Cover Page

BARD Project Number: US-3949-06

Date of Submission of the report: September 2, 2010

Project Title: *Salmonella enterica* Interactions with Fresh Produce

Investigators

Institutions

Principal Investigator (PI): Brandl, Maria T.

USDA, ARS, WRRC

Co-Principal Investigator (Co-PI): Sela, Shlomo

ARO, The Volcani Center

Collaborating Investigators:

Parker, Craig T.

USDA, ARS, WRRC

Rodov, Victor

ARO-Volcani Center

Keywords: food safety, produce, lettuce, cilantro, soft-rot, *Dickeya dadantii*, *Salmonella*, enteric pathogens

Budget: IS: \$162,000

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Total: \$324,000

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Final Scientific Report

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	1	1	1	3
Submitted, in review, in preparation	1	1		2
Invited review papers			1	1
Book chapters				
Books				
Master theses			1	
Ph.D. theses				1
Abstracts	4	1		5
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant. N/A

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		1	2	3
Longer Visits (Sabbaticals)

Final Scientific Report

Abstract

The emergence of food-borne illness outbreaks linked to the contamination of fruits and vegetables is a great concern in industrialized countries. The current lack of control measures and effective sanitization methods prompt the need for new strategies to reduce contamination of produce. Our ability to assess the risk associated with produce contamination and to devise innovative control strategies depends on the identification of critical determinants that affect the growth and the persistence of human pathogens on plants. *Salmonella enterica*, a common causal agent of illness linked to produce, including lettuce and other leafy vegetables and herbs, has the ability to colonize and persist on plants. Our main objectives were to identify *S. enterica* plant-inducible genes that are involved in 1) the growth and/or survival of *S. enterica* on postharvest lettuce, and 2) its interaction with *Dickeya dadantii* (*Erwinia chrysanthemi*), a plant pathogen that is commonly responsible for soft rot in postharvest produce and is known to increase the incidence of *Salmonella* contamination of produce.

Our results under objective 1 suggest that *in-vitro* biofilm formation tests may provide a suitable model to predict the initial attachment of *Salmonella* to cut-romaine lettuce leaves. We observed that *Salmonella* association with lettuce increases its acid-tolerance, a trait which might be correlated with an enhanced ability of the pathogen to pass through the acidic barrier of the stomach. We have demonstrated that *Salmonella* can internalize leaves of iceberg lettuce through open stomata. We found for the first time that internalization is an active bacterial process mediated by chemotaxis and motility toward nutrient produced in the leaf by photosynthesis. These findings may provide a partial explanation for the failure of sanitizers to efficiently eradicate foodborne pathogens in leafy greens and may point on a novel mechanism utilized by foodborne pathogens and perhaps plant pathogens to infect leaves. Using resolvase *in vivo* expression technology (RIVET) we identified multiple *Salmonella* genes, some of which of unknown function, that are involved in attachment to and persistence of *Salmonella* on lettuce leaves. The precise role of these genes in *Salmonella*-leaf interactions is yet to be elucidated. Our findings advance the understanding of how *Salmonella* persist in the plant environment and suggest a potential effect of contaminated cut lettuce tissue on *Salmonella* survival in humans.

Under objective 2, we demonstrated that *S. enterica* achieved large population sizes and formed complex biofilms on leaves infected with *D. dadantii*. Microarray analysis of the gene expression profile in *S. enterica* cells colonizing lettuce and cilantro soft rot lesions caused by *D. dadantii* revealed that the human pathogen deploys a large part of its transcriptional machinery for anaerobic metabolism and the utilization of propanediol (pdu) and ethanolamine (eut), along with cobalamine synthesis (cob), an essential co-factor in these pathways. Global transcriptional analysis indicates that the pdu and eut pathways, which function in the human intestine, are activated in lettuce and cilantro by the presence of substrates resulting directly from the degradation of plant cells by the soft rot pathogen. Genes involved in the transport of quorum sensing autoinducers were induced also in soft rot lesions, indicating cross-talk between the plant and human pathogens. Additionally, *S. enterica* cells recovered from soft rot lesions were more invasive of eukaryotic cells than when grown in culture. Our observations suggest that soft-rot pathogens contribute to creating a familiar and hospitable niche for human pathogens on plants, which may increase their prevalence in produce as well as affect their infectious dose in humans. This indicates that soft-rot disease is a high risk factor in the transmission of *S. enterica* from raw fruit and vegetables to humans and should be controlled throughout the food production chain.

Final Scientific Report

Achievements

Epidemiologic data collected by the CSPI in 1998-2007 in the U.S. revealed that among single food vehicles associated with outbreaks, produce caused the most cases of illness (23%) and came second only to seafood in total number of outbreaks (<http://cspinet.org/>). The association of produce with outbreaks in Israel is not well monitored; however, previous data linked ca. 20% of all outbreaks with a known agent to vegetable products in 1993-1998. Importantly, produce grown in Israel has been recalled in the US and the UK due to contamination with *Salmonella*. The current lack of control measures prompt the need for new strategies to reduce contamination of produce. Our ability to assess the risk associated with produce contamination and to devise innovative control strategies depends on the identification of critical determinants in the growth and persistence of enteric pathogens on plants. Thus, our overall objective was to elucidate the interactions between *Salmonella enterica* and leafy vegetables, first on healthy fresh-cut lettuce, and second, in softrot lesions on leafy vegetables infected with the common postharvest plant pathogen *D. dadantii*.

1.1 Attachment to and biofilm formation on lettuce, and effect of colonization on acid tolerance

Various *Salmonella enterica* serotypes and strains were tested for their capacity to form biofilm in a 96-wells plate model. Strong biofilm producers attached better to intact romaine lettuce leaf compared to weak biofilm producers. These findings imply that *in vitro* biofilm assays may be suitable to predict initial attachment of *Salmonella* to lettuce. Using confocal microscopy and viable count studies, we showed that *Salmonella* attaches preferentially to cut-regions of the leaf after 2 h at 25 °C. These findings suggest that bacteria prefer the nutrient-rich environment of the injured tissue for attachment and underscore the safety implications of fresh-cut lettuce.

Exposure of lettuce-associated *Salmonella enterica* serotypes and planktonic cells to acidic conditions (pH-3.0) revealed increased tolerance to acid of the leaf-attached versus planktonic cells. Increased acid-tolerance is an important virulence trait that may increase the ability of the pathogen to pass through the acidic barrier of the stomach. Therefore, association of *Salmonella* with lettuce might be linked to the capacity of the pathogen to infect in relatively low doses.

1.2 Internalization through stomata Using *gfp*-tagged *Salmonella*, we found that the pathogen aggregates near open stomata and internalizes into the substomatal cavity. Our studies suggest

Final Scientific Report

that the pathogen is attracted to nutrients in the stomata produced *de novo* by photosynthetically active leaf cells. Indeed, isogenic mutations affecting *Salmonella* motility (*fliGHI*) and chemotaxis (*cheY*) significantly inhibited bacterial internalization. The elucidation of the mechanism by which *Salmonella* invades intact leaves has important implications for both pre- and postharvest handling of lettuce. Inhibition of internalization may limit bacterial colonization to the leaf surface and enhance the effectiveness of surface sanitizers. Our findings may also be applicable to plant pathogen interaction with leaves since chemotaxis and motility may be important mechanisms used by plant pathogens to infect leaves through stomata.

1.3. Identification of *Salmonella* genes that affect its colonization of fresh-cut lettuce leaves. We have constructed a resolvase-based in vivo expression technology (RIVET) library which enable us to screen for *Salmonella* promoters differentially expressed under particular environmental conditions. Screening of the *S. enterica* promoter library was performed on both intact and injured lettuce leaves stored for 7 days at 8°C, or for 3 days at 20°C, and yielded 169 clones that potentially contain inducible promoters. The details of the identified genes obtained for each storage condition are given in Appendix, Table 1, p. 29. Four of eight mutants (Appendix, Table 2, p. 31) that we constructed, namely, *stfC*, *yhjO*, *yidR*, and *misL* had impaired persistence on intact (but not injured) lettuce after 7 days at 8°C (Appendix, Fig. 1A, p. 28). Except for the *stfC* mutant, all three mutants had significantly lower attachment rate to intact leaf (Appendix, Fig. 1B, p. 28) and also were impaired in biofilm production in vitro (Appendix, Fig. 1C, p. 28). Further studies are required to elucidate the function of these genes in attachment and persistence of *Salmonella*.

2.1 *D. dadantii* soft-rot promotes growth of *S. enterica* on lettuce and cilantro We observed that *S. enterica* achieves populations sizes 100-fold greater when co-inoculated into the phyllosphere of cilantro plants with the wild-type pathogenic *D. dadantii* strain than with its non-pathogenic outD⁻ mutant, which does not cause soft rot disease (Appendix, Fig. 2A, p. 32). *D. dadantii* also increased the population sizes of *E. coli* O157:H7 by 3- to 6-fold on symptomatic Romaine lettuce leaves compared with non-infected healthy leaves. Confocal microscopy revealed that *S. enterica* produced large aggregates even in microscopic lesions (Appendix, Fig. 2B, p. 32), suggesting that *D. dadantii*-infected produce may pose a significant risk to public health already at the initial and undetectable stages of the disease. During advanced stages of rot, the human and the plant pathogen formed large and thick biofilms (Appendix, Fig. 2C, p. 32). The presence

Final Scientific Report

of *S. enterica* in such biofilms may greatly promote its survival and spread to other produce or inert surfaces throughout the food production/retail chain. Therefore, the ubiquitous nature of soft rot pathogens on leafy greens and the considerable growth of enteric pathogens that they promote in macerated plant tissue, warrant the food industry's and consumers' vigilance about symptoms of this disease on produce in order to prevent or minimize food-borne illness.

