CENTER *for* PRODUCE SAFETY

CPS 2010 RFP
FINAL PROJECT REPORT

Project Title
Influence of the pre-harvest environment on the physiological state of *Salmonella* and its impact on increased survival capability

Project Period
January 1, 2010 – December 31, 2012

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Objectives
1. To evaluate the impact of pre-and post-harvest environmental factors on the formation of aggregative fimbriae and cellulose
2. To characterize the role of thin aggregative fimbriae and cellulose
    a. in the desiccation tolerance and long term survival of *Salmonella*
    b. in acid tolerance and resistance to chlorine

Funding for this project provided by the Center for Produce Safety through:
CDFA SCBGP grant #SCB10063 and the California Almond Board

FINAL REPORT (5 to 15 pages)

Abstract

Salmonella has been implicated in numerous outbreaks of foodborne illness tied to the consumption of fresh produce. Multistate outbreaks of salmonellosis due to consumption of fresh produce and raw almonds have highlighted the ability of *Salmonella* to persist in a wide range of pre- and postharvest environments. Exposure to large swings in moisture, temperature, and nutrient levels are expected in these environments. The relative tolerance to these conditions is known to differ among strains of *Salmonella* but likely plays an important role in the survival of this organism to the point of consumption. Introduction of *Salmonella* may occur at any point in the farm-to-fork continuum and the contamination matrix may be in one of many forms: dry (e.g., dust), wet (e.g., decaying material), solid (e.g., food-contact surface), liquid (e.g., water). In this study the impact of pre-and post-harvest environmental factors on desiccation tolerance was evaluated. Strain, growth temperature, medium composition and form (solid surface or broth) were evaluated. All 14 *Salmonella* strains evaluated survived better during desiccation and persisted for a longer time when cultured on solid agar surfaces than when cultured in liquid medium. *Salmonella* strains that are able to produce cellulose and aggregative fimbrae (also called rdar morphotype) survived better during desiccation than strains that did not. Loss of the rdar phenotype impacted long-term survival of *Salmonella* on almonds but in model systems, survival for up to 7 days was not significantly reduced. Growth conditions that enhance desiccation tolerance (rdar morphotype, growth on agar) did not confir chlorine or acid tolerance. Rdar-positive strains should be included for research studies that involve desiccation of *Salmonella* and strains should be cultured and collected from agar medium.

Background

Since the mid 1990s, *Salmonella* has been implicated in multiple outbreaks of foodborne illness tied to the consumption of fresh fruits, vegetables, and tree nuts. Exposure to even low levels of *Salmonella* are thought to be sufficient to cause illness thus survival of the organism from contamination to the point of consumption is an important risk factor.

Although the *Salmonella* serovars associated with produce outbreaks have differed, their capability of long-term pre- and postharvest environmental persistence under a broad range of moisture levels has been well documented. For example, *Salmonella* Enteritidis PT 30 was isolated from the almond production environment for a period of 6 years (Isaacs et al., 2005; Uesugi et al., 2007); survival for 1.5 years with little to no reduction of the organism has been observed in almonds stored under ambient, refrigerated, or frozen conditions (Uesugi et al., 2006). *Salmonella* Enteritidis PT30 can grow in almond hull and shell slurries (Uesugi and Harris, 2006), in wetted hulls (Danyluk et al., 2008a), and in wetted dusts that are prevalent in the almond production and processing environments (Du et al., 2010). When hull extract is added to soil, multiplication of *Salmonella* can also be demonstrated providing an additional route of contamination (Danyluk et al., 2008b).

Previous studies in our laboratory demonstrated that cultures of *Salmonella* Enteritidis PT30 grown on petri plates (plate-grown or sessile cells) are physiologically different from the broth-grown culture (broth-grown or planktonic cells); the plate-grown cultures are more desiccation tolerant than broth cultures (Keller et al., 2012; Uesugi et al., 2006). *Salmonella* cells grown on solid media have higher attachment ability and pathogenicity than cells from liquid cultures (Wang et al., 2004) and have a greater degree of thermal tolerance (Harris, unpublished, Keller et al., 2012). This phenomenon appeared to be linked to the rdar phenotype (red, dry and rough) which is related to cellular production of cellulose and fimbriae.

