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Metagenome tracking biogeographic agroecology: Phytobiota of tomatoes from Virginia, Maryland, North Carolina and California

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ABSTRACT

Describing baseline microbiota associated with agricultural commodities in the field is an important step towards improving our understanding of a wide range of important objectives from plant pathology and horticultural sustainability, to food safety. Environmental pressures on plants (wind, dust, drought, water, temperature) vary by geography and characterizing the impact of these variable pressures on phyllosphere microbiota will contribute to improved stewardship of fresh produce for both plant and human health. A higher resolution understanding of the incidence of human pathogens on food plants and co-occurring phytobiota using metagenomic approaches (metagenome tracking) may contribute to improved source attribution and risk assessment in cases where human pathogens become introduced to agro-ecologies. Between 1990 and 2007, as many as 1990 culture-confirmed *Salmonella* illnesses were linked to tomatoes from as many as 12 multistate outbreaks (Bell et al., 2012; Bell et al., 2015; Bennett et al., 2014; CDC, 2004; CDC, 2007; Greene et al., 2005a; Gruszynski et al., 2014). When possible, source attribution for these incidents revealed a biogeographic trend, most events were associated with eastern growing regions. To improve our understanding of potential biogeographically linked trends in contamination of tomatoes by *Salmonella*, we profiled microbiota from the surfaces of tomatoes from Virginia, Maryland, North Carolina and California. Bacterial profiles from California tomatoes were completely different than those of Maryland, Virginia and North Carolina (which were highly similar to each other). A statistically significant enrichment of Firmicutes taxa was observed in California phytobiota compared to the three eastern states. Rhizobiaceae, Sphingobacteriaceae and Xanthobacteraceae were the most abundant bacterial families associated with tomatoes grown in eastern states. These baseline metagenomic profiles of phyllosphere microbiota may contribute to improved understanding of how certain ecologies provide supportive resources for human pathogens on plants and how components of certain agro-ecologies may play a role in the introduction of human pathogens to plants.

1. Introduction

From 1990 to 2007, as many as 1990 culture-confirmed *Salmonella* illnesses were linked to tomatoes from as many as 12 multistate outbreaks (Bell et al., 2012; Bell et al., 2015; Bennett et al., 2014; CDC, 2004; CDC, 2007; Greene et al., 2005a; Gruszynski et al., 2014). Because the majority of *Salmonella* infections are not culture-confirmed, the Centers for Disease Control (CDC) estimates that many more illnesses occurred than were actually reported (CDC, 2007). From 2002 to 2012, more than 10 outbreaks, documented in PulseNet, (CDC's molecular subtyping network for bacterial foodborne diseases), involved *Salmonella* Newport, and were linked to the eastern shore of Virginia (Delmarva Peninsula) (Swaminathan et al., 2001). Additionally, a subtype of Newport, known as “Pattern 61” (name derived from its

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PulseNet identifier: Pulsed Field Gel Electrophoresis (PFGE) *XbaI* pattern JJPX01.0061) was reported in 2002, 2005, 2006, and 2010, associated with the Delmarva Penninsula of the eastern shore of Virginia (Bell et al., 2015; Bennett et al., 2014; CDC, 2007). All in all, at least nineteen *Salmonella* – tomato associated outbreaks occurred between the years of 1990 and 2014, causing thousands of illnesses and making clear the need for higher resolution research aimed at understanding environmental parameters that may play a role in how *Salmonella* becomes associated with tomatoes in agro-ecologies (Bell et al., 2012; Bell et al., 2015; Bennett et al., 2014; CDC, 2004; CDC, 2007; Greene et al., 2005a; Ottesen et al., 2013). Survival of enteric bacterial pathogens such as *Salmonella* on food plants is well known to be affected by a wide range of abiotic environmental factors (Brandl, 2006).

