CENTER for PRODUCE SAFETY

CPS 2012 RFP
FINAL PROJECT REPORT

Project Title
Avirulent Salmonella strains and their use to model behavior of the pathogen in water, composts, in and on vegetables

Project Period
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Objectives
1) Construct non-pathogenic mutants in Salmonella ATCC 14028 and isolates from produce-related outbreaks and test their virulence in a mouse and check model.
2) Determine whether the surrogate strains can be detected with common isolation and identification protocols.
3) Test attachment of the avirulent strains to surfaces and their responses to common sanitizers under laboratory conditions.
4) Test the field fitness of the surrogate strains in soil and irrigation water.

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FINAL REPORT

Abstract
Outbreaks of gastroenteritis linked to pre- and post-harvest contamination of produce continue to raise questions about the ecology of human enteric pathogens in the production environment. Although experiments using nonpathogenic indicator organisms have been instrumental in the development of the environmental safety metrics, important biological differences exist between *Salmonella,* Shiga toxin-producing *E. coli* (STEC)*,* fecal coliforms and other organisms used as indicators. These differences are reflected in the pathogens' persistence in the pre- and post-harvest production environments even when indicator organisms are not detected. Because all outbreak strains of STEC and *Salmonella* are pathogenic, field studies with these organisms in an unaltered state are not feasible. Two naturally occurring Shiga toxin-defective mutants of EHEC have been used in field studies in California and Georgia. No such avirulent *Salmonella* surrogate suitable for field studies has ever been developed. With this CPS grant we developed an avirulent surrogate strain of *Salmonella* and tested its suitability for field studies. Several precautions were taken to generate this construct: Pathogenicity islands were precisely excised, the strain does not harbor virulence plasmids, the strain is free of features increasing its resistance to antibiotics. The strain was designed to fail PCR-based *Salmonella* detection approaches, however, it can still be identified using culture-based techniques. The surrogate strain can be distinguished (using PCR or culture-based techniques) from the wild type *Salmonella* because of its loss of function *phoN* mutation. The surrogate strain was shown to be avirulent in two animal models.

Background
Behavior of common indicator organisms and Salmonella in soil, organic amendments and water. The need for suitable non-pathogenic indicators of microbiological safety of the environment has long been recognized. However, even after a 40-year-long search for an ideal non-pathogenic indicator, the behavior of which correlates with that of pathogens, it has not been found (57). In general, *Salmonella* is commonly accepted as being better able to survive in the environment than *E. coli* (rev. (56), although *Salmonella* is not as common in the environmental samples as *E. coli* or coliforms. *Salmonella* can better persist in processed organic waste and amended soils than fecal coliforms, although its persistence appears to be strain- and/or site-dependent (38, 58, 59). The persistence pattern of *Salmonella* on surfaces of peaches, cantaloupes, lettuce, and bell pepper has been shown to be very different from that of *E. coli* (and/or select microbial indicators) (7, 49). These differences in survival and re-growth under field conditions and in the vegetable production raise important questions about the suitability of indicators for predicting behavior of *Salmonella* in manure, irrigation water and in the association with plants.

Field release studies using attenuated pathogens. The recognition that coliforms and other indicator organisms are poor predictors of the behavior of pathogens under field conditions led to studies in which attenuated EHCE and *Salmonella* strains were released in the research fields in California and Georgia (14, 15, 22, 23, 34). EHEC mutants lacking *stx* genes (encoding the Shiga toxin), sometimes containing a plasmid-borne green fluorescent protein, were used. These seminal experiments with attenuated EHEC strains allowed an assessment of the impact of the pathogen on the overall numbers of phyllosphere bacteria (34); and the role of irrigation types and exposure to the sun in the ability of the pathogen to colonize specific sites on plants and to persist on crops (14, 15, 34). The availability of attenuated EHEC strains was instrumental in defining these aspects of pathogen ecology under the conditions that are relevant in the produce industry.

In the US, attenuated *Salmonella* appears to have been used in only two field studies, where a strain of *S.* Typhimurium (originally developed as a live oral vaccine) lacking the *cya/crp* global regulatory system was released (22, 23). While incapable of eliciting disease symptoms in mice, this strain attaches to and persists in gut-associated lymphoid tissues and is fully capable of eliciting immune responses in animals (10). Most vaccine strains, including *cya/crp* mutants, have severe growth defects due to lesions in major catabolic and regulatory pathways (2, 6, 8, 10, 25, 26, 42, 51, 55). Under laboratory conditions, phenotypes of *cya* and *crp* mutants are unstable, and second site spontaneous compensatory mutants arise and become dominant very quickly, often already after overnight culture (2, 51). Because virulence genes are otherwise intact, spontaneous second site mutations (for example in promoter regions controlled by CRP-cAMP) or gene conversions may arise and restore virulence to the released strains. With this propose research, we planned to develop mutants in which multiple loci of *Salmonella* pathogenicity genes are cleanly excised, thus making them incapable of invading animal hosts and eliciting disease, while maintaining those genes needed for normal growth in manure, soil, irrigation water and in or on plants.