Production of multicellular structures has also been shown to play a role in the long-term survival of *Salmonella* (Vestby et al., 2009). These multicellular structures can be identified on solid agar medium; *Salmonella* is categorized by colony morphology into rdar (red, dry and rough) or cells expressing fimbriae and cellulose. Genes responsible for fimbriae and cellulose synthesis are usually expressed during late stationary phase and/or under environments with low osmolarity (Romling, 2005).

We hypothesized that both pre- and post-harvest environmental factors directly impact the physiological state of *Salmonella* and the physiological state drives the strain-dependent production of multicellular structural components, such as fimbriae and cellulose. These structural components play a key role in the ability of *Salmonella* to survive stresses such as desiccation and also contribute to long-term persistence in the production and processing environments. They also provide enhanced tolerance to post-harvest stresses such as sanitizers, and increase *Salmonella*'s capability to resist acidity resulting in increased likelihood of illness at lower doses.

Our objectives were:

1. To evaluate the impact of pre-and post-harvest environmental factors on the formation of aggregative fimbriae and cellulose and
2. To characterize the role of thin aggregative fimbriae and cellulose
    a. in the desiccation tolerance and long term survival of *Salmonella*
    b. in acid tolerance and resistance to chlorine

Research Methods and Results

***Salmonella* strains, rdar morphotype and desiccation tolerance.** Rdar morphotype is linked to production of thin aggregative fimbriae and cellulose in *Salmonella*. *Salmonella* isolates (Appendix Table 1) were screened for rdar morphotype using a standard method of plating broth cultures onto LB-no-salt agar (LBSNA). To determine desiccation tolerance, cell suspensions were inoculated onto glass coverslips and held in desiccator at a relative humidity of 72-74% for up to 7 days. All *Salmonella* grown (rdar+ and rdar- strains) on agar medium survived significantly better during desiccation and persisted for a longer period of time than when grown in broth (Fig. 1). Rdar+ strains (broth or plate cultures) were significantly more tolerant to desiccation stress than rdar- strains.

Effect of substrate on expression of the *adr*A gene. Low nutrient broth (0.1% LBB), broth (LBB), agar (LBA), and low osmotic strength agar (LBNSA) were used to culture SEPT30. Cells grown in 0.1% LBB and on LBA and LBNSA showed significantly up-regulated expression of the *adr*A gene after 12 h of incubation at 28°C compared to cells grown in LBB (baseline) (Fig. 2). The relative expression of *adr*A for cells grown on LBNSA was 22-fold that observed on LBB and much higher than observed for either 0.1% LBB or LBA. These data suggest that growth under conditions of nutrient and osmotic stress as well as growth on solid medium trigger the expression of *adr*A.

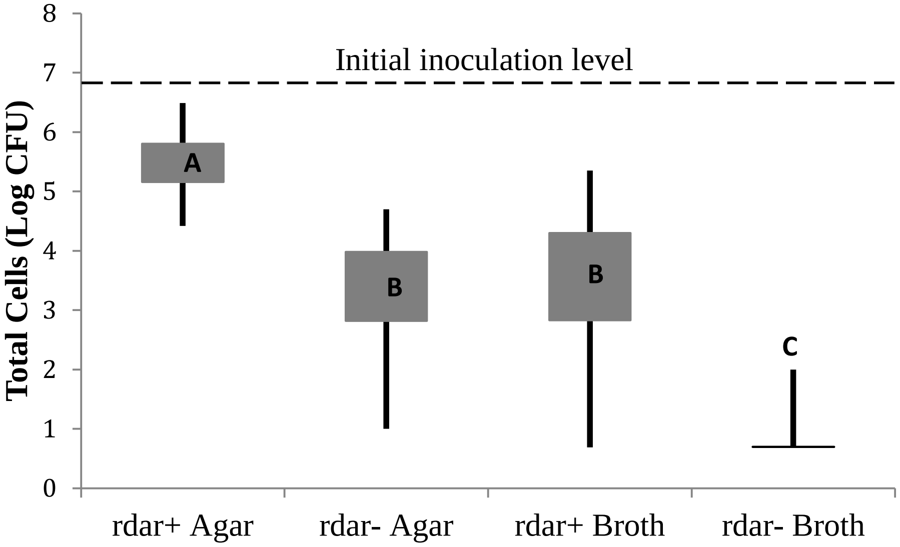


Fig. 1. Average survival of seven rdar+ and seven rdar- *Salmonella* strains grown on tryptic soy agar (TSA) or tryptic soy broth (TSB) and dried at 72% RH for 7 days. Values show the 95 percentile (box) and maximum and minimum values (bars) for each strain; *n* = 6. Means with different letters are significantly different (*P* < 0.05).