California and Florida lead the nation in production of fresh market and processing tomatoes, followed by North Carolina, South Carolina, Ohio, Michigan and Virginia vying for third place. Fresh market production for California in the time frame examined in this study (~2009) was the highest in the country, with approximately 37,000 acres of fresh market tomatoes (and 279,000 acres of processing tomatoes). The majority of these were grown in the Central San Joaquin Valley, with almost 9000 acres in fresh market and more than 110,000 acres in processing tomatoes in Fresno County alone (Fresno Department of Agriculture). Despite this hefty production, California saw only one tomato related *Salmonella* outbreak in a time frame in which 10 were reported in Virginia and at least 7 more were traced to other states along the Southeastern coast (Bell et al., 2012; Bell et al., 2015; Bennett et al., 2014; CDC, 2004; CDC, 2007; Greene et al., 2005a; Gruszynski et al., 2014; Gupta et al., 2007). In 2009, the National Agriculture Statistics Service of the United States Department of Agriculture (USDA) estimated total acreage of fresh market tomatoes in Virginia to be 4800. The majority (80%) of which, were grown on the Delmarva Peninsula of eastern Virginia. Thus, from acreage, 1/7th the size as California tomato acreage, a disproportionate number of outbreaks were reported (Fig. 1). From 2002 to 2014, almost yearly, an incident involving *S.* Newport and tomatoes grown in Virginia was documented in PulseNet (Bell et al., 2015; Swaminathan et al., 2001).

Certain cases of *Salmonella* - tomato contamination are thought to occur in agricultural field settings. Multistate incidents have been traced to specific geographic growing regions suggesting that contamination occurs early in the distribution chain, potentially in the field (Bennett et al., 2014; Greene et al., 2005b). Environmental source tracking in Virginia by state and federal agencies, following contamination events, identified the same PFGE Pattern 61 of *Salmonella* Newport from Virginia irrigation pond water, suggesting that the agroecology was the source of the contamination (Greene et al., 2005b). *Salmonella* is often part of a complex ecology in the gut microbiota of birds, amphibians, mammals, insects, and protozoal species. It has been shown to survive in aquatic sediments, protozoal food vacuoles, insect eggs and soils for extended periods (Brandl and Mandrell, 2002; Creel, 1912). Thus, examination of agricultural microbial ecologies including phyllosphere microbiota, soils, air and water, may provide an epidemiological methodology for improved food safety risk assessment via careful characterization of biogeographical microbiota using metagenome tracking. Programs like Genometrakr, coordinated by the Food and Drug Agency (FDA), National Center for Biotechnology Information (NCBI) and CDC, use whole genome sequencing (wgs) of bacterial pathogens for revolutionary precision in public health and food safety source tracking initiatives (Stevens et al., 2017). The next frontier of public health response to illness outbreaks will involve metagenome tracking approaches like the one presented here. MetagenomeTrakr is currently a fledgling initiative out of the Center for Food Safety and Applied Nutrition (CFSAN), designed to provide more comprehensive ecological fingerprints of food and food ecologies to improve source attribution, risk mitigation and rapid response to foodborne outbreaks using metagenomic DNA.

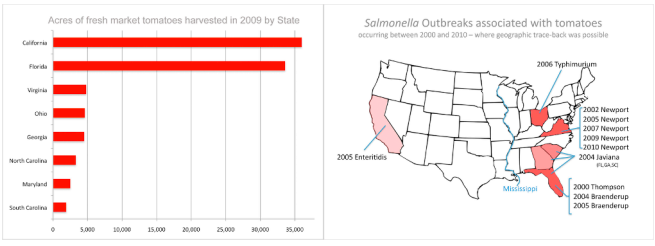

Fig. 1. Acres of fresh market tomatoes harvested in 2009 by state and *Salmonella* outbreaks associates with tomatoes between 2000 and 2010, where geographic trace-back was possible.

2. Materials and methods

2.1. Field sampling

VA: Tomatoes and leaves were collected from Painter, VA (Latitude 37.58, Longitude −75.78) on August 16th, 2013; CA: Tomatoes and leaves were collected from Five Points, CA in the San Joaquin Valley (Latitude 36.42, Longitude −120.10) on August 16th, 2013. NC: Tomatoes were collected from Salisbury, NC (Latitude 35.67 and Longitude −80.47) on August 16th, 2013. MD: Tomatoes were collected from Queenstown, Maryland (Latitude 38.99°, Longitude −76.15°) on August 16th, 2013. All tomato cultivars were large round fresh market tomatoes (BHN602 for VA, unknown for CA (Cultivar information was not available for California tomatoes but they were most likely 'Quality 21'). For North Carolina, the cultivar was 'Amelia' and for Maryland the tomato cultivar used was BHN 602). Approximately 3 tomatoes and 5 leaves were collected from fresh market cultivars of tomatoes planted in typical 22 m growing rows. Samples were collected by walking down a 22 m row and picking a leaf and a tomato approximately every 3 m. The leaves and tomatoes were placed in ziplock bags (using gloves) and 300 ml of sterile water was added to each bag. Five replicates were taken from each site. The mixture of leaves, tomatoes and sterile water was sonicated for 6 min and the resulting wash off water was centrifuged at 4000 rpm (3220 rcf) using an Eppendorf 5810R to create a pellet which was frozen at −20 °C for subsequent DNA extraction.