2.2 *S. enterica* gene expression profile in *D. dadantii* soft-rot lesions We have demonstrated by microarray analysis of the global transcriptome of *S. enterica* that the pathogen engages in anaerobic metabolism in soft-rot lesions on lettuce and cilantro leaves, as revealed by the upregulation of its numerous operons that function in nitrate reduction and hydrogenase activity and by its broad induction of pathways for the utilization of propanediol (pdu) and ethanolamine (eut), as well as that for cobalamine (cob) synthesis, a co-factor in those pathways (Appendix, Table 3, p.33; and Fig. 3, p. 34). Increased transcription of the *rha* and *fuc* operons provides further support that rhamnose and fucose are being funneled into the pdu pathway by *S. enterica* due to their increased availability by *D. dadantii* maceration of the plant cell wall. The induction of ethanolamine catabolism in *S. enterica* in that environment results likely also from plant cell maceration since it is a component of the cell wall matrix. *S. enterica* mutants defective in propanediol and ethanolamine utilization, and cobalamine synthesis were significantly less fit in soft rotted leaf tissue than the parental strain (Appendix, Fig. 4, p. 35). Propanediol and ethanolamine utilization in *S. enterica* takes place mostly in the human gut due to the presence of necessary substrates. Thus, our results suggest that, along with anaerobic metabolism occurring in soft rot lesions and in the intestinal milieu, there is a considerable overlap in physicochemical conditions in these two habitats. Additionally, we have demonstrated that *S. enterica* cells recovered from soft rot lesions on cilantro leaves are more invasive of Hela cells than when cultured in growth medium (Appendix, Fig. 5, p. 35).

Overall, our study reveals that soft rot pathogens contribute to creating a hospitable niche for *S. enterica* on plants, which may increase its prevalence in produce as well as affect its infectious dose in humans. This provides public health agencies with data for risk assessment and the produce industry and retailers with information to develop good agricultural practices and identify hazard critical control points of intervention in relation to soft rot disease.

Final Scientific Report

Details of Cooperation

Collaboration between the Israeli and US parties was done via email and telephone exchanges, meetings at international conferences, and a meeting in Berkeley, CA. More specifically, the collaborators met at the Food Microbiology 2008 Conference in Aberdeen, Scotland, September 1-6, 2008, where each co-PI gave an invited oral presentation about the studies performed with the BARD grant, and at the Phyllosphere 2010 Conference in Corvallis, OR, August 15-18, 2010. Dr. Brandl, who was a member of the Phyllosphere 2010 conference organizing committee, invited Dr. Sela to present the results of the investigations funded by the BARD grant. Dr. Brandl also gave invited oral presentations at several conferences about her BARD-funded investigations: ICEID, Atlanta, GA, March 2008; IFT Annual Meeting, New Orleans, LA, June 2008; HPOP Symposium - Colorado State University, Fort Collins, CO, October 2008; ICFST meeting, UC Davis, CA, August 2009; and a seminar at UC Davis, CA, September 2010. In addition, poster presentations of results from the BARD-funded studies were given at several conferences throughout the years. Funding from BARD was acknowledged during all of the above presentations. Several experimental protocols were exchanged between the two laboratories. Additionally, Dr. Sela's laboratory constructed mutants which were sent to Dr. Brandl to be tested in her model system and were found to be functionally defective. Dr. Brandl sent a complementation plasmid that she constructed to Dr. Sela for complementation of a *Salmonella* cellulose biosynthesis mutant, which was impaired in fitness on lettuce. Three papers have been published so far: one regarding *S. enterica* attachment to processed lettuce leaves is coauthored by the co-PIs; a second about the multiplication of *E. coli* O157:H7 in lettuce lesions was published by Dr. Brandl; and a third about a novel finding regarding internalization of *Salmonella* in stomata and which was featured in the ASM Microbe Magazine was published by Dr. Sela. At least three more articles about the studies supported by this BARD grant are expected to be published in the coming year.

List of publications

- Brandl, M.T. 2008. Plant lesions promote the rapid multiplication of *E. coli* O157:H7 on post-harvest lettuce. *Appl. Environ. Microbiol.* 74:5285-5289
- Kroupitski, Y., Pinto, R., Brandl, M., Belausov, E., and S. Sela. 2009. Interactions of *Salmonella enterica* with lettuce leaves. *J. Applied Microbiol.* 106:1876-1885.
- Kroupitski, Y., D. Golberg, E. Belausov, R. Pinto, D. Swartzberg, D. Granot, and S. Sela. 2009. Internalization of *Salmonella* in leaves is induced by light and involves chemotaxis and penetration through open stomata. *Appl. Environ. Microbiol.* 75:6076-6086.