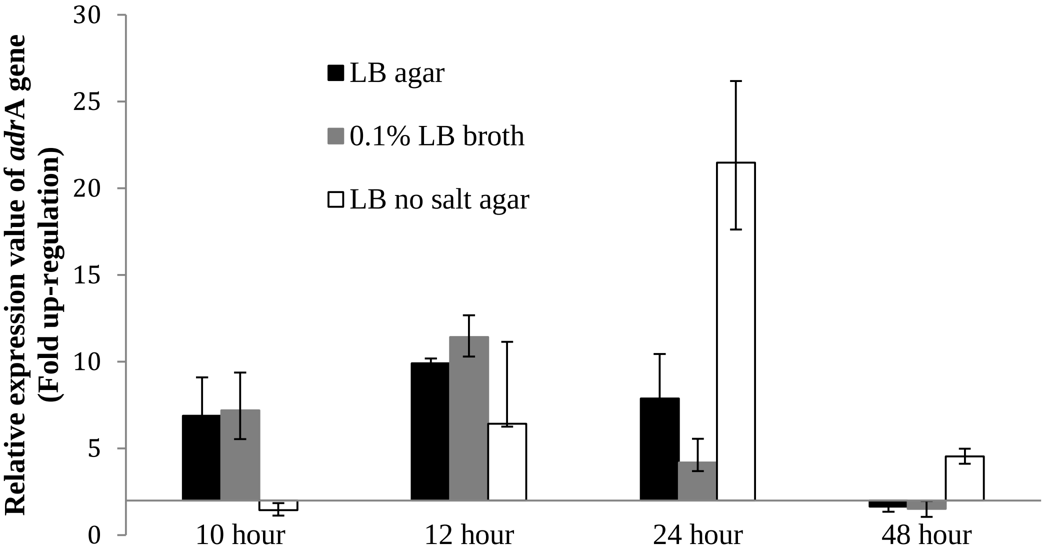


Fig. 2. Expression of *adr*A gene in *Salmonella* Enteritidis PT30 cells grown on different media at 28°C for 2 days relative to cells grown in LB broth.

Construction and evaluation of an rdar-negative mutant. In order to better understand the mechanisms of rdar morphotype and its effect on desiccation, a targeted rdar-morphotype negative derivative of SEPT30 was constructed (SEPT30D). A regulatory gene (*adr*A) associated with the rdar morphotype (production of cellulose and aggregative fimbrae) was targeted. A gene conferring kanamycin-resistance was inserted into the adrA gene. Previous studies had demonstrated that *adr*A mutants were not able to form rdar morphotype on LBNSA (Zogaj et al., 2001; Da Re and Ghigo, 2006).

We used several methods to confirm that the insertion was successful. SEPT30D had a negative rdar morphotype on LBNSA (Appendix Fig 1). SEPT30 and SEPT30D were cultured in LB broth, diluted and plated on LBNSA; plates were incubated at 28°C for 2 days to encourage the expression of the rdar morphotype. Cells were examined by Scanning Electron Microscopy (SEM). The SEPT30 (Fig. 3 A) appeared to be embedded in significant amounts of extracellular substances while SEPT30D was free of this extracellular material (Fig. 3 B). SEPT30D produced equivalent amounts of fimbriae but significantly lower amounts of cellulose than SEPT30 on LBNSA (Table 1).