2.2. 16S rRNA amplicon sequencing

16S rRNA gene amplicon sequencing was performed on all samples according to Illumina's "*Overview of tailed amplicon sequencing approach with MiSeq*" protocol. Genomic DNA extracted from each sample was used as template for PCR. Sequence specific primers were used for the first round of PCR as follows: 16S rRNA gene primers for theV4 region (515f, 805r), GTGCCAGCMGCCGCGGTAA (forward) GGACTACHVGGGTWTCTAAT (reverse) (Caporaso et al., 2012) and the V1-3 region PCR for 16S targeing the first 330 bases of the V1-V3 region 27F AGAGTT TGATCMTGGCTCAG (forward) 336R ACTGCTGCS YCCCGTAGGAGTCT (reverse). PCR conditions: 94 °C for 2 min, (94 °C for 40 s, 56 °C for15 s, 68 °C for 40 s) x25 cycles, 68 °C for 5 min, 4 °C Hold. The amplicons were indexed based on manufacturer's instructions, using Nextera library preparation (Illumina, SanDiego, CA, USA). 16S amplicon products were approximately 390 bps and 330 bps respectively. Clean PCR product was obtained using AMPure XT Beads (Beckman Coulter Inc. Brea, CA) to remove fragments smaller than 100 bp. Two microliters (2 μl) of product from the first round of PCR was used as template for the second round of PCR. One microliter (1 μl) of each index N50X and N70X were added to the PCR reaction. Each sample had a different combination of N50X and N70X indices and there were no repeats. Product obtained from the second round of PCR was cleaned using AMPure XT beads (Beckman Coulter Inc. Brea, CA). Sample DNA concentration was determined using Qubit High-Sensitivity Assay (Life

Technologies, Grand Island, NY). Samples were diluted to 2 nM with EB buffer (Qiagen, Hilden, Germany) and pooled using 10 µl of each sample. Ten microliters (10 µl) were taken from the amplicon multiplex sample and denatured with 10 µl 0.2 N NaOH. This process was performed simultaneously for a 2 nM PhiX sample (Illumina, San Diego, CA) in a separate tube. Samples were incubated at room temperature for 5 min then 980 µl HT1 buffer (Illumina, San Diego, CA) was added to each sample to create a final concentration of 20pM. PhiX and amplicon multiplex samples were diluted to 5pM in 500 µl, and pooled together at a 1:1 ratio for a final volume of 1000 µl. Six hundred microliters (600 µl) of the sample was loaded on a MiSeq V2 cartridge for sequencing (Illumina, San Diego, CA).

2.3. Bioinformatic methods

Raw fastq files reflecting forward reads output by the MiSeq platform were initially filtered for quality and length (≥200bp) using QIIME (Yilmaz et al., 2011; Caporaso et al., 2010a), and spurious hits to the PhiX control genome were identified using BLASTN and removed. Sequences passing the quality metrics described above were trimmed of the forward primer, and evaluated for chimeras using UCHIME (*de novo* mode) (Edgar et al., 2011), and filtered for host-related chloroplast DNA using the RDP Bayesian classifier (Wang et al., 2007). Next a large-scale BLASTN search of the GreenGenes database (v13_05) was performed to identify unknown contaminant sequences. Sequences without a database match of at least 70% identity along 60% of their length were removed. (Contaminants included a substantial number of mitochondrial DNA and chloroplast DNA). The final dataset of high-quality 16S rRNA gene amplicon sequences from each region were characterized for diversity and taxonomic composition using QIIME with the GreenGenes database. Sequences were clustered into operational taxonomic units (OTUs) using UCLUST (*de novo*) (Edgar, 2010) with a 97% identity threshold. Representative sequences of each cluster were assigned to a taxonomic lineage by the RDP classifier (trained on the GreenGenes 16S database, v13_05) using a minimum threshold of 0.50. Representatives were input to PYNAST (Caporaso et al., 2010b) to generate a multiple sequence alignment, which was subsequently used to construct a neighbor-joining phylogenetic tree with FastTree (Price et al., 2009). After full characterization of the clean sequence dataset, sampling depth was normalized by rarefaction to 2500 sequences per sample. Beta-diversity distance metrics (Bray-Curtis) were computed from rarefied OTU tables and visualized using principal coordinate analysis in QIIME. Hierarchical clustering and visualization were performed in R (v.2.12.0).