Virulence determinants in Salmonella spp. The evolutionary divergence of *Salmonella* from *E. coli* 40-60 million years ago was associated with the acquisition of the *Salmonella* Pathogenicity Island (SPI)-1 (43). The presence of SPI-1 is a distinguishing characteristic of all of the 2,200+ strains of *Salmonella*, and this feature is commonly used in *hilA*- and/or *inv*-based PCR protocols for the detection of *Salmonella* in environmental samples and produce. Genes located on SPI-1 are involved in the early events of the interaction of the pathogen with the host gut epithelial cells. SPI-1 is also present in generally avirulent *Salmonella bongori,* and genes located on it are not sufficient to cause disease (3, 19, 29, 32, 43-45, 53, 60). Sequential acquisition of SPI-2, SPI-3, SPI-4 and SPI-5 by *Salmonella enterica* led to strains that are virulent in multiple animal hosts (13, 17, 19, 31, 37, 43, 53, 60). In addition to the Pathogenicity Islands, genes located on the *Salmonella* virulence plasmid contribute to virulence of some serovars (1, 18). All these horizontally acquired virulence genes (on SPI's or virulence plasmids) are tightly integrated into bacterial regulatory cascades. Therefore, deleting many housekeeping regulators (such as *crp* and *rpoS*) reduces expression of virulence genes and results in attenuated pathogenicity. The type strain *S. enterica* sv Typhimurium LT2 (sequenced by the McClelland's group) contains a spontaneous mutation in *rpoS*, which is at least in part responsible for its avirulence (32). For the purpose of this proposal, we use a narrow definition of "virulence genes" as only those that are present in *Salmonella* (and not in its closest relative *E. coli*) and are directly involved in the interactions with animal hosts. This definition, therefore, excludes pleiotropic regulators, housekeeping and metabolic genes that are also present in avirulent sister lineages (other members of *Enterobacteriacea*) and indirectly contribute to virulence. Evolutionary genomics analyses conducted by the McClelland's group have already defined many of these virulence genes, and led to the identification of targets for the deletion in the surrogate strains.

Research Methods and Results

Mutant construction. One-step PCR mutagenesis (11) was used to replace entire SPI's with *frt-kan-frt* cassettes. Subsequently, the antibiotic markers were removed as in (11). This approach allowed us to precisely excise entire pathogenicity islands, leaving only a ~20-nucleotide "scar" in place of each pathogenicity island. The *phoN* gene was removed similarly. Excisions were confirmed by PCR (Fig. 1). Once mutants were constructed, they were all first tested on Congo Red plates to check for the *rdar* phenotype, and if deviations from the expected phenotype were observed, mutants were remade prior to phenotypic characterization.

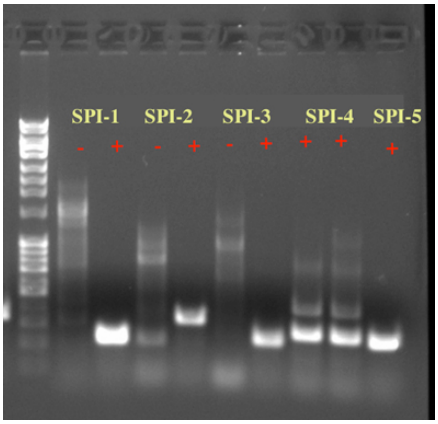


Fig. 1. PCR confirmation of *Salmonella* Pathogenicity Island (SPI) deletions in the surrogate strain. Primers flanking deleted regions were used to confirm deletions of each of the SPI's. "+" denote *Salmonella* wild type template, and "-" is surrogate. Note that SPI1, SPI-2 and SPI3 are over 10Kb, and the observed smearing in the wild type lanes is a result of non-specific binding.

Even though a strain of *S.* Typhimurium lacking its virulence plasmid pSLT was already constructed (1) and was shown to have no fitness defect in tomatoes (35), we designed and constructed a strain lacking the pSLT plasmid in the same background as the rest of the mutations. This was done to ensure that all mutations were in the same background. Even though we had no problems cloning *traStraT Salmonella* strains overexpressing it had reduced colony size and altered colony morphology, likely due to the fact that TraS, TraT are membrane proteins their overexpression was likely deleterious to the bacterium.

Animal virulence studies. Mutations in any of the *Salmonella* Pathogenicity Islands reduce virulence of the pathogen in animal models. Therefore, it was expected that the strain lacking all these mutations will be avirulent. We tested virulence of the strain in two murine models (female BALB/c, most commonly used for *Salmonella* infections, and black C57BL/6, most commonly used to model human disease). Mice were infected by oral gavage, animals were observed for a week, surviving animals were sacrificed, and *Salmonella* cells were recovered from liver, spleen, intestine and Peyer's patches (lymphatic tissue in the small intestine invaded by *Salmonella* when it crosses the intestinal barrier) on a selective medium. As expected, the surrogate strain was fully avirulent, and no *Salmonella* was recovered from any of the organs of mice infected with the surrogate strain (Table 1).

Table 1. Recovery of the wild type *Salmonella* and surrogate from BALB/c mouse organs

Infectious dose	**1.20E+07**			**1.20E+05**		**1.20E+03**	
	Wild type		***Surrogate***	***Wild type***	***Surrogate***	***Wild type***	***Surrogate***
	Mean	St. deviation	Mean	Mean	Mean	Mean	Mean
Spleen	2.00E+05	8.42E+04	0.00E+00	1071.43	0.00E+00	1.50E+01	0.00E+00
Liver	5.91E+04	2.42E+04	0.00E+00	1.02E+02	0.00E+00	7.50E-01	0.00E+00
Large Intestine	1.58E+05	7.67E+04	0.00E+00	7.50E-01	0.00E+00	0.00E+00	0.00E+00
Peyer's Patches	3.18E+06	3.89E+06	0.00E+00	20.00	0.00E+00	0.00E+00	0.00E+00

The surrogate strain was fully avirulent in mice: even at doses that exceed wild type LD50 by ten fold, the surrogate did not cause a disease in mice (Fig. 2).

Table 2. 7-day Survival of mice infected with the wild type and surrogate strains of *Salmonella*

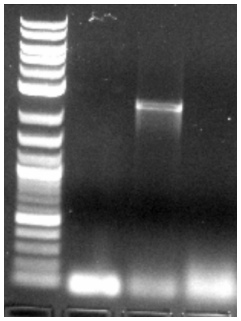
Strain	**Dose**	**Survival**
wild type	1.20E+07	0
wild type	1.20E+05	3
wild type	1.20E+03	3
surrogate	1.00E+08	3
surrogate	1.00E+06	3
surrogate	1.00E+04	3

Objective 2. *Determine whether the surrogate strains can be detected with common isolation and identification protocols.*

Culture-based detection. It would significantly *streamline the identification* of the surrogate if we can engineer it to behave just like the wild type under all environmental conditions, but display a somewhat different phenotype on detection media. Engineering the surrogate to contain a fluorescent protein or an antibiotic resistance marker is un-acceptable to environmental health and safety offices (34). The use of mercury resistance was approved for human volunteer studies with *Vibrio cholerae* vaccine strains (24, 27), and the *mer* Hg-resistance operon could be similarly used to mark *Salmonella* surrogate strains. However, the tightening regulation of mercury use and disposal of Hg-containing wastes may mean that this would not be the optimal way to mark the surrogates. Instead, we used a *phoN* (*an alkaline phosphatase*) mutant for such a purpose. The *phoN* mutants are unable to use the chromogenic substrate XP (5-bromo-4-chloro-3-indolylphosphate). This mutant was moved into the surrogate strain. Because the *phoN* mutation resulted in a loss of function, we expect that this way to mark the surrogate will be broadly acceptable to EHS boards.

Fig. 2. Culture-based detection and identification of the *Salmonella* surrogate. Surrogate and wild type strains on the XLD medium (indistinguishable). Surrogate and the wild type strain on LB medium with 5-bromo-4-chloro-3-indolylphosphate

As shown here, the *phoN* mutant behaves just like the wild type *Salmonella* on XLD by forming black colonies. XLD is the medium that is used most commonly for the isolation of Salmonella. However, as shown on the right image, it can be easily distinguished from the wild type on a secondary plate containing a substrate for phosphatase. This is a loss-of-function mutant, and therefore it allows to identify the surrogate strain without using any foreign markers.



PCR-based detection of the surrogates. Because our mutant strain lacks SPI-1, it will not be detectable using common primers for *Salmonella* detection (which target *hilA* or *inv* genes present in SPI-1).

Fig. 3. PCR with *hilA*-specific primers. Lane 1: ladder, Lane 2: negative controls (no template), Lane 3: wild type. The band corresponding to *hilA* is clearly visible, lane 4: avirulent mutant

Objective 3. *Test attachment of the avirulent strains to surfaces and their responses to common sanitizers under laboratory conditions.*

Attachment to abiotic surfaces. Biofilm assays were performed as before (50) on polystyrene and polypropylene surfaces. The ability of the bacteria to form biofilms was estimated using crystal violet following a 24-hr incubation at 22C.

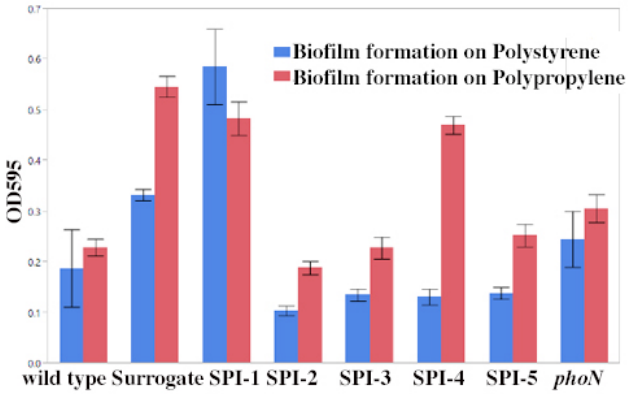


Fig. 4. Biofilm formation by the wild type and avirulent strains on abiotic surfaces.

The SPI-1 mutation (and to a lesser extent the SPI-4) mutations appear to have increased biofilm formation by the surrogate strain (Fig. 4). The increase in biofilm formation on polystyrene is not statistically significant compared to the wild type, however, the increase in biofilm formation on polypropylene is about 2.5 fold.

Attachment to leafy greens. The constructed avirulent strains and their wild type parents were inoculated onto plant surfaces and persistence of the surrogate strain, individual avirulent mutants and the wild type were tested on cut romaine leaves at 4°C and 10°C.

Table 3. Inoculated *Salmonella* SPI mutant populations on cut romaine leaves at 4°C.

	Time (d)											
train	0	1		2		5		7		14		21	
NT	6.1 ± 0.0	6.1 ± 0.0	BC[1,2]b[3]	5.8 ± 0.1	Ba	5.7 ± 0.1	Ba	5.6 ± 0.2	Ba	5.7 ± 0.2	Ba	5.5 ± 0.2	Ba
PI 1	6.0 ± 0.1	6.1 ± 0.1	Cd	5.5 ± 0.0	Ba	5.9 ± 0.1	Bcd	5.6 ± 0.2	Bab	5.8 ± 0.1	Bbc	5.8 ± 0.1	Bbc
PI 2	6.1 ± 0.1	5.2 ± 0.4	Ab	4.8 ± 0.4	Ab	3.0 ± 0.8	Aa	3.1 ± 0.4	Aa	2.8 ± 0.3	Aa	3.0 ± 0.2	Aa
PI 3	6.1 ± 0.2	5.8 ± 0.1	BCb	5.9 ± 0.1	Bb	5.9 ± 0.1	Bb	5.7 ± 0.2	Bab	5.9 ± 0.1	Bb	5.3 ± 0.4	Ba
PI 5	6.2 ± 0.1	6.1 ± 0.1	BCc	6.0 ± 0.1	Bbc	6.0 ± 0.1	Bbc	5.8 ± 0.1	Bbc	5.9 ± 0.3	Bbc	5.3 ± 0.2	Ba
hoN	5.9 ± 0.2	5.8 ± 0.1	BCa	5.8 ± 0.2	Ba	5.7 ± 0.1	Ba	5.4 ± 0.1	Ba	5.4 ± 0.3	Ba	5.6 ± 0.2	Ba
PI 4	6.2 ± 0.1	5.9 ± 0.1	BCb	5.9 ± 0.2	Bb	5.8 ± 0.2	Bb	5.8 ± 0.3	Bb	5.4 ± 0.3	Ba	5.2 ± 0.1	Ba
urrogate	6.1 ± 0.0	6.0 ± 0.2	BCb	5.9 ± 0.2	Bab	5.8 ± 0.2	Bab	5.9 ± 0.1	Bab	5.7± 0.1	Bab	5.9 ± 0.1	Bab

Values are means (log CFU/leaf) ± standard deviations (n=6).
[1]Means calculated based on normalized data, accounting for differences in initial concentration at d0.
[2]Means with the same capital letter in each column are not significantly different (P > 0.05).
[3]Means with the same lowercase letter in each row (i.e., same strain) are not significantly different (P > 0.05).

Table 4. Inoculated *Salmonella* SPI mutant populations on cut romaine leaves at 10°C.

	Time (d)								
Strain	0	1		2		5		7	
WT	6.1 ± 0.1	5.9 ± 0.5	B[1,2]a[3]	5.8 ± 0.3	Ba	5.8 ± 0.3	Ba	5.8 ± 0.1	Da
SPI 1	6.2 ± 0.1	5.8 ± 0.1	Bc	5.5 ± 0.2	Bc	5.4 ± 0.1	Bb	5.0 ± 0.2	Ba
SPI 2	6.4 ± 0.1	5.5 ± 0.1	Ab	3.9 ± 0.4	Aa	3.9 ± 0.8	Aa	4.5 ± 0.3	Aa
SPI 3	6.3 ± 0.1	5.8 ± 0.2	ABa	5.8 ± 0.2	Ba	5.7 ± 0.2	Ba	5.7 ± 0.1	CDa
SPI 5	6.4 ± 0.1	6.0 ± 0.2	ABa	5.9 ± 0.1	Ba	6.0 ± 0.1	Ba	5.9 ± 0.1	CDa
phoN	6.2 ± 0.1	6.0 ± 0.3	Ba	5.9 ± 0.3	Ba	5.8 ± 0.2	Ba	5.6 ± 0.1	CDa
SPI 4	6.5 ± 0.0	6.1 ± 0.3	ABb	6.0 ± 0.1	Bab	5.8 ± 0.1	Bab	5.7 ± 0.3	Ca
Surrogate	6.5 ± 0.1	6.1 ± 0.1	ABa	6.1 ± 0.2	Ba	5.8 ± 0.3	Ba	6.0 ± 0.1	Da

Values are means (log CFU/leaf) ± standard deviations (n=6).
[1]Means calculated based on normalized data, accounting for differences in initial concentration at d0.
[2]Means with the same capital letter in each column are not significantly different (P > 0.05).
[3]Means with the same lowercase letter in each row (i.e., same strain) are not significantly different (P > 0.05).

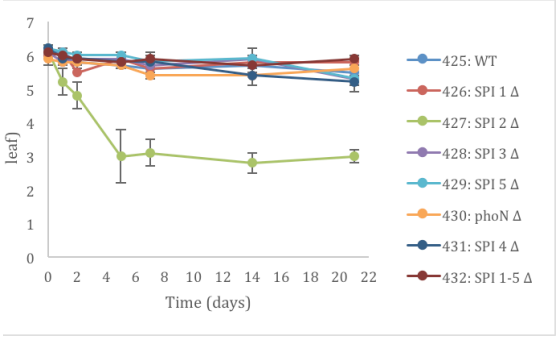


Figure 4. Fate of *Salmonella* SPI mutants on cut romaine leaves

It appears that while SPI2 mutant was less fit on cut romaine leaves, however the rest of the mutations in the final surrogate strain (shown in burgundy) were statistically not distinct from the wild type (dark blue).

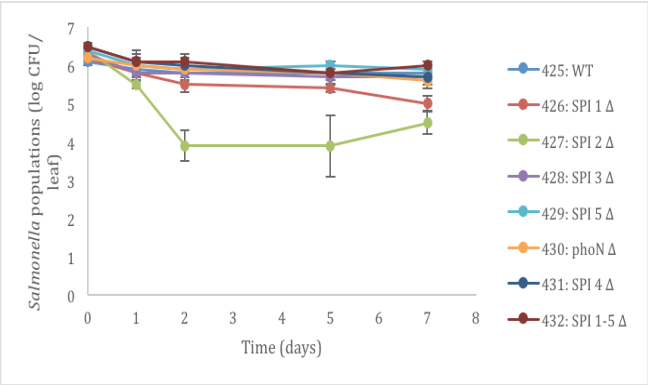
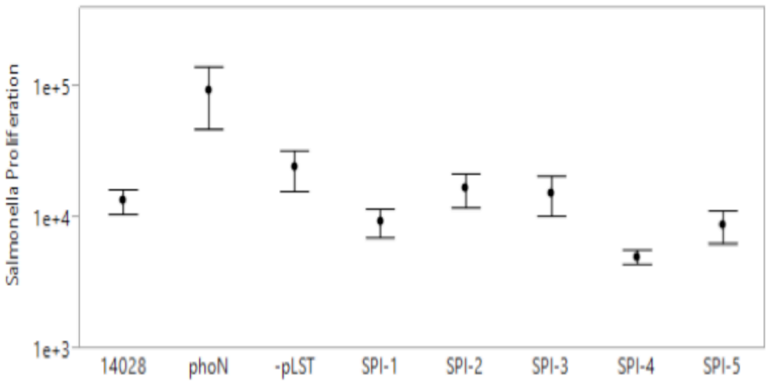


Figure 5. Fate of *Salmonella* SPI mutants on cut romaine leaves at 10°C.

Persistence red and green tomatoes. Shallow wounds in tomato surfaces were seeded with ~ 100-1,000 cells of each mutant. Fruits of three tomato varieties grown in the field under conventional production conditions were tested in for these experiments. Tomatoes supplied by our industry partner as well as store-bought tomatoes "Campari" were tested. Inoculated tomatoes were incubated at 18° -21°C. *Salmonella* were recovered from internal tissues at the end of a one week-long incubation period. Infected produce was macerated by stomaching in PBS and dilution-plated on a selective XLD medium.



Level			Mean
phoN	A		96517.5
-pLST	A	B	24681
SPI-2	A	B	17088
SPI-3	A	B	15788
14028		B	13729
SPI-1		B	9497
SPI-5		B	8933
SPI-4	A	B	5134

Fig. 5. Proliferation (measured as a ratio of recovered/inoculated CFU) of the avirulent strains and the parental wild type inside red tomatoes.

Even though differences between persistence of individual mutants in tomatoes were not strong (Fig. 5), the final surrogate strain was approximately 10-fold less fit than the wild type in tomatoes (Fig. 6 and Appendix 1).

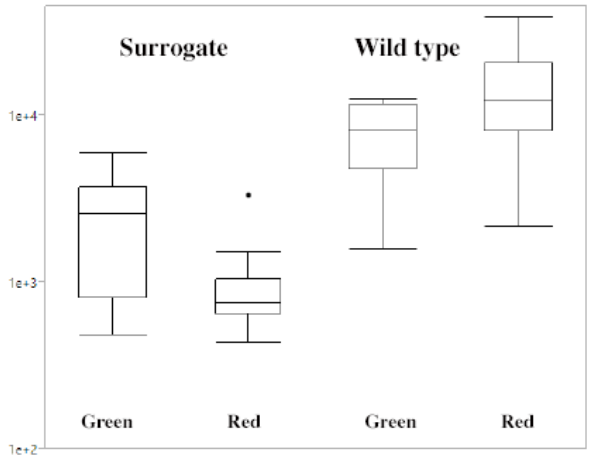


Fig. 5. Proliferation (measured as a ratio of recovered/inoculated CFU) of the surrogate strain and the parental wild type inside red and green tomatoes.

Sensitivity to common chemicals and chlorine. To establish how deletion of virulence genes affects susceptibility to common chemicals in the post-harvest environment, the wild type strain and an avirulent strain MHM56 lacking SPI's were inoculated onto cantaloupes, and then exposed to a variety of post-harvest treatments (Fig. 6). For the ease of the detection, a wild type strain was marked with a kanamycin-resistance cassette in a neutral site, resulting in CEC1000. Survivability studies in response to post-harvest treatment with 200 ppm chlorine and two commercial produce washes were conducted with spot-inoculations using previously developed protocols either directly or with slight modifications (5). Experiments presented in Fig. 6 were carried out at UF in collaboration with a commercial partner.

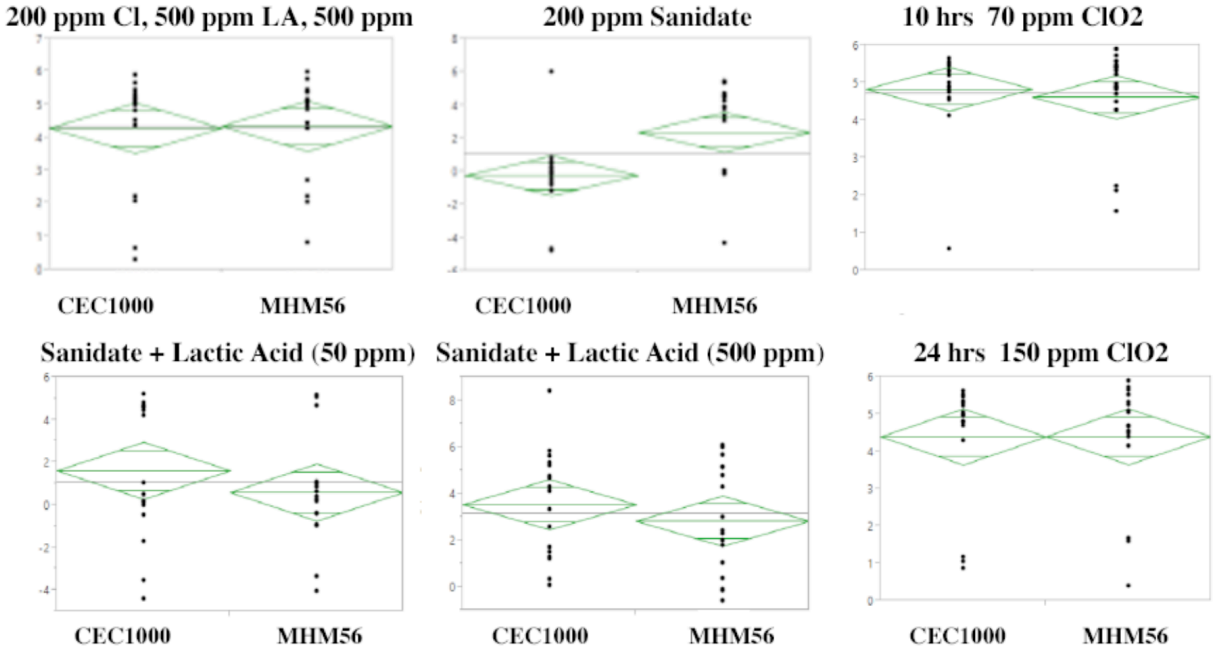


Fig. 6. Susceptibility of the avirulent (lacking SPIs) strain MHM56 and a wild type strain CEC1000 to common disinfectants.

Generally, the wild type and the surrogate strain responded similarly to the surface disinfection treatments commonly used in the industry.

Objective 4. *Test the field fitness of the surrogate strains in soil and irrigation water*.

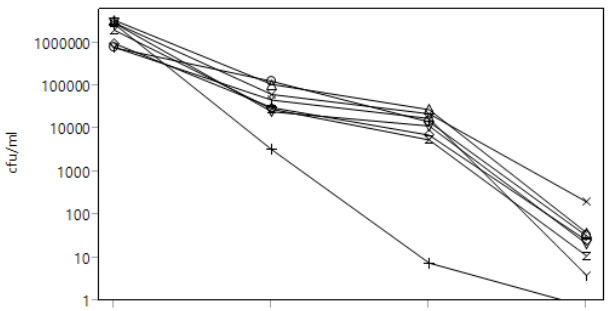


Fig. 7. Survival of the avirulent strains in well water at 22C. Key: empty circle is *Salmonella* Typhimurium 14028 (wild type), plus sign is *Salmonella phoN* mutant, diamond depicts a strain lacking the virulence plasmid pSLT, an "x" depicts a SPI-1 mutant, upward triangle is a SPI-2 mutant, a "y" is a SPI-3 mutants, a downward triangle is SPI-4, while a "z" is a SPI-5 mutant

Table 5. Inoculated *Salmonella* SPI mutant populations in EPA worst-case water at 25°C.

	Time (d)											
train	0	1		2		5		7		14		21	
ild type	5.7 ± 0.1	5.8 ± 0.1	AB[1,2] d[3]	6.0 ± 0.1	Dd	5.7 ± 0.1	Ed	5.1 ± 0.1	BCc	4.6 ± 0.2	Cb	3.8 ± 0.2	DEa
PI 1	4.8 ± 0.2	4.1 ± 0.4	Ad	3.6 ± 0.4	ABcd	3.0 ± 0.3	BCDEbc	2.2 ± 0.2	Aa	2.1 ± 0.3	ABab	1.2 ± 0.7	ABCa
PI 2	5.1 ± 0.2	4.6 ± 0.1	ABe	3.5 ± 0.4	Ad	2.9 ± 0.2	ABCcd	2.4 ± 0.5	Abc	2.0 ± 0.7	Aab	0.7 ± 0.7	Aa
PI 3	5.4 ± 0.2	5.1 ± 0.9	ABb	5.0 ± 0.1	BCDb	4.4 ± 0.9	CDEab	4.4 ± 1.0	Bab	4.0 ± 1.1	BCab	3.3 ± 1.0	CDEa
PI 5	5.5 ± 0.1	5.3 ± 0.3	ABb	5.3 ± 0.2	BCDb	3.6 ± 0.4	ABCDa	4.0 ± 0.3	Ba	3.8 ± 0.6	BCa	3.5 ± 0.3	CDEa
hoN	5.6 ± 0.0	5.9 ± 0.1	Be	6.1 ± 0.0	Df	5.4 ± 0.1	DEc	5.7 ± 0.1	Cd	4.8 ± 0.1	Cb	4.3 ± 0.1	Ea
PI 4	5.4 ± 0.2	5.0 ± 0.7	ABc	4.7 ± 0.9	ABCbc	3.4 ± 0.5	Aa	4.1 ± 0.6	Babc	3.4 ± 1.1	BCabc	2.5 ± 1.5	BCDab
urrogate	5.0 ± 0.2	4.6 ± 0.7	ABcd	5.3 ± 0.2	CDd	3.0 ± 0.0	ABab	4.0 ± 0.3	Bcd	3.4 ± 0.6	BCbc	0.9 ± 1.0	ABa

Values are means (log CFU/g) ± standard deviations (n=6).
[1]Means calculated based on normalized data, accounting for differences in initial concentration at d0.
[2]Means with the same capital letter in each column are not significantly different (P > 0.05).
[3]Means with the same lowercase letter in each row (i.e., same strain) are not significantly different (P > 0.05).

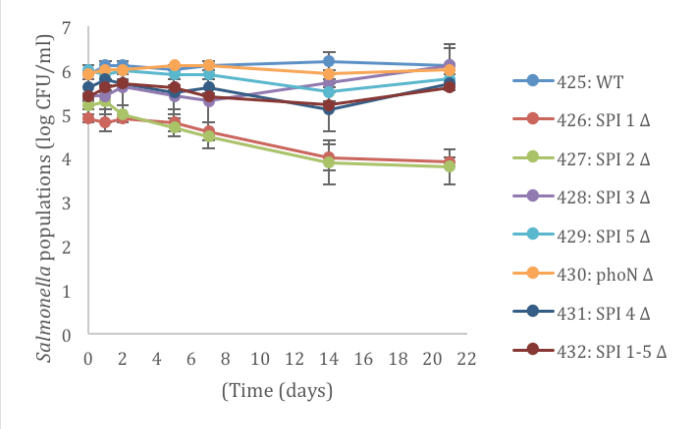


Fig. 8. Survival of individual *Salmonella* mutants and avirulent surrogate in EPA worst-case water at 25C.

Table 1. Survival of *Salmonella* SPI mutant populations in soil at 25°C.

	Time (d)											
train	0	1		2		5		7		14		21	
VT	5.9 ± 0.1	6.1 ± 0.1	A[1,2] a[3]	6.1 ± 0.0	BCa	6.0 ± 0.0	Ba	6.1 ± 0.1	Ca	6.2 ± 0.2	Ba	6.1 ± 0.5	BCa
PI 1	4.9 ±0.1	4.8 ± 0.2	Ab	4.9 ± 0.1	ABCb	4.8 ± 0.2	ABb	4.6 ± 0.2	ABb	4.0 ± 0.3	ABa	3.9 ± 0.1	Aa
PI 2	5.2 ± 0.1	5.3 ± 0.2	Ad	5.0 ± 0.2	Acd	4.7 ± 0.2	Abc	4.5 ± 0.3	Ab	3.9 ± 0.5	ABa	3.8 ± 0.4	Aa
PI 3	5.4 ± 0.4	5.4 ± 0.6	Aab	5.6 ± 0.4	BCab	5.4 ± 0.6	Bab	5.3 ± 0.8	BCab	5.7 ± 0.2	Aa	6.1 ± 0.4	Cb
PI 5	6.0 ± 0.1	5.9 ± 0.1	Ab	6.0 ± 0.1	ABCb	5.9 ± 0.1	Bb	5.9 ± 0.1	BCb	5.5 ± 0.2	ABa	5.8 ± 0.2	Bb
hoN	5.9 ± 0.1	6.0 ± 0.0	Aa	6.0 ± 0.2	ABa	6.1 ± 0.0	Ba	6.1 ± 0.0	Ca	5.9 ± 0.1	ABa	6.0 ± 0.1	BCa
PI 4	5.6 ± 0.2	5.8 ± 0.3	Ab	5.7 ± 0.1	ABCab	5.5 ± 0.4	ABab	5.6 ± 0.2	BCab	5.1 ± 0.5	ABa	5.7 ± 0.1	BCab
urrogate	5.4 ± 0.1	5.6 ± 0.1	Ab	5.7 ± 0.1	Cb	5.6 ± 0.1	Bb	5.4 ± 0.2	Cab	5.2 ± 0.3	ABa	5.6 ± 0.1	BCb

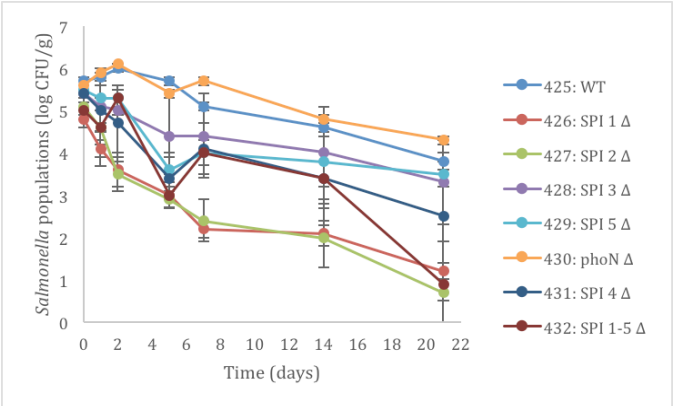


Fig. 9. Survival of individual *Salmonella* mutants and avirulent surrogate in non-sterile soil at 25C.

It appears that there is significant variability in survival of the avirulent mutants in soils. For the first 14 days, persistence of the surrogate in the soil (burgundy line) was statistically indistinguishable from the wild type, however populations of the surrogate declined rapidly by day 22. We are uncertain what the basis for this phenotype are, however, these observations indicate that in non-sterile soils, the surrogate faithfully represents the wild type for at least 14 days.

Even though there were differences between individual mutants in terms of their persistence in untreated well water and EPA worst-case water, wild type and the surrogate were indistinguishable throughout the duration of the three-week experiment. When tested for persistence in tap water and in water from a highly eutrophied body of water, both the wild type and surrogate *Salmonella* strains were non-detectable following 2-6 hours of incubation.

Outcomes and Accomplishments
With this study, we constructed and validated the first avirulent *Salmonella* strain that is suitable for field studies under FSMA. This strain is avirulent, does not carry antibiotic resistance genes, does not carry the self-transmissible virulence plasmid. If used in field-release studies, it will not be detected with common DNA-based detection techniques, however, it is detectable on common laboratory media used for the detection of *Salmonella* (such as XLD, HE, etc). It could be distinguished from the wild type strains of *Salmonella* on plates containing the substrate for PhoN.

Summary of Findings and Recommendations
This avirulent surrogate should be suitable for on-site experimentation under FSMA. However, those using the surrogate strain should be cautioned, as the large deletions (we removed almost 100 Kb of *Salmonella* genome) resulted in loss of fitness under some conditions (e.g. persistence in tomatoes) and increased biofilm formation on polypropylene. Impressively, the avirulent strain was not significantly different from the wild type under a number of other conditions (persistence in well water and EPA-worst case scenario water; on cut romaine leaves at 4C and 10C), it responded similarly to the wild type when exposed to chemicals commonly used for the surface disinfection of cantaloupes.

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APPENDICES

Publications and Presentations (required)

Publications:

1. Moraes, M., Danyluk, M., McClelland, M, Teplitski, M. *Salmonella* surrogate strain suitable for field release studies*. In preparation*
2. Brandl, M.T, Cox, C.E., Teplitski, M. 2013. *Salmonella* interactions with plants and their associated microbiota. *Phytopathology* 103: 316-325
3. Marvasi, M., Cox, C.E., Xu, Y., Noel, J. , Giovannoni, J., Teplitski, M. 2013. Differential regulation of *Salmonella* Typhimurium genes involved in O-antigen capsule production and their role in persistence within tomatoes. *Mol Plant Microbe Interact.* 26: 793-800.

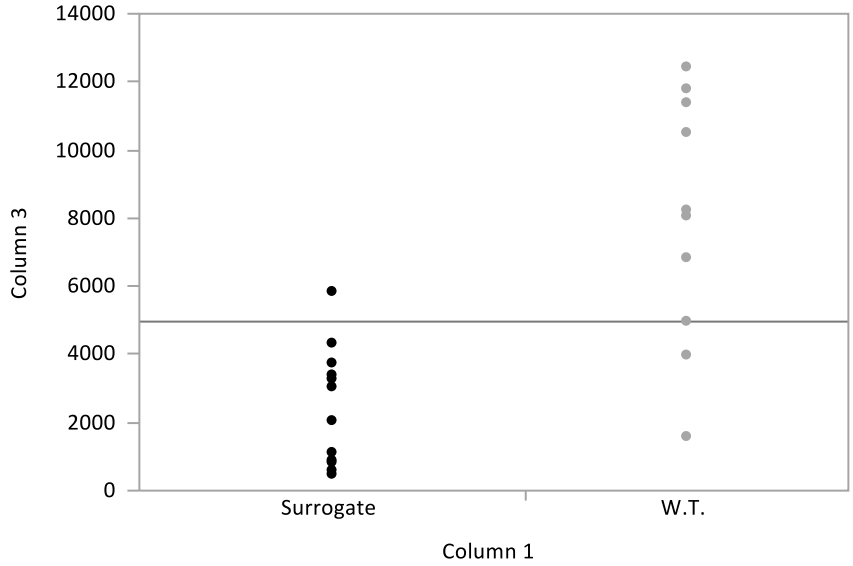
Presentations (discoveries partially or fully supported by this funding were presented at the following venues):

1. Teplitski, M. *Salmonella* interactions with crop plants: mechanisms and practical consequences. Food Science and Human Nutrition Department, University of Florida. Feb 10, 2015. Gainesville, FL
2. Moraes, M.H., McClelland, M., Teplitski, M. 2014. Mutant library screening reveals that *Salmonella* virulence genes are involved in the interactions with tomatoes. Southeastern Microbiology Summit – Joint Meeting of Southeastern and Florida Branches of ASM, Ponte Vedra, Fl, September 5-7, 2014.
3. Salas Gonzalez, I., Moraes, M.H., Teplitski, M. 2014. Comparative genomic analysis of *Salmonella enterica* sv Newport and a common generalist *S. enterica* sv Typhimurium. Southeastern Microbiology Summit – Joint Meeting of Southeastern and Florida Branches of ASM, Ponte Vedra, Fl, September 5-7, 2014.
4. Farias, M., Jenkins, K., Marvasi, M., Teplitski, M. 2014. Contribution of the *Salmonella* capsular transcriptional regulators *rcsA* and *rcsB* to the persistence of *Salmonella* in tomatoes. Southeastern Microbiology Summit – Joint Meeting of Southeastern and Florida Branches of ASM, Ponte Vedra, Fl, September 5-7, 2014.
5. Kirkpatrick, E., Marvasi, M., Teplitski, M. 2014. A carnitine metabolism key gene is involved in *Salmonella* persistence within tomatoes. Southeastern Microbiology Summit – Joint Meeting of Southeastern and Florida Branches of ASM, Ponte Vedra, Fl, September 5-7, 2014.
6. Teplitski, M. 2014. Avirulent *Salmonella* strains and their use to model behavior of the pathogen in water, composts, in and on vegetables. Center for Produce Safety Research Symposium, Newport Beach, CA. June 25, 2014
7. Farias, M., Jenkins, K., Marvasi, M., Teplitski, M. 2014. Contribution of the *Salmonella* capsular transcriptional regulators *rcsA* and *rcsB* to the persistence within tomatoes. Florida Association for Food Protection Annual Educational Conference, Clearwater Beach, FL, May 28-30, 2014.
8. Salas Gonzalez, I., Moraes, M.H., Teplitski, M. 2014. Comparative genomic analysis of *Salmonella enterica* sv. Newport Type 3 (prevalent in tomatoes) and a common generalist *S. enterica* sv. Typhimurium. Florida Association for Food Protection Annual Educational Conference, Clearwater Beach, FL, May 28-30, 2014.
9. Moraes, M.H., Teplitski, M. 2014. The role of *Salmonella enterica* virulence determinants in its proliferation in tomato fruits. Florida Association for Food Protection Annual Educational Conference, Clearwater Beach, FL, May 28-30, 2014.
10. Parker, K., Moraes, M.H., Teplitski, M. 2014. The effect of *Salmonella* Pathogenicity Islands on biofilm formation. Florida Association for Food Protection Annual Educational Conference, Clearwater Beach, FL, May 28-30, 2014.
11.

Budget Summary (required)

Tables and Figures (optional)

Oneway Analysis of *Salmonella* persistence in green tomatoes

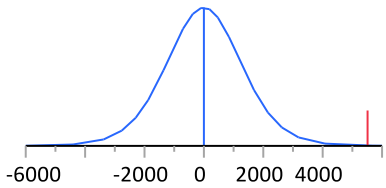


t Test

W.T.-Surrogate

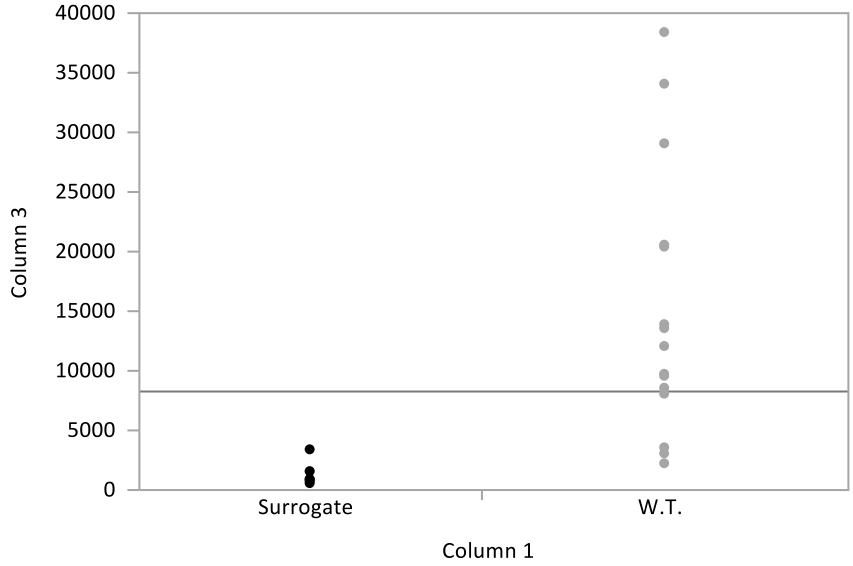
Assuming unequal variances

Difference	5512.08	t Ratio	4.382035
Std Err Dif	1257.88	DF	12.36206
Upper CL Dif	8243.90	Prob > |t|	0.0008*
Lower CL Dif	2780.27	Prob > t	0.0004*
Confidence	0.95	Prob < t	0.9996



Max Teplitski, University of Florida
Avirulent Salmonella strains and their use to model behavior of the pathogen in water, composts, in and on vegetables

Oneway Analysis of *Salmonella* persistence in Red tomatoes

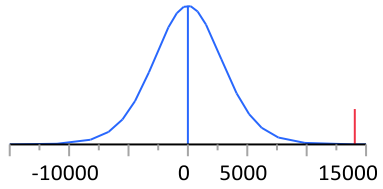


t Test

W.T.-Surrogate

Assuming unequal variances

Difference	14051.5	t Ratio	4.837128
Std Err Dif	2904.9	DF	14.12732
Upper CL Dif	20276.7	Prob > |t|	0.0003*
Lower CL Dif	7826.3	Prob > t	0.0001*
Confidence	0.95	Prob < t	0.9999



Suggestions to CPS (optional) none