Rdar and desiccation tolerance. The influence of rdar morphotype on desiccation tolerance was evaluated by culturing SEPT30 and SEPT30D on LBNSA at 37°C for 1, 3, and 5 days. The 1-, 3- and 5-day old cultures were collected and suspended in sterile MilliQ water; the OD_{600} values were adjusted to 0.60 ± 0.05 before inoculating 10 µl onto glass coverslips. The inoculated glass coverslips were dried in a desiccator with relative humidity (RH) adjusted to 72% at room temperature. No significant difference ($P<0.05$) was seen for the numbers of recovered cells between 1-, 3-, and 5-day old cultures after 2 days of drying (Fig. 4).

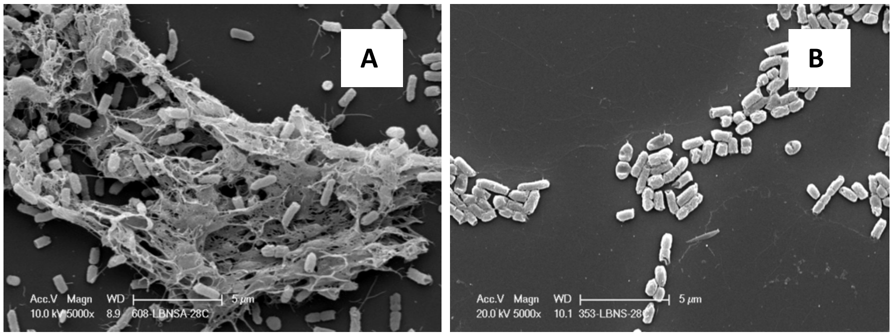


Figure 3. SEM of 2-day old LBNSA grown (A) SE PT30 and (B) SE PT30 mutant.

Table 1. Direct measurement of the fimbriae and cellulose produced by SE PT30 and SE PT30M (2-day old LBNSA cultures).

		Fimbriae[a]	**Cellulose**
Culture	Media	A_{500}	µg/10^9 CFU cells
SE PT30	LBNSA	0.98 a	19.8 ± 3.12 A
SE PT30M	LBNSA	0.97 a	10.2 ± 2.62 B

[a] Within columns, values with different letters are significantly different (n=6; $P < 0.05$).

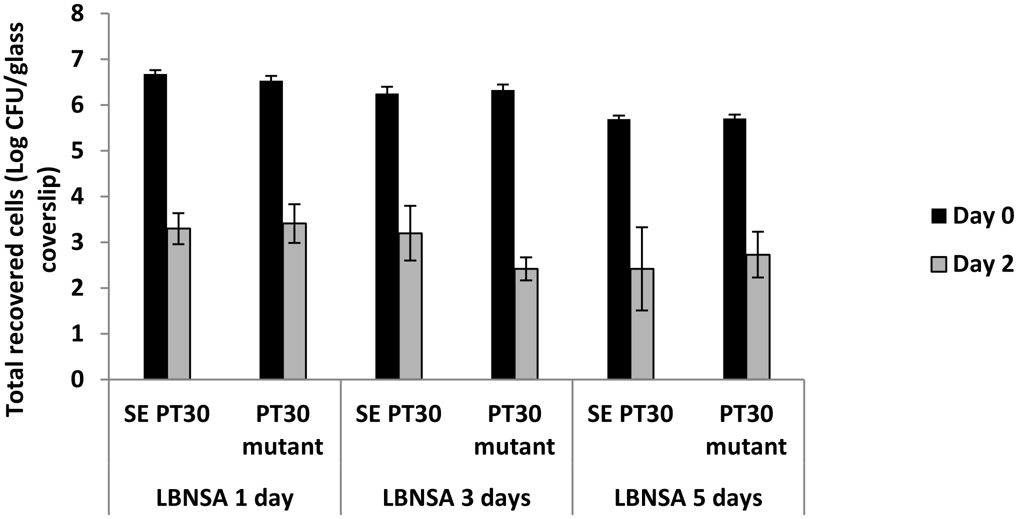


Fig. 4. Desiccation tolerance comparison between SE PT30 and SE PT30 mutant. Cultures were grown on LBNSA for 1, 3, and 5 day(s) before drying in the desiccator for 2 days (72% RH). N=6.

Survival of SEPT30 and SEPT30D on inoculated almonds. *Salmonella* SEPT30 and SEPT30D were cultured on TSA for 48 h, cells were collected, diluted to a standard OD_{600}, almonds were inoculated and dried for 3 days. Almonds were stored at 23ºC and 72% RH for up to 5 months. Counts on almonds inoculated with SEPT30D were 0.8 log CFU/g lower than for SEPT30 after 3 days of drying. At 2 months of storage and beyond, SEPT30D population densities declined more rapidly than SEPT30 (Fig. 5).

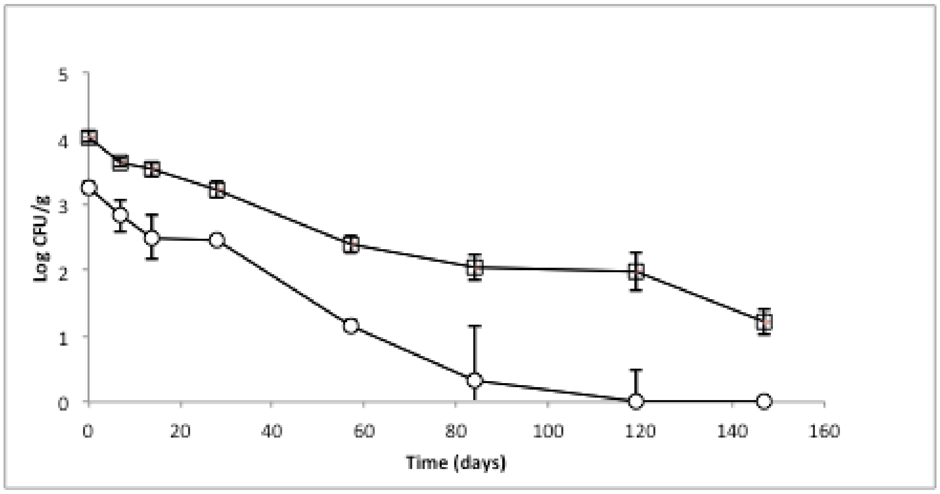


Fig 5. Survival of SEPT30 (squares) and SEPT30D (circles) on inoculated almonds stored at 23C and 72% RH.

Sensitivity of rdar+ and rdar- strains to chlorine and acid. The influence of rdar morphotype on chlorine and acid sensitivity was evaluated by culturing SEPT30, SEPT30D, and rdar negative *Salmonella* Oranienberg (rdarNeg) in TSB and on TSA in at 37°C for 24 h. The cells were collected and suspended in sterile MilliQ water. The OD_{600} values were adjusted to 0.60 ± 0.05 before inoculating 10 µl onto glass coverslips. The inoculated glass coverslips were dried in a desiccator with relative humidity (RH) adjusted to 72% at room temperature. No significant difference was observed in sensitivity to 5 % citric acid (Fig. 5) or 5 ppm free chlorine (Appendix Fig. 2) between broth or agar-grown cultures or among the rdar-positive or rdar-negative strains.

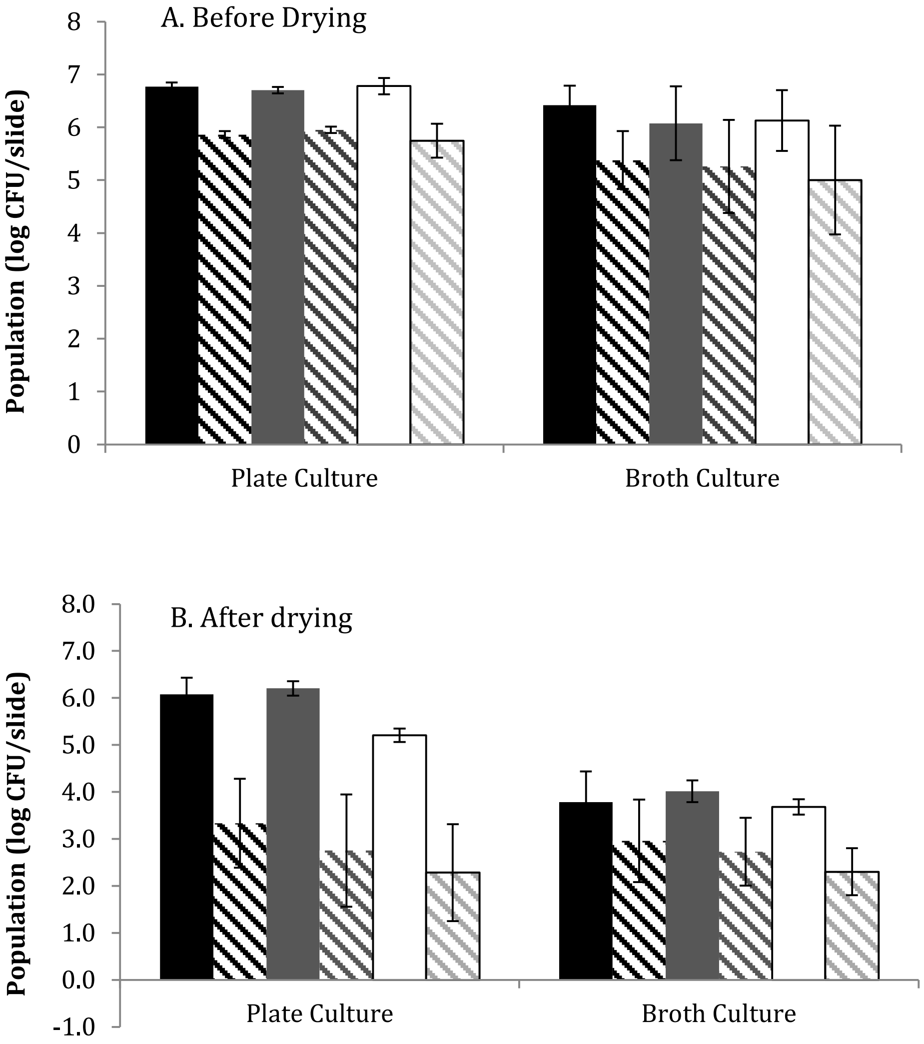


Fig. 6. Population levels of plate and broth grown *Salmonella* SEPT30 (black bars), SEPT30D (grey bars), and rdarNeg (white bar) before (A) and after (B) drying and after a 2 min soak in either D/E Broth control (solid bar) or 5% citric acid (cross hatch bar), n=6.

Influence of growth temperature on desiccation tolerance. *Salmonella* may be exposed to a wide range of temperatures in the environment. Although the optimum temperature for growth of this organism is 37°C, rdar expression is often measured at 28°C. SEPT30, SEPT30D, and rdarNeg were cultured in TSB and on TSA at 23, 28, and 37°C for 48 h (23 and 28°C) or 24 h (37°C). The cells were collected and suspended in sterile MilliQ water. The OD_{600} values were adjusted to 0.60 ± 0.05 before inoculating 10 µl onto glass coverslips. The inoculated glass coverslips were held in a desiccator with relative humidity (RH) adjusted to 72% at room temperature for up to 5 days.

Trends at all three temperatures were the same; at all temperatures significantly better survival was observed for all plate-grown cultures. The greatest separation of survival of broth and plate-grown cultures was observed at 23°C (Appendix Fig. 3). Survival of all three plate-grown strains was similar through day 2 but by day 5, the population density of the rdarNeg strain was significantly lower than that of the SEPT30 or SEPT30D. Survival of SEPT30 or SEPT30D was similar for broth-grown cultures and slightly better than that of the rdarNeg strain.

Outcomes and Accomplishments

1. An rdar-negative derivative of *Salmonella* Enteritidis PT30 was constructed by insertion into the adrA gene (a regulatory gene for cellulose production).
2. The rdar-negative derivative produced significantly lower amounts of cellulose during growth on agar medium.
3. While the rdar-negative derivative was not significantly more desiccation tolerant on glass surfaces, decreased survival was observed on almonds, particularly during longer storage periods.

Summary of Findings and Recommendations

Key findings:

1. *Salmonella* strains that have an rdar-positive morphotype (produce cellulose and aggregative fibmbrae) are more tolerant to desiccation.
2. *Salmonella* strains cultured on agar surfaces are more desiccation tolerant (survive better during drying) than those cultured in broth. This phenenomon was observed for both rdar-positive and rdar-negative strains of *Salmonella*.
3. Loss of the rdar phenotype and possibly reduced cellulose production impacted long-term survival of *Salmonella* on almonds but in model systems, survival for up to 7 days was not significantly impacted.
4. The rdar morphotype alone does not explain the increased desiccation tolerance triggered by growth on agar medium.
5. Growth conditions that enhance desiccation tolerance (rdar morphotype, growth on agar) do not appear to confir enhanced chlorine or acid tolerance.

Recommendations:
Rdar-positive strains should be included for research studies that involve desiccation of *Salmonella*, and strains should be cultured and collected from agar medium.

APPENDICES

Publications and Presentations (required)

Publications:

None

Presentations:

Wang, L., and L.J. Harris. 2011. Rdar Morphotype and its relationship to desiccation tolerance in *Salmonella* spp. IAFP Annual Meeting, Milwaukee, WI, July 31-August 3. (P3-31)

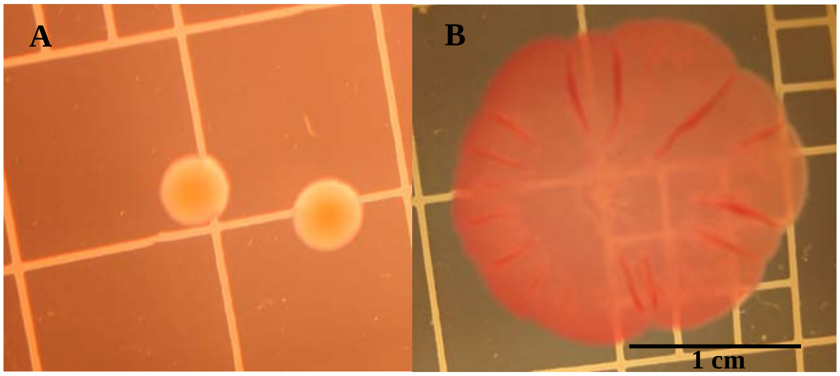
Budget Summary (required)

The funds expended were for salary and benefits for Dr. Luxin Wang (postdoctoral fellow leading the research, construction and evaluation of the mutant), Anuja Ganpule (for the study on long-term survival of the SEPT30 and SEPT30D on almonds), Chris Theofel (for studies pertaining to survival during acid exposure) and Vanessa Morales (for studies pertaining to survival during chlorine exposure and impact of temperature on desiccation tolerance). Several undergraduate students were also paid for their support in media preparation and laboratory analysis. Funds were also expended on microbiological media and supplies as well as for charges for use of the scanning electron microscope.

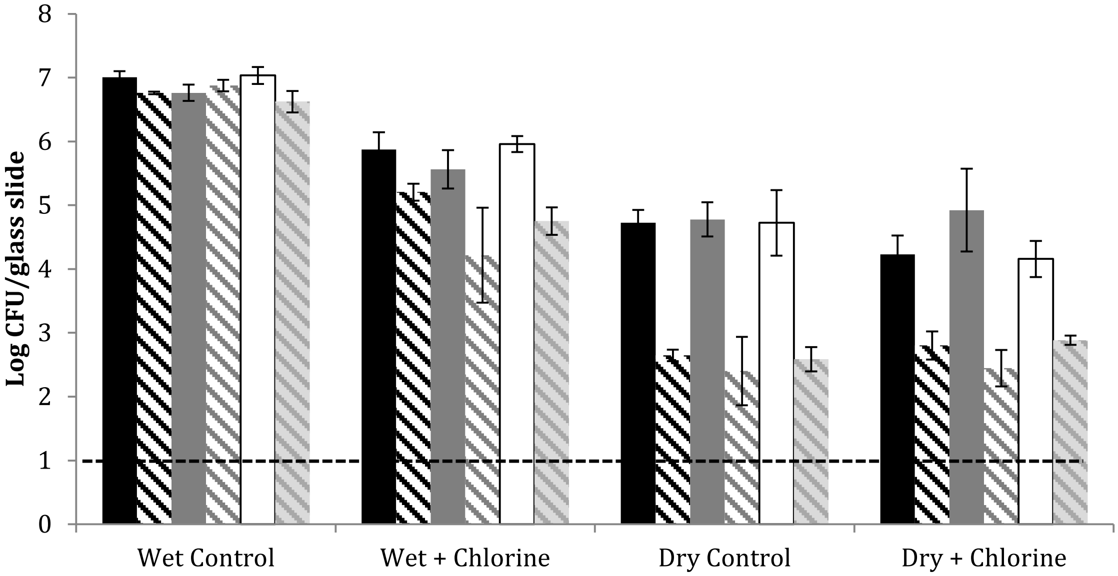
Tables and Figures (optional)

Appendix TABLE 1. Source and rdar morphotype of *Salmonella* strains used in this study

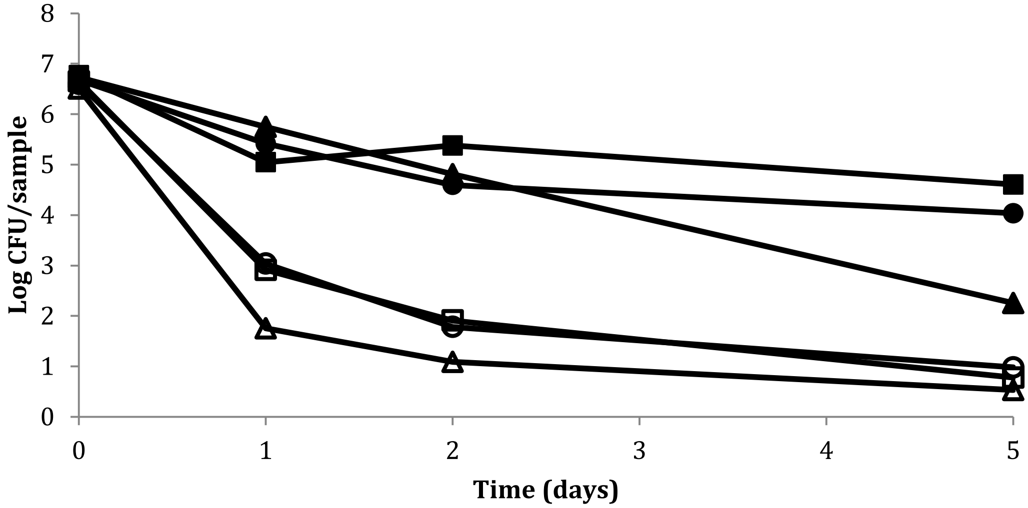
Rdar morphotype	*Salmonella* strain	Source
Positive	Anatum	Almond, survey
	Enteritidis PT 8	Egg outbreak, clinical isolate
	Enteritidis PT 9c	Almond outbreak, clinical isolate
	Enteritidis PT 30	Almond outbreak, almond isolate
	Garminara	Orange juice
	Saintpaul	Jalapeno outbreak, clinical isolate
	Typhimurium DT104	Almond, survey
Negative	Michigan	Cantaloupe outbreak
	Montevideo	Tomato outbreak, clinical isolate
	Montevideo	Pistachio isolate, recall
	Oranienberg	Pecan, survey
	Poona	Cantaloupe, clinical isolate
	Senftenberg	ATCC 43845 (775W)
	Tennessee	Peanut butter outbreak, clinical isolate



Appendix Fig. 1. Colony morphotype of *Salmonella* Enteritidis PT30 **(**PT 30 Δ*adr*A-) (A) and *Salmonella* Enteritidis PT30 wildtype (B) on LBNSA after incubation at 28 °C for 7 days.



Appendix Figure 2. Population levels of plate and broth grown *Salmonella* SEPT30 (black bars), SEPT30D (grey bars), and rdarNeg (white bar) before (wet) and after drying (dry) and after a 2 min soak in either D/E Broth control (solid bar) or 5 ppm chlorine (cross hatch bar), n=6.



Appendix Figure 3. Survival during desiccation and storage at ambient temperature and 72% RH. SEPT30 (squares), SEPT30D (circles) and rdarNeg (triangles) cultured at 23°C on plates (solid symbols) or broth (open symbols).

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Suggestions to CPS (optional)

None