2.4. Data submission

All 16S rRNA gene fastq files have been deposited in the SRA of NCBI associated with accession number PRJNA506919 http://www.ncbi.nlm.nih.gov/bioproject/506919. All metadata has been submitted according to MIMARKS (minimum information about a marker gene sequence) (Yilmaz et al., 2011).

3. Results

One of the most intriguing differences between California and Eastern U.S. tomato samples was the high prevalence of Enterobacteriaceae spp. that appeared to dominate California samples in contrast to eastern samples. Using amplicons from the V4 region of the 16S rRNA gene (Fig. 2), we were only able to assign taxonomy at a family level for the Enterobacteriaceae, however using V1-V3 region (known to be more discriminatory for Enterobacteriaceae) (Fig. 3), *Erwinia* sp. could be assigned using the methods described above. Another interesting difference between east and west was a higher incidence of Firmicutes species in California bacterial profiles. For example, *Bacillus* and *Weissella spp*. are interesting members of

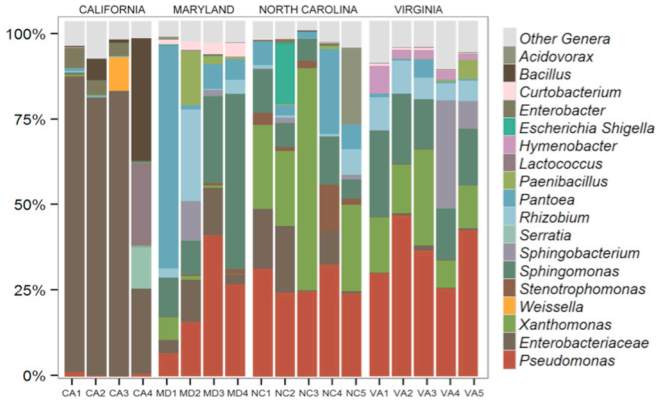

Fig. 2. Relative abundance of dominant bacterial taxa from California (CA), Maryland (MD), North Carolina (NC) and Virginia (VA) tomato phytobiota, described using amplicons from the V4 region of the 16S rRNA gene.

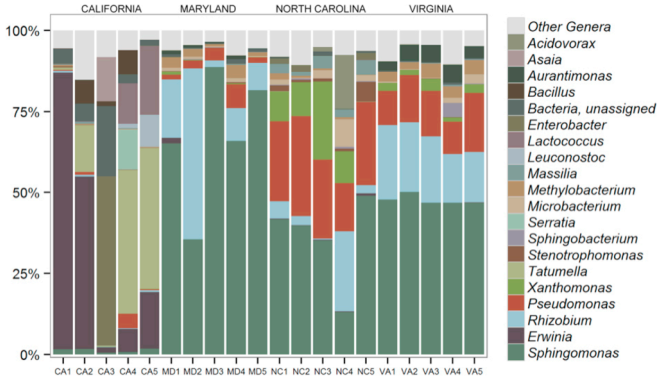

Fig. 3. Relative abundance of bacterial taxa in tomato phytobiomes from California (CA), Maryland (MD), North Carolina (NC) and Virginia (VA) described using amplicons from the V1/V3 region of the 16S rRNA gene.

Leuconostocaceae, a family of lactic acid producing heterofermentative Gram-positive Lactobacillales. These genera were abundant in California phytobiomes in contrast to Maryland, Virginia and North Carolina. Lactic acid bacteria and *Weissella spp.* in particular (Campana et al., 2017; Trias et al., 2008) demonstrate strong antibiotic activity against Gram negative bacteria including certain human pathogens and uropathogens such as *Escherichia coli, Pseudomonas aeruginosa*, *Enterobacter cloaceae, Citrobacter freundii* and *Proteus mirabilis.* One species *of Weissella, Weissella cibaria* has been shown to disrupt oral biofilms (Kang et al., 2006) so the possibility exists that antibiotic activity from these taxa or other *Bacilli* species may actually disrupt biofilms that occur in the California phyllosphere environment, thereby potentially functioning as a natural bio-control of potentially pathogenic species (Shafi et al., 2017). Conversely, the predominance of Rhizobiaceae (*Rhizobium spp.),* which are known to generate biofilms with complex and diverse quorum sensing physiologies, was described in Virginia, Maryland and North Carolina phytobiota. These may create more hospitable niches for *Salmonella* via provision of resources from epiphytic biofilms. Dominant bacterial clades in eastern tomatoes; *Xanthomonas, Stenotrophomonas*, *Rhizobium*, and *Sphingomonas*, are all known to be proficient at generation of biofilms under the right conditions. Taxa that were unique or more prominent in Virginia phytobiomes included *Hymenobacter* of the (Flavobacteriaceae) Flavobacterialeas and *Aurantimonas* of the (Aurantimonadaceae) Rhizobiales. *Aurantimonas* species have been reported from caves and associated with corals, which might have been introduced from the ocean, or the sandy loam soils of the Virginia fields, or components of fertilizers applied in this area to tomato phyllosphere. Interestingly, the genus *Tatumella* was reported in the California tomato samples using V1/V3

16S rRNA gene amplicons. *Tatumella ptyseos* strains have been isolated from powered infant formula milk and linked to neonatal sepsis, bacteremia and urinary tract infections – as well as cultured from many food sources around the world (Mardaneh et al., 2014).

4. Discussion

In contrast to some work, our data suggest that for tomatoes, microbial communities are not consistent across geographic locales on the same genus and species of host plants. Redford et al. described microbial communities associated with pine trees in diverse geographic regions like Australia and Colorado and hypothesized that the epiphytic microbiota was more influenced by phylogenetics of host plant (*Pinus sp.)* than by environmental pressures in diverse geographic regions. For example, microbial communities in Australia and Colorado on the same genus of tree were more similar than microbial communities in the same geographic location on different tree genera (Redford et al., 2010). Yang et al. also found with denaturing gradient gel electrophoresis (DGGE) profiles that different agricultural crops such as citrus, sugar beets, corn and grapefruit harbored distinct microbial populations even when grown in the same geographic region (Yang et al., 2001). In contrast to those results, other work, such as studies of glass slides placed near leaves demonstrated that the slides captured a similar microbiota to what was present on the leaves, supporting the idea that phyllosphere microbiota is influenced by the environment (Andrews and Harris, 2000). Additional support for this idea is provided by research that described microbiota on surfaces of tomato plants contrasted to abiotic polyurethane “control” surfaces placed adjacent to plants, that shared almost 97% of the same bacterial taxa (Ottesen et al., 2016).

The afore mentioned results and the results presented here support the hypothesis that much of the bacterial diversity associated with the phylloplane of crop plants may be introduced by environmental, non-host plant sources. Many additional factors such as anthropogenic agricultural inputs (pesticides and fertilizers), plant host genetics, soil composition, etc., contribute to temporal and biogeographic microbiota associated with plant surfaces but environmental pressures are clearly important drivers of phyllosphere ecology. Many studies have suggested that seasonal and/or biogeographic factors have stronger influences on the composition of phyllosphere microbiota than pesticides (Ottesen et al., 2014), water sources (Telias et al., 2011), and other agricultural management practices (Perazzolli et al., 2014; Thompson IPBMJF et al., 1993; Marine et al., 2015). Work by Gales et al., actually demonstrated that plant surfaces can serve as sentinels to evaluate bioaerosol emission from neighboring composting facilities because of their accurate representation of how far airborne microbiota will travel in the air (Galès et al., 2014).

The description of agro-ecologies along the farm to fork continuum has begun to establish microbial baselines that will contribute to an improved understanding of precisely where and how human pathogens become associated with food plants in agricultural settings, and which components of these ecologies pose the greatest risk. Water, wind, soil and dust will always play important roles in agriculture and each of these have vastly different compositions according to geographic region of origin. Water has always been one of the most important agricultural resources. Scarcity of water in certain geographical regions is already requiring use of water that has been exposed to numerous biological and chemical pollutants before being reclaimed for agricultural uses. Because of the vast complexity of phytobiomes, increased sample replication, increased sequencing depth and expanded temporal and geographic description will provide more complete understandings of microbiota on tomatoes and other food crops, to better assess whether certain biogeographic regions pose greater risks for contamination by pathogens and if so, how to mitigate those risks.

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