**Involvement of the *Salmonella* Typhimurium Rcs regulon in the persistence within tomatoes.**

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**Abstract**

It is becoming clear that human enteric pathogens, like *Salmonella*, can efficiently colonize vegetative and reproductive organs of plants. Even though this ability of human pathogens to proliferate within plant tissues has been linked to outbreaks of salmonellosis, little remains known about regulatory and physiological adaptations of human pathogens to their persistence in plants. A screen of *Salmonella* large deletion mutants identified *rcsA* and *rcsB* genes as important for its proliferation within tomatoes. Within tomato fruits, populations of *Salmonella rcsB* mutants were up to 2 log10 lower than those of the wild type, competitive fitness of the *rcsA* and *rcsB* mutants was strongly reduced in tomatoes. Bioinformatics predictions identified a putative *Salmonella* RcsAB binding box (TTMGGAWWAABCTYA) and revealed an extensive putative RcsAB regulon, of which at least 70 members were differentially fit within tomatoes.

**INTRODUCTION**

Outbreaks of human salmonellosis linked to the consumption of fresh fruits and vegetables have increased over the past decade {Jackson, 2013 #2425; Gould, 2013 #2426; Painter, 2013 #2427}. Production practices, pre- and post-harvest management, environmental conditions, cultivar selection, broader distribution and an increase in consumption of fresh produce coupled with improved surveillance likely have contributed to the increased incidence (or reporting) of the outbreaks. However, it is also clear that the ability of opportunistic human pathogens (such as non-typhoidal strains of *Salmonella* *enterica*) to persist outside of their animal hosts, to colonize and multiply within plants is an important factor leading to the outbreaks of human salmonellosis from the consumption of fruits and vegetables {Martinez-Vaz, 2014 #2418; Brandl, 2013 #2016}. *Salmonella* can persist in manure-amended soils for over 7 months, and the pathogen was detected on above-ground parts of leafy greens, and root vegetables grown in these amended soils for 2-3 months {Islam, 2004 #1712;Islam, 2004 #1713; Hofmann, 2014 #2428}. These field reports are consistent with the laboratory studies that demonstrated that plant-associated *Salmonella* and pathogenic *E. coli* ingested by vertebrate and invertebrate herbivores were shed by the animals with feces and were capable of re-colonizing plants once excreted into the environment {Semenov, 2010 #1714}.

Under laboratory conditions and in the field, *Salmonella* can colonize plant surfaces {Berger, 2009 #1756; Kroupitski, 2011 #1733; Cevallos-Cevallos, 2012 #2026; Klerks, 2007 #969}. Attachment to plant surