

454 Pyrosequencing. Massively Parallel bacterial tag-encoded FLX-Titanium amplicon pyrosequencing (bTEFAP) Titanium was performed as described previously (Dowd et al. 2008a,b,c; Wolcott et al. 2009a,b; Bailey et al. 2010). Briefly, the samples were lysed in RLT/β-mercaptoethanol buffer, a 5-mm steel bead (Qiagen, Valencia, CA) and 500 μl of sterile 0.1-mm glass beads (Scientific Industries, Inc., Bohemia, NY). DNA was recovered from a DNA spin column by following the QIAamp DNA Mini Kit - Tissue Protocol (Qiagen, Valencia, CA) beginning with step 5. Samples were quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France) then diluted to 100 ng/μl with sterile nuclease free water.

One hundred ng (1 μl) of each DNA sample was used in a 50-μl PCR reaction by using 16S universal Eubacterial primers 530 F (5′-GTG CCA GCM GCN GCG G) and 1100R (5′-GGG TTN CGN TCG TTG) for amplification of a 600-bp region of the 16S gene. HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was used under these conditions: 94°C for 3 min; 32 cycles of 94°C for 30 s, 60°C for 40 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. Another PCR was performed for FLX (Roche, Nutley, NJ) amplicon sequencing under the same conditions but with special fusion primers (LinkerA-Tags-530 F and LinkerB-1100R), which prevent amplification of any bias potentially caused by tag and linker inclusions during initial amplification. All secondary PCR amplicons were mixed in equal concentrations and purified with Agencourt Ampure beads (Agencourt Bioscience Corporation, MA).

DNA size (bp) and concentration were measured by using DNA chips under a Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, CA) and a TBS-380 Fluorometer (Turner Biosystems,

CA) in preparation for FLX sequencing (Roche, Nutley, NJ). DNA with a concentration of 9.6 by 10^6 molecules/μl and an average size of 625 bp was combined with 9.6 million DNA capture beads and amplified by emulsion PCR. After recovery and enrichment, the bead-attached DNAs were denatured with NaOH and primers for sequencing were annealed. A two-region 454 sequencing run was performed on a 70 by 75 GS PicoTiterPlate (PTP) using a Genome Sequencer FLX System according to the manufacturers protocol (Roche, Nutley, NJ).

Data Analysis. After sequencing, failed reads, low quality ends and tags, and nonbacterial ribosome sequences were removed (Dowd et al. 2008a). The remaining sequences were queried against 16S rDNA bacterial sequences derived from NCBI (Cole et al. 2009) by using a distributed BLASTn.NET algorithm (Dowd et al. 2005). BLASTn results were compiled and validated as described previously (Dowd et al. 2008a,b; Wolcott et al. 2009) and rarefaction estimates of maximum diversity were performed according to Acosta-Martinez et al. 2008. BLASTn results were assigned to the appropriate taxonomic classification based on their identity scores to known 16S sequences. Those with <3% divergence were resolved at the species level, those divergent between 3 and 5% to the genus level, between 5 and 10% to the family level, and those divergent between 10 and 20% to the order level.

A principal component analysis, PCA, was performed using PC-ORD 5.0 software (http://home.centurytel.net/≈mjm/) to compare the bacterial genera present in the surface sterilized and nonsterile lifestage insect samples. Three separate PCA tests were performed—the Correlation Cross-Product Matrix, the Non-Centered Cross-Product Matrix, and the Variance-Covariance Cross-Product Matrix. All distance-based biplots included a randomization test for resampling.

Genera Overlap. When reviewing the bTEFAP sequence data, concern was raised that some of the bacteria identified could have been artifacts of artificial rearing or contaminants from the lab acquired by the insects. Attention was focused on overlaps in the sequence data (genera) between the surface sterilized and nonsurface sterilized samples. Eight such genera (*Achromobacter*, *Bacteroides*, *Methylobacterium*, *Microbacterium*, *Niastella*, *Nocardioides*, *Rhodoferax*, *and Pseudomonas*) were selected. Sequences from both sets of samples were aligned using 10 iterations of the MUSCLE alignment tool in Geneious (Drummond et al. 2010, v5.1.6). From these consensus sequences, primers (Table 2) were designed (Geneious, Primer3) to investigate the presence of these genera in previously extracted potato psyllid DNA from wild populations in Texas and Washington State, with the assumption that a positive identification in both locations implied a positive identification in locations in between. From each location, DNA was extracted from individual field trapped potato psyllids by using the DNeasy Kit (Qiagen) according the manufacturer's protocol; five samples from each location were combined. Amplification was performed, according to the manufacturer's protocol, using the Amplitaq Gold 360 PCR kit and genera presence per location was noted.

Table 2. Primers Designed for Genera Overlap Analysis

Primers
Achromobacter F1-TAGCGGCGGACGGGTGAGT
Achromobacter R1-TTCCCCACTGCTGCCTCCCG
Product size: 265
Achromobacter F2-GCGGCGGACGGGTGAGTAAT
Achromobacter R2-TTCCCCACTGCTGCCTCCCG
Product size: 263
Bacteroides F1-GGTCCAAACTCCTACGGGAGGC
Bacteroides R1-ACGACCCATAGAGCCTTCATCCTTCA
Product size: 100
Methylobacterium F1-TGGCTCAGAGCGAACGCTGG
Methylobacterium R1-ATCAGACGCGGGCCGATCCT
Product size: 197
Methylobacterium F2-ACGCTGGCGGCAGGCTTAAC
Methylobacterium R2-CCCGTAGGAGTCTGGGCCGT
Product size: 288
Microbacterium F1-AACGCTGGCGGCGTGCTTA
Microbacterium R1-GTCGACGCCTTGGTGAGCCA
Product size: 248
Microbacterium F2-GCTCGCGGCCTATCAGCTTGT
Microbacterium R2-CCCCACTGCTGCCTCCCGTA
Product size: 131
Niastella F1-TCAGCCACACGGGCACTGAG
Niastella R1-TCCTTCACGCGGCATGGCTG
Product size: 103
Niastella F2-ACACGGGCACTGAGACACGG
Niastella R2-TCCTTCACGCGGCATGGCTG
Product size: 97
Nocardioides F1-CTCAGGACGAACGCTGGCGG
Nocardioides R1-TCAGTCCCAGTGTGGCCGGT
Product size: 298
Nocardioides F2-GGTACTCGAGCGGCGAACGG
Nocardioides R2-CCCCACTGCTGCCTCCCGTA
Product size: 273
Rhodoferax F1-AGACCTCGCGCGATTGGAGC
Rhodoferax R1-ATCCTGCACGCGGCATTGCT
Product size: 200
Rhodoferax F2-CGGAACGTGCCCAGTCGTGG
Rhodoferax R2-ATCCTGCACGCGGCATTGCT
Product size: 285
Pseudomonas F1-AAGCGCGCGTAGGTGGTTCG
Pseudomonas R1-GTCCAGGTGGTCGCCTTCGC
Product size: 172

Sequences recovered from sterile and non-sterile sample sets were aligned using 10 iterations of the MUSCLE alignment tool in Geneious. From these consensus sequences, primers were designed to determine the presence of the genera in wild populations from Texas and Washington State.

Results

In total, just over 20,000 sequences were recovered from the various potato psyllid lifestage samples—13,046 from the nonsterile group and 7,081 from the sterilized group. Potato psyllid excrement yielded 203 sequences and a water sample, taken from the source used for the plants, resulted in 108 sequences. Across all the samples, the average number of sequences was 2044 (min – 98, max – 4,155). In the nonsterile sample, *Wolbachia* and *Staphylococcus* were recovered from all lifestages. In the sterilized samples, *Ralstonia* and *Staphylococcus* were recovered from all lifestages.

The eggs had the highest richness of bacteria. Several prominent genera of bacteria including *Mycobac-*

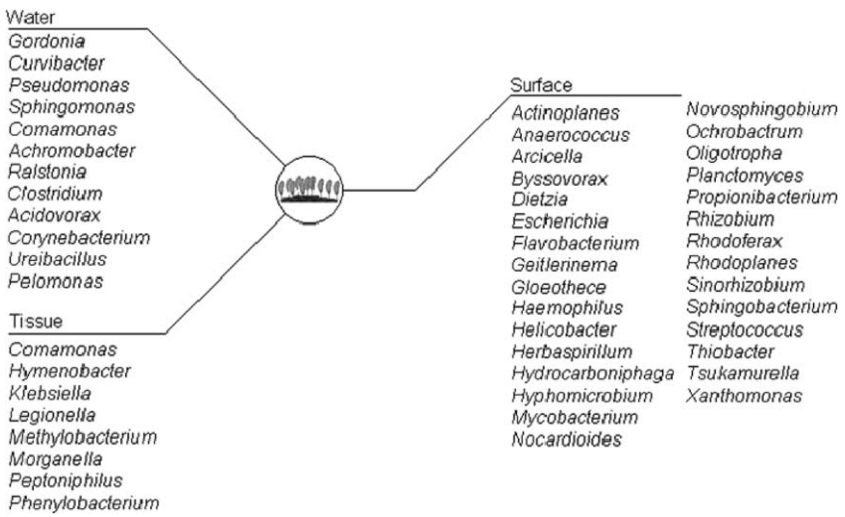

Fig. 1. Probable Location of 16S rDNA Sequences Recovered from Potato Psyllid Eggs. "Tissue" is determined by removing genera which were identified in a) "Water" and b) "Psyllid Sugars". "Surface" is determined by removing a) "Water" b) "Psyllid Sugars" and c) "Tissue" from NSS.

terium (0.67% in "nonsterilized" and 0% in "sterilized"), *Wolbachia* (41.65% in "nonsterilized" and 12.76% in "sterilized"), and *Microbacterium* (2.65% in "nonsterilized" and 0.82% in "sterilized") showed a marked reduction. Sequences aligning to *Rhizobium* (9.10%), *Propionibacterium* (18.91%), *Gordonia* (10.13%), and *Xanthomonas* (0.99%) were only recovered in "nonsterilized" but *Ralstonia* (19.75%) and *Legionella* (7.82%) were only found in the sterilized sample. *Bradyrhizobium* had a modest increase from 1.19% in "nonsterilized" to 4.53% in "sterilized" and *Staphylococcus* sequences (5.46% in "nonsterilized" and 30.86% in "sterilized") increased with sterilization. The survey of bacteria found in the eggs also indicated an overlap of genera between the surface sterilized and nonsterilized samples: *Achromobacter*, *Bradyrhizobium*, *Microbacter*, *Niastella*, *Staphylococcus*, and *Wolbachia.*

In the early-instar nymphs, sequences aligning to *Wolbachia* only were recovered in "nonsterilized" (95.62% and 0% in "sterilized"). *Geitlerinema* and *Gloeothece* were found in fifth instars, early instars, and eggs (not sterile) and sequences of *Staphylococcus* increased (1.59% in "nonsterilized" and 14.29% in "sterilized"). *Ralstonia* (30.61%) and *Delftia* (7.14%) only were found in "sterile" and *Mycobacterium* (0.06%) was only found in "not sterile." Five genera were common between surface sterilized and nonsterile early instars: *Bacteroides*, *Methylobacterium*, *Pseudomonas*, *Sinorhizobium*, and *Staphylococcus.*

In the fifth-instar nymphs *Wolbachia* accounted for >95% of the sequences recovered without or with surface sterilization (3,155/3,164 in "nonsterilized" and 3,091/3,137 in "sterilized"). *Staphylococcus* (0.09% in "nonsterilized" and 0.61% in "sterilized") and *Bradyrhizobium* (0.03% in "nonsterilized" and 0.29% in "sterilized") increased slightly. *Ralstonia* (0.13%) and *Legionella* (0.03%) only were found in "sterile." Three genera were common between the sterile and nonsterile fifth instars: *Bradyrhizobium*, *Staphylococcus*, and *Wolbachia.*

Many of the sequences recovered from the adult potato psyllids were identified as a variety of *Rhizobium*-related genera. The sequences aligned to *Rhizobium* (80.60% in "nonsterilized" and 0% in "sterilized") and *Mesorhizobium* (0.12% in "nonsterilized" and 0% in "sterilized"); *Bradyrhizobium* (0.11%) and *Sinorhizobium* (0.08%) only were recovered from "sterile." Sequences aligning to *Ralstonia* (0.06%) and *Gordonia* (0.03%) were recovered from "sterile" and *Devosia* (0.39%) was only found in "not sterile". Two noteworthy genera *Wolbachia* (17.11% in "nonsterilized" and 84.18% in "sterilized") and *Staphylococcus* (1.49% in "nonsterilized" and 10.69% in "sterilized") increased in response to surface sterilization. Two genera were common between the sterile and nonsterile adults: *Staphylococcus* and *Wolbachia.*

Controls and Comparisons. Using a method of subtractive comparisons of the various samples, the putative locations of the bacteria were identified in this study. To identify surface bacteria on a particular lifestage, the genera identified in the psyllid sugars and the water were removed from those found in the nonsterilized insects (Figs. 1 and 2, 3 and 4). Comparisons of the surface bacteria of the various lifestages indicated that some genera were present in two (*Bradyrhizobium*, *Corynebacterium*); three (*Geitlerinema*, *Rhizobium*); or all four (*Wolbachia*, *Staphylococcus*) stages. To isolate the bacteria located in the tissues but not in the gut, the genera from the excrement and the water were removed from those found in the surface sterilized insects (Figs. 1 and 2, 3 and 4). Comparison of these genera showed *Tepidimicrobium*, *Methylobacterium*, *Sporocytophaga*, *Niastella*, and *Legionella* were present in at least two lifestages.

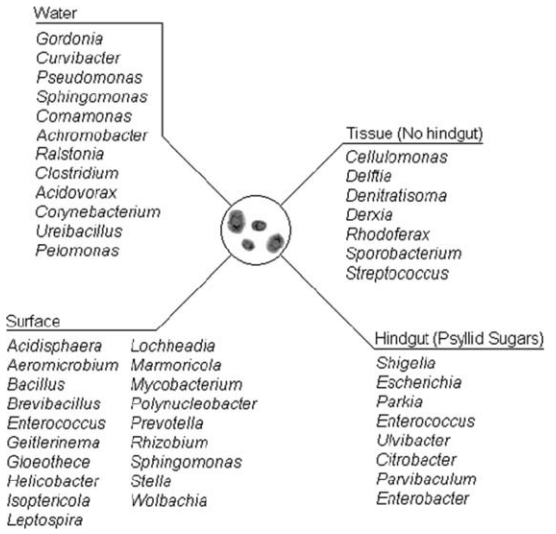

Fig. 2. Probable Location of 16S rDNA Sequences Recovered from Early Instar Potato Psyllids. "Tissue" is determined by removing genera which were identified in a) "Water" and b) "Psyllid Sugars". "Surface" is determined by removing a) "Water" b) "Psyllid Sugars" and c) "Tissue" from NSS. "Hindgut" was determined by removing genera identified in "Water". "Hindgut" here is less certain; most of the psyllid sugars collected were likely from fifth instars and/or adults so the early instar flora could be different.

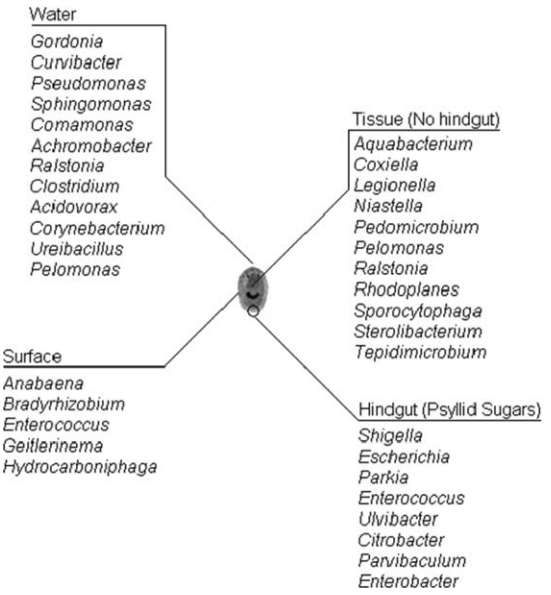

Fig. 3. Probable Location of 16S rDNA Sequences Recovered from fifth Instar Potato Psyllids. "Tissue" is determined by removing genera which were identified in a) "Water" and b) "Psyllid Sugars". "Surface" is determined by removing a) "Water" b) "Psyllid Sugars" and c) "Tissue" from NSS. "Hindgut" was determined by removing genera identified in "Water".

A sample was taken from the water source used for the plants to serve as a type of control with the assumption that the water may harbor bacteria transmissible to insects. There were 12 genera identified; *Ralstonia* (32%), a common phytopathogen, was identified in all life stages. In surface sterilized eggs, sequences homologous to *Ralstonia* were a significant fraction (53%) of those recovered but tapered off in adults (2%). *Corynebacterium* (19.4%), a widely common bacterium was found only in the fifth instars and *Pelomonas* (23.15%), was recovered from only nonsterilized samples. Nine of the genera were recovered in single digits but were not notably represented in any of the insect samples. A single *Ureibacillus* sequence was recovered from the water but not from the others and *Gordonia*, recovered six times from the water, was only found a single time in the adult surface sterilized samples. In total, eight genera present in the water also were present in various lifestages of surface sterilized insects leaving eggs with 12 possibly unique genera, early instars with 11, fifth instars with 12, and adults with 22 (Fig. 5).

In an attempt to survey the microbial contents of the potato psyllid hindgut, a sample of psyllid sugars was taken from the top surface of the leaves. Several genera recovered from the excrement were found in the other nonsterile samples. *Clostridium*, possibly from the water, was identified in the nonsterilized adult insects, and *Escherichia* was found in the adults and fifth-instar nymphs. *Enterococcus* (72.91%) also was found in the adults and fifth instars. *Citrobacter* (12.81%) only was found in adults. Comparing the genera data from the water and the psyllid sugars indicated (Fig. 6) an overlap of two, *Clostridium* and *Pelonomas*, leaving sequences of seven unique genera from the excrement, many of which are commonly isolated from animal guts. Sequences homologous to *Pelomonas* and *Enterobacter* were recovered from the surface sterilized fifth instars and adults. *Pelomonas* was present in the water (23%), which could have carried through to contaminate the likeliest cause, residual excrement in the hindgut. Because *Enterobacter* was not present in the water that brings the total to eight (*Shigella, Escherichia, Parkia, Enterococcus, Ulvibacter, Citrobacter, Enterobacter,* and *Parvibaculum*).

The hindgut contents also were compared with the sterilized insects (Fig. 7) to check the surface sterilization procedure and nonsterilized lifestages (Fig. 8) to determine if the psyllids are surface contaminated with fecal matter. Two genera from the excrement were identified in the surface sterilized lifestages, *Enterobacter* (0.44%) from the adults and a single sequence of *Pelomonas* (3.19×10^{-4} %) from the fifth instars. Both of these genera were present in the water and so cannot be discounted as contaminants. A single sequence of *Escherichia* and another single sequence of *Enterococcus*, both recovered exclusively from the feces, were found in the eggs and fifth instars, respectively.

Genera Overlap. All of the bacterial genera tested (*Achromobacter, Bacteroides, Methylobacterium, Microbacterium, Niastella, Nocardioides, Rhodoferax, and Pseudomonas*) were present in DNA previously ex-

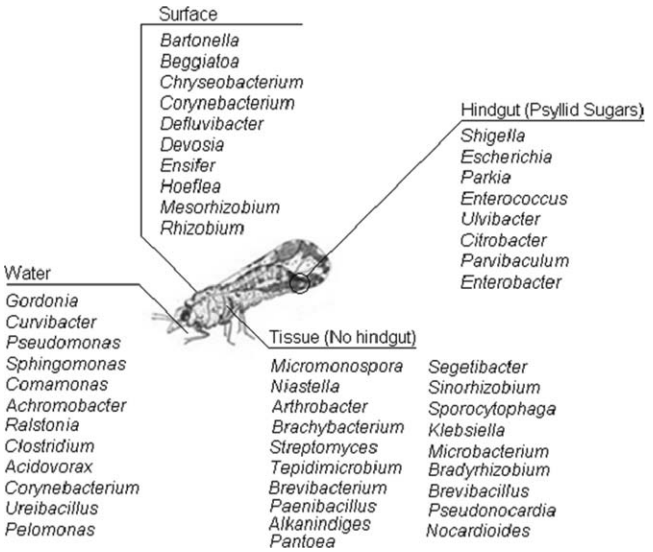

Fig. 4. Probable Location of 16S rDNA Sequences Recovered from Adult Potato Psyllids. "Tissue" is determined by removing genera which were identified in a) "Water" and b) "Psyllid Sugars". "Surface" is determined by removing a) "Water" b) "Psyllid Sugars" and c) "Tissue" from NSS. "Hindgut" was determined by removing genera identified in "Water".

tracted from potato psyllids in both Texas and Washington State potato fields. Assuming that psyllids at all points in between also would have tested positive for the genera in question, it is unlikely that this suite of bacteria was introduced during colony rearing in the lab.

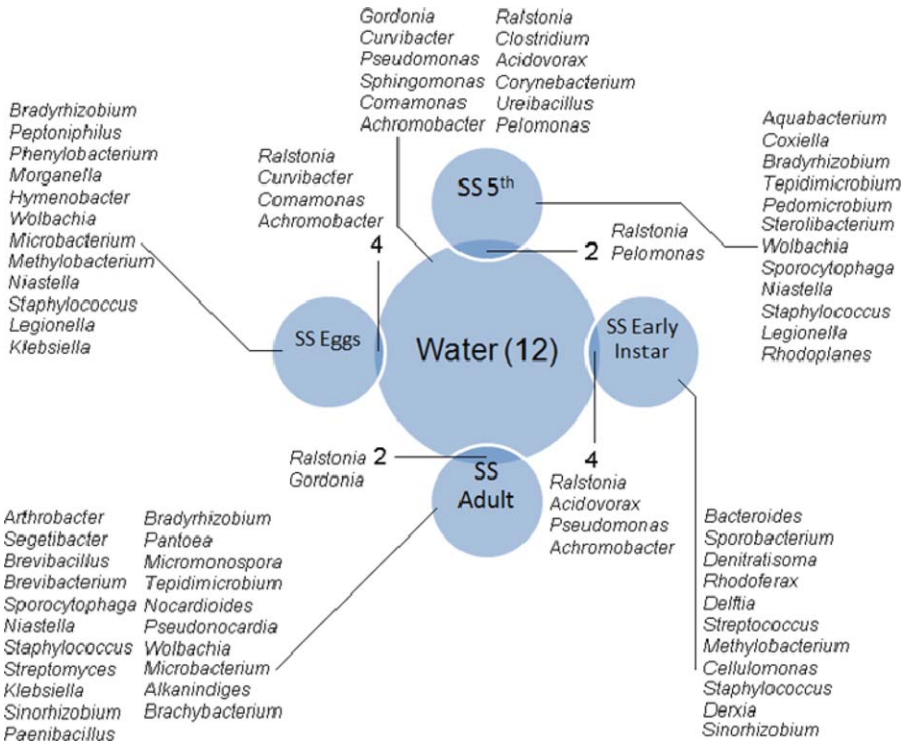

Fig. 5. Comparison of Genera Identified in Water Control as Compared with Those in Surface Sterilized Lifestages. Comparison showed eight genera in the surface sterilized lifestages that were also in the water. Because of this, *Ralstonia*, *Curvibacter*, *Comamonas*, *Achromobacter*, *Acidovorax*, *Pseudomonas*, and *Gordonia* are possible carry-over contaminants. This leaves eggs with 12, early instars with 11, fifth instars with 12, and adults with 22 possibly unique genera. (Online figure in color.)

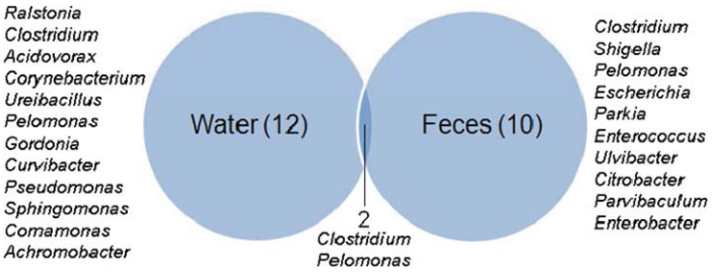

Fig. 6. Comparison of Genera Identified in Water Control as Compared with Those in Psyllid Sugars. Comparison showed two genera in the excrement that were also in the water. Because of this, *Clostridium* and *Pelomonas* are possible carry-over contaminants. Several genera identified in the excrement (*Escherichia*, *Enterobacter*, *Citrobacter*, *Enterococcus*, *Shigella*, etc.) are common flora in animal guts. (Online figure in color.)

Discussion

In a principal component analysis performed on the surface sterilized and nonsterile insects, there was unexpected grouping of samples with respect to sterilization and lifestage when using Correlation Cross-Product Matrix- , Non-Centered Cross-Product Matrix- or Variance-Covariance Cross-Product Matrix- based biplots. In the Correlation Cross-Product Matrix, the sample grouping indicates 1) surface sterilized insects are similar to nonsterilized insects but are more similar to one another, 2) among the surface sterilized insects, the early and fifth instar lifestages are more similar to each other than to the adult and egg pair, and 3) the nonsterilized insects were very dissimilar on the whole but adults and fifth instars were the most alike. In the Non-Centered Cross-Product Matrix, the sterilized adults and fifth instars (sterilized and nonsterile) clustered the closest and the sterilized eggs and early instars grouped together but away from the others. In the Variance-Covariance Cross-Product Matrix, sterilized adults and fifth instars grouped with nonsterilized fifth instars and early instars and, as the Non-Centered Cross-Product Matrix, the sterilized eggs and early instars grouped together but away from the others.

In the Non-Centered and Variance-Covariance analyses, there was a consistent grouping of the fifth-instar nymphs, the nonsterile early instars, and the

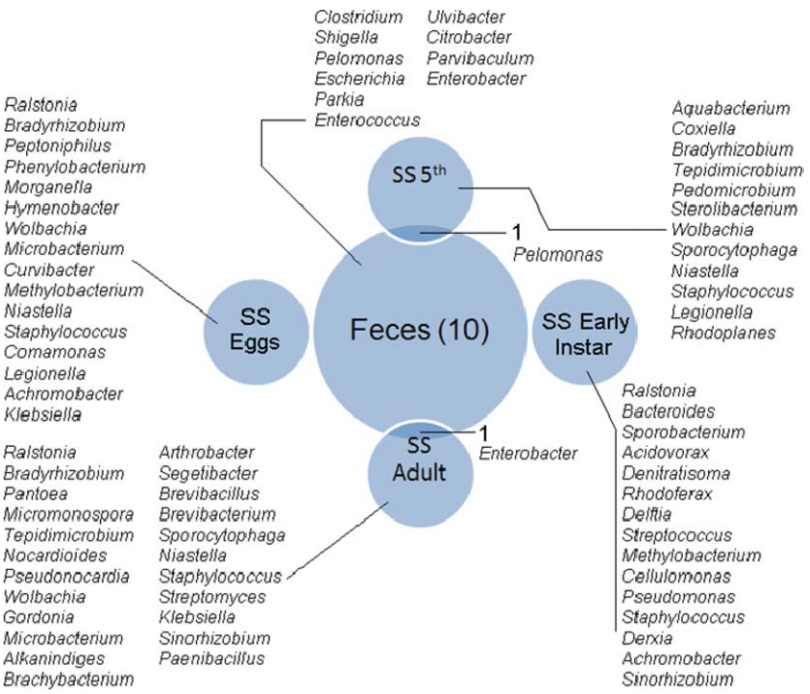

Fig. 7. Comparison of Genera Identified in the Surface Sterilized Lifestages as Compared with Those in Psyllid Sugars. Comparison showed two genera in the excrement that were also in the surface sterilized lifestages. Because of this, *Enterobacter* and *Pelomonas* are possible carry-over contaminants; the points of overlap in the fifth instar and the adult were (0.44%) and (3.19×10^{-4} %) respectively, also testing the sterilization protocol. (Online figure in color.)

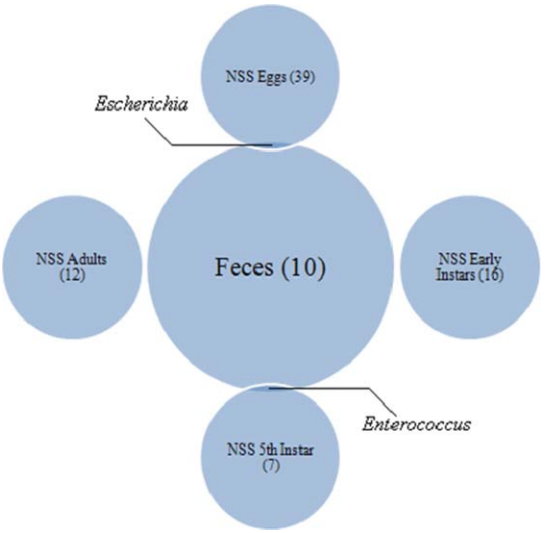

Fig. 8. Comparison of Genera Identified in the Non-Sterilized (NSS) Lifestages as Compared with Those in Psyllid Sugars. Comparison showed two genera in the excrement that were also in the unsterilized lifestages. Because of this, *Enterococcus* and *Escherichia* are possible carry-over contaminants; the points of overlap in the fifth instar and the egg were from single sequences, and determined if the psyllids were surface contaminated with fecal matter. *Escherichia* and *Enterococcus* were both recovered exclusively from the feces. (Online figure in color.)

sterilized adults. The fifth instars and the nonsterile early instars had high percentages of *Wolbachia* but the sterilized adults did as well. After the total removal of *Rhizobium* (80.60% in "nonsterilized" and 0% in "sterilized"), the percentage of *Wolbachia* increased nearly five-fold (17.11% in "nonsterilized" and 84.18% in "sterilized").

Across all the PCA biplots, the samples that grouped the closest were the fifth-instar nymphs. This result is most likely because of the overwhelming presence (≈98%) of sequences homologous to *Wolbachia.* After sterilization, sequences identified as *Wolbachia* were reduced, i.e., the non-*Wolbachia* sequences increased, by only 1.19%. In early-instar nymphs, *Wolbachia* was removed completely by surface sterilization (95.62% and 0% in "sterilized") and therefore significantly skewed the sample groupings. Surface sterilization removed 23 genera from the eggs and contributed to their failure to group. Although grouped closely in the Correlation Cross-Product Matrix Distance-Based Biplot, the adult samples were widely separated in the other analyses.

As indicated by the PCA data, surface sterilization appears to be necessary for determining the specific locations of bacteria present in or on the various lifestages of the potato psyllid. For example, although the egg material is very soft and moist, it is protected by a more rigid shell. Bacteria growing on the shells were washed away by surface sterilization to reveal fewer total genera.

Three washes in 70% ethanol, one wash in 10% bleach, then three washes in nanopure water were used to surface sterilize the insects in this study. Although some surface contaminants were certainly lysed by the 70% ethanol and washed away by the nanopure water, junctions of body segments, wing, and leg attachments and overlapping segments of the abdomen are possible sites of persistent contamination with either bacteria or DNA. Contamination also could have resulted from exposure of samples to air while transferring solutions between washes, however, Fig. 8 seems to indicate this did not occur.

In a study by Davidson et al. (1994) silverleaf whitefly (*Bemisia argentifolii*) pupae and eggs were surface-sterilized using soapy water, a water rinse, a 70% ethanol dip, and then 2-min agitation in 20% household bleach solution. Although this treatment may be sufficient for soft-bodied life stages like eggs and pupae, there is no uniformly accepted method for surface sterilization of all insect life stages. That said, the two most commonly used methods of surface sterilization are ethanol and bleach (Connell 1981), the latter being used only when development, life stage viability, or both are not a concern (Vail et al. 1968, Leppla et al. 1974).

To acquire as much information as possible as to the constituents of the potato psyllid microbiome, many insect samples were taken. Sampling all lifestages and using overlapping controls takes great advantage of the large data set from even a single sequencing run. Analysis of the outer surface, inner tissues, and gut flora is possible by comparing the bacterial sequences identified in 1) a water sample to those in the feces, 2) the feces to those in the surface sterilized insects, 3) the surface sterilized insect to those in the nonsurface sterilized, and 4) the water to those in the surface sterilized insects. A clear understanding of the bacterial flora of the 1) inside of the insect, excluding the gut; and 2) the gut, excluding other organs, identified insect-specific pathogens, and commensal or mutualistic bacteria, excellent targets for reducing potato psyllid fitness.

The genera chosen for this experiment are known or suspected to associate with insects and are of interest as these organisms could 1) serve as infectious agents, 2) allow infectious agents to flourish in their absence, or 3) serve as candidates for delivering designer molecules.

Although not insects, various species of spiders native to Japan were found to have species of *Nocardioides* in their webs, cuticles, and egg sacs (Iwai 2009). Although there are no other reports of *Rhodoferax ferrireducens* being present in any insect species, the completion of its genome sequence showed a sequence homologous to a portion of the carotenoid biosynthesis pathway in insects. Similarly, *Niastella* has not been reported in insects but was recovered from the lab reared and wild psyllids.

Although associated with the water, *Achromobacter*, known (Poinar 1967) to infect the Greater Wax Moth [*Galleria mellonella* (L.)], was chosen because of its association with the surface sterilized eggs and pre-

vious association with the Asian Citrus Psyllid (*Diaphorina citri*), a sister species of the potato psyllid. Anaerobic species in the genus *Bacteroides* have been identified as constituents of the gut flora (Breznak 1982) of the eastern subterranean termite [*Reticulitermes flavipes* (Kollar)]. If truly anaerobic in the potato psyllid, *Bacteroides* also may be performing a similar function; that is, cross-feeding lactate with the facultatively anaerobic *Lactococcus lactis* (formerly *Streptococcus lactis;* Schultz and Breznak 1979), also identified in the adult lifestage.

The citrus endophyte *Methylobacterium mesophilicum* has been shown to be transmitted by the xylem feeding leafhopper *Bucephalogonia xanthophis* (Gao 2009). Potato psyllids are phloem feeders, and if *Methylobacterium* can live in the phloem, they may be able to function as transports for molecules designed against either the potato psyllid or the root cause of Zebra Chip, *Candidatus* Liberibacter solanacearum. *Pseudomonas* is a ubiquitous genus but one species in particular, *P. entomophila*, is a known *Drosophila* pathogen (Vodovar 2006) and routinely kills insects from other orders as well. A genome sequence of this entomopathogenic bacterium revealed that it has catabolic gene homologies with *P. putida*, a common soil microbe. Genes for the type III secretion system and its associated toxins were not identified in *P. entomophila* but the bacterium has many proposed virulence factors (insecticidal toxins and proteases) and secondary metabolites like hydrogen cyanide to kill insects (Vodovar et al. 2006).

This study was a first attempt to investigate the bacteria associated with the potato psyllid. 16S microbiome sequencing provided thousands of sequences and gave an estimate of the bacterial constituents of several parts of the insect. To develop a complete picture, future studies could focus on how various things can potentially change the bacterial flora: infection by pathogens, infection by transmissible phytopathogens, geographic location of wild populations or tomato fed versus potato fed insects. Long-term goals should be to disrupt more and more of an insect's life processes. Anaerobic bacteria in the genus *Bacteroides* have been identified here and in the gut flora of termites. The facultative anaerobe *Lactococcus lactis*, also identified here, is known to interact with *Bacteroides* and is known to have lytic phages. With the manipulation of a phage genome, dsRNA against a gut epithelial target in the insect (Desmosomes, tight or gap junctions, adheren) can be designed in and delivered by infection of the gut flora. As the bacteriophage replicates in the bacteria of the target insect's gut, some will lyse and release fragments of dsRNA. In Hemipterans like the potato psyllid, these molecules are taken up by a receptor-mediated mechanism and result in RNA interference. Simultaneously, the disruption of *Lactococcus lactis* populations and their interactions with other bacteria could lead to secondary infection by insect pathogens. Many other possibilities exist and having a thorough knowledge of the genome, bacteria and viruses in the potato psyllid could provide important insights into designing novel targeted management strategies for the insect.

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