CPS CENTER for PRODUCE SAFETY

CPS 2011 RFP
FINAL PROJECT REPORT

Project Title

DNA-based identification of foliar microbiota with potential to predict or preclude pathogen establishment on field-grown leafy greens

Project Period

April 1, 2012 – December 31, 2012

Principal Investigator

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Objectives

1. *Determine to what extent differences in bacterial populations on leaves between cultivars of lettuce and spinach correlate with the ability of these populations to resist invasion by pathogens, including EcO157:H7 and Xcv.*

2. *Address the temporal variability in bacterial community composition by longitudinal sampling of lettuce fields in the Salinas valley.*

Funding for this project provided by the Center for Produce Safety through:

CPS Campaign for Research and California Leafy Greens Research Program

FINAL REPORT

Abstract
We gathered and interpreted DNA-based information on the identity and relative abundance of members that make up the microbial communities ('microbiota') that are naturally associated with leafy greens. We observed large differences between lettuce and spinach in the bacterial diversity that they carried on their foliage, but less so between different cultivars of lettuce. As the season progressed, the bacterial community on leaves of lettuce and spinach changed dramatically towards increased representation by bacteria from the family Enterobacteriaceae, which includes environmental (i.e. nonfecal) coliforms. Spray-inoculation of lettuce leaves with *Escherichia coli* did not reveal differences in the ability of different cultivars to facilitate *E. coli* survival, i.e. DNA- and CFU-based estimates of *E. coli* dropped rapidly following inoculation on all cultivars, despite the differences in community composition among some of those cultivars. Our findings demonstrate practical utility of microbiota-based metrics to inform the industry about pathogen survival and detection in a complex background of naturally associated leaf bacteria and their DNA. A specific recommendation based on our findings is that the design, use, and interpretation of culture-dependent or -independent protocols for the detection of fecal contamination on produce should embrace an appreciation for the temporally variable but typically high abundances of nonfecal coliforms that naturally associate with lettuce and spinach leaves, so as to avoid or minimize false-positives in said protocols and the consequences (costs, distress) that come with such false-positives.

Background
In a previous project, funded jointly by the Center for Produce Safety (CPS) and the California Leafy Greens Research Program (CLGRP), we generated a database of the bacteria associated naturally with the foliage of field-grown lettuce at time of harvest (Rastogi et al, 2012, ISME Journal 32:1-11). For this analysis, we used a methodology known as 454 pyrosequencing of 16S rRNA gene amplicons, which paints a detailed, culture-independent picture of the bacterial diversity on leaf surfaces and other microbial habitats. The goal of that project was to identify bacterial index species that can be used to highlight samples with increased probability of contamination with *Ec*O157:H7 or other pathogens. The absence of confirmed or suspected pathogen-positive leaf samples among those that we analyzed introduced a bias into our dataset, so that we learned much about the bacterial diversity on '*E.coli*-free' lettuce leaves ('*E.coli*-free' representing a common event) but very little about the diversity on '*E.coli*-positive' leaves ('*E.coli*-positive' representing a rare event). With this new project, we took an alternative approach, by controlled recreation of a contamination event to reveal how persistence of the pathogen (we used a surrogate for *E. coli* O157:H7) is a function of the bacterial community composition and to extract from these data bacterial species or genera with the greatest potential as index organisms (objective 1). We used the same approach to ask the same question for the foliar plant pathogen, *Xanthomonas campestris* pv *vitians*, causal agent of bacterial leaf spot of lettuce (objective 1). In the previous project, we had looked at community composition on harvest-ready leaves only, leaving us with no insight into the variation in community composition during the course of the growing season. Therefore, we also performed a longitudinal analysis of leaf microbiota from lettuce plants sampled between time of planting and harvest (objective 2).

Johan Leveau was PI on this project. Other participants include Gitta Coaker (co-PI), Gurdeep Rastogi, Jan Tech, Trevor Suslow, Steve Koike, Laura Murphy, Tom Williams, and Maria Marco.

We also acknowledge support from the cooperative grower who prepared, planted and maintained the field where we grew and sampled our leafy greens.

Research Methods and Results

We addressed objectives 1 and 2 simultaneously by sampling of a single field in Soledad (CA) made available by a cooperative grower through collaboration with Steve Koike. Given the evidence in the literature for a cultivar impact of bacterial community composition, we decided to grow different cultivars to generate different bacterial communities on the leaves of these plants, which would allow us to test hypothesis 1.

Eight different cultivars of lettuce were planted from seed in the field during the summer of 2012 (planting 06/04/2012, harvest 08/10/2012). Five cultivars were Romaine (Green Forest, Darkland Cos, Heartbreaker, Sunbelt, Frontier), two were Iceberg (Inspire, Vandenberg), and one was Greenleaf (Big Star). Plants were thinned at the 4-6 true leaf-stage, i.e. 29 days after planting. All beds were irrigated with overhead sprinklers to promote the germination at day of planting followed by periodic irrigation as per commercial schedule until the end of the trial. Each cultivar bed was 400 feet long with a width of 5 feet. Each bed was divided into 4 blocks (60 feet each) and each block was equally sub-divided into 3 sections corresponding to three different treatments (water, *E. coli*, *X. campestris* pv *vitians* or *Xcv*), allowing 4 replicates of each treatment per cultivar. The sections within a bed were arranged using a complete randomized design. A 20- and 40-feet buffer zone was established in the starting and ends of each bed, respectively and on each side of the entire field that was under trial, an unplanted buffer zone was retained. In addition to 4 blocks in a bed, each bed at its far end also contained 4 sections (20 feet each) that did not receive any treatment.

In the same field, four different cultivars of spinach (Bejo 2866 F1, Corfu, Whale RZ F1, Emu RZ F1) were planted from seed (planting on 06/19/2012, harvest on 07/28/2012). Each spinach bed was 300 ft long and 5 ft wide. Each bed was divided into 4 blocks (40 ft each) and each block was equally divided into 2 sections each with a different treatment (water or *E. coli*), allowing 4 replicates of *E. coli* treatment per cultivar. The sections within a bed were arranged using a randomized design. A 20-feet buffer zone was established in the starting and ends of each bed. On each side of the field an unplanted buffer zone was retained. Seeds were sown following normal commercial practices for spinach plantation in Salinas Valley. All beds were irrigated with overhead sprinklers to promote the germination at day of planting followed by periodic irrigation as per commercial schedule until the end of trial.

A cocktail of three generic rifampin resistant *E. coli* strains (TVS353, TVS354, and TVS355) were used for spray inoculation of lettuce and spinach cultivars. These strains have been described previously (Gutiérrez-Rodríguez et al, 2010, Journal of Applied Microbiology 112: 109-118) and were provided by project collaborator Trevor Suslow. At 40 days after planting, lettuce plants were spray-inoculated in the morning between 7 and 10 AM. Inoculum for spray was prepared from overnight-grown *E. coli* LB rifampin (Rif50) plates. *E. coli* biomass was scraped from the plates and suspended in water at a concentration of 10^8 CFU/ml (an OD_{600} of 0.75 approximates 10^9 CFU/ml). An aliquot of *E. coli* ready-to-spray inoculum was serially diluted and plated on LB rif50 plates yielding 1.4×10^8 CFUs/ml after overnight incubation at 37°C. Six plants growing in the center of sections labeled *E. coli* were spray-inoculated, each with about 10 ml of bacterial suspension, using a hand-held pressurized pump. Recovery of *E. coli* from inoculated lettuce plants was done at day 1, 2, 7, and 8 post-inoculation. For recovery,

whole lettuce heads were weighed and then washed thoroughly in wash buffer solution as described elsewhere (Rastogi et al, 2012, ISME Journal 32:1-11). Leaf washings were plated on CHROMagar ECC and LB rif50 medium and incubated at 37°C for 24 hrs.

The same *E. coli* cocktail was used to inoculate spinach plants. Ten ml of a 10^8 CFU/ml bacterial suspension was spray inoculated onto individual spinach plants at day 31 after planting. Only plants that were in the center of section were spray inoculated. *E. coli* recovery was done at day 7 and 8 from different spinach cultivars. Three inoculated plants were taken from each *E. coli* sprayed section in a block and vortexed for 1 min in wash buffer. Leaf washings were plated on ECC and LBrif 50 medium and incubated at 37°C for 24 hrs.

We used a cocktail of two rifampicin resistant *Xcv* strains (*Xcv*9837 and *Xcv*9805) for field inoculation trial on different cultivars of lettuce. These strains were provided by Bob Gilbertson. *Xcv* cells were scraped from overnight-grown LBrif50 plates and suspended in water at the concentration of 10^4 CFU/ml. An aliquot of *Xcv* ready-to-spray inoculum was serially diluted and plated on LB rif50 plates yielding 2.4 x 10^4 CFUs/ml after overnight incubation at 37°C. About 10 ml of Xcv suspension was spray inoculated on six plants each growing in the center of the sections designated for *Xcv* inoculation. *Xcv* recovery from inoculated or water sprayed plants was done using LBrif50 medium at day 1, 2, 14, and 15 after inoculation.

We also extracted total microbial DNA from foliage of lettuce on days 38, 46, 47, 53, 54, and 67 after planting. From spinach cultivars, we extracted DNA on 24, 31, 32, 38, and 39 days after planting. We also extracted total microbial DNA from *E. coli*/*Xcv*/water sprayed treatments of lettuce and spinach cultivars. Leaf washings from these samples were plated for total culturable bacteria and coliforms. From the DNA samples, a selection was used for community analysis by sequencing of 16S rRNA gene amplicon sequencing. Details for this procedure and the analysis of DNA sequence data are described previously (Rastogi et al, 2012, ISME Journal 32:1-11).

Outcomes and Accomplishments

Major outcomes and accomplishments of this project are summarized below. We are preparing a manuscript in which these findings are documented in much greater detail (including figures) and submitted for review, revision and ultimately publication in an appropriate scientific journal.

In the context of the first objective (Determine to what extent differences in bacterial populations on leaves between cultivars of lettuce and spinach correlate with the ability of these populations to resist invasion by pathogens, including *Ec*O157:H7 and *Xcv*), we established that lettuce and spinach plants that were grown at the same time in the same field under the same management practices appeared to carry very different bacterial communities on their leaves. This finding is consistent with the idea that the plant has ‘control’ over which bacteria it allows to colonize its foliage. It is likely that such ‘control’ is underpinned by genetic factors, which opens up the future possibility to make leaf communities a breeding target. This is of obvious interest if one knows what makes a healthy microbiota (e.g. one that prevents the establishment of unwanted pathogens). Among different lettuce cultivars, the bacterial communities were similar in that for most cultivars the variation between plants from the same cultivar was not significantly different from the variation between plants from different cultivars. However, upon closer inspection of the data, there certainly were exceptions to this, for example, communities on cultivar Green Forest were clearly different from those on cultivar Inspire. This suggests that even among

different cultivars of a single crop (lettuce) there is sufficient genetic variation to sustain different bacterial communities on their leaf surfaces.

The demonstration that at least some lettuce cultivars carried different communities on their leaf surfaces provided the desired starting point for asking the question whether such differences impacted the outcomes of foliar inoculation with *E. coli*. In short, we did not observe such differences in outcomes: the mix of rifampin-resistant *E. coli* bacteria that was sprayed onto the foliage showed a typical death curve that was not significantly different between plants representing different cultivars. Survival was quantified by spreading of leaf washings onto plates containing rifampin and counting the colony-forming units (CFUs) per gram of plant tissue. One and two days after inoculation, we were still able to detect in most samples between 10^2 and 10^4 CFUs per gram, while on days 7 and 8, the majority of samples no longer yielded CFUs at a detection limit of 10^2 per gram. Similar experiments with *Xanthomonas* showed that this foliar pathogen (which was inoculated onto lettuce foliage at much lower densities than was *E. coli*), remained detectable for at least 15 days post inoculation at levels between 10^2 and 10^5 CFUs per gram of leaf tissue, demonstrating much higher epiphytic fitness under these conditions than *E. coli*.

In the lettuce inoculation experiment, we found a striking correlation between the number of colony-forming units of *E. coli* and the relative abundance of *Escherichia* sequences in the culture-independent data. These *Escherichia* sequences disappeared from the community at a half-life rate of 1.4 days, indicating that not only do these bacteria become unculturable (as the plating experiments show), their DNA also disappears, suggesting that at least under these conditions *E. coli* bacteria died and disintegrated rather than remained in some sort of viable but nonculturable form.

For the second objective (Address the temporal variability in bacterial community composition by longitudinal sampling of lettuce fields in the Salinas valley), we sampled uninoculated plants from the same field at high temporal resolution. We discovered that both on lettuce and spinach leaves, bacterial communities changed dramatically over the course of the growing season. The most striking change was represented by bacteria from the family Enterobacteriaceae, which includes such genera as *Pantoea* and *Erwinia*, two of the most ubiquitous environmental (i.e. nonfecal) coliform bacterial genera associated with plants. For spinach, these Enterobacteriaceae started to dominate the community quite early on (our first sampling point was 24 days after planting, and for some plants, 60% of the bacterial sequences were identified as Enterobacteriaceae). For lettuce, their abundances were relatively low (<30%) but increased considerably at time of harvest. These culture-independent data were supported by CFU countings on CHROMagar plates, which allow the quantification of total coliforms, including *Pantoea* and *Erwinia*: we saw clear domination of these bacteria early (spinach) or later (lettuce) in the season. We explain this as the ability of bacteria from genera such as *Pantoea* and *Erwinia* to utilize leaf surface resources such as photosynthates for growth; in other words, the epiphytic fitness of these bacteria contributes to their abundant presence on the leaf surfaces of lettuce and spinach under field conditions. This finding has implications for the design and interpretation of protocols that assess fecal coliform contamination of produce, as such protocols may be vulnerable to the undesirable generation of false-positives as a result of high levels of other (i.e. environmental) coliforms.

Summary of Findings and Recommendations

We demonstrated clear differences in the leaf microbiota of lettuce and spinach plants growing at the same time and in the same field, suggesting that the plant is a major determinant in the outcome of bacterial colonization of plant foliage.

We found partial evidence for cultivar-dependent differences in the bacterial communities on lettuce leaves, but there were no major differences among cultivars in their ability to support *E. coli* survival, suggesting that cultivar choice is an unlikely contributor to pathogen persistence in case of a contamination event.

Overall, the decline in *Escherichia* sequences on *E. coli*-inoculated plants mirrored the decrease in culturable *E. coli* that could be recovered from these plants. This agreement between culture-independent and -dependent observations suggests a rapid turnover of sprayed *E. coli* cells and their DNA on contaminated plants.

In the course of the growing season, leaves of lettuce and spinach accumulated high levels of Enterobacteriaceae and culturable coliforms. An important question for the future is whether these contribute to an increased probability of false-positive outcomes in tests for *E. coli* O157:H7 and other enteropathogens.

APPENDICES

Publications and Presentations (required)

Publications

2013, Rastogi, G., G.L. Coaker, J.H.J. Leveau. New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches. FEMS Microbiology Letters, 348(1): 1-10.
2012, Rastogi, G., A. Sbodio, J.J. Tech, T.V. Suslow, G.L. Coaker and J.H.J. Leveau. Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. The ISME Journal, 6(10): 1812-1822.

Presentations by Johan Leveau (PI)

2014, August, 'Genomics-enabled discovery of novel adaptations to bacterial life in the phyllosphere', invited session chair and speaker at the 15th International Symposium on Microbial Ecology, Seoul, Korea, August 24-29, 2014.
2014, April 9, 'Microbial associations with plant foliage', invited speaker in the UC Merced Environmental Systems seminar series, Merced, CA.
2013, December 3, 'Bacterial communities on lettuce', 2013 Vegetable Crops Workgroup Meeting, UC Davis campus.
2013, July 24, 'Turning over a new leaf in phyllosphere microbiology', Keynote address at the FEMS 2013 5th congress of European Microbiologists, Leipzig, Germany, July 21-25, 2013.
2013, June 25, 'DNA-based identification of foliar microbiota with potential to predict or preclude pathogen establishment on field-grown leafy greens' (poster presentation), 4th Annual Produce Research Symposium, Wegmans Conference Center, Rochester NY.
2013, May 14, 'Structure and function of plant leaf-associated microbiota', invited lecture at Novozymes, Davis CA.
2013, March 19, 'DNA-based census of bacteria on foliage of field-grown lettuce and spinach', Annual research meeting of the California Leafy Greens Research Board, Harris Ranch, Coalinga CA, March 19 2013.
2012, June 29, 'Bacterial colonization of plant leaf surfaces: of communities and single cells', University of Goettingen, Germany, June 29, 2012.
2012, June 27, 'DNA-based identification of foliar microbiota with potential to predict or preclude pathogen establishment on field-grown leafy greens' (poster presentation), 3rd Annual Produce Research Symposium, UC Davis campus, June 27, 2012.
2012, May 19, 'Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce' (poster presentation), 28th New Phytologist Symposium, Rhodes, Greece, May 18-21, 2012.

Budget Summary (required)

Total budget was $96,973, split between the California Leafy Green research Board and Center for Produce Safety as $90,000 and $6,973, respectively. Budget was expended with little deviation from the original proposed budget. A more detailed budget summary is attached.

Tables and Figures (optional)

Suggestions to CPS (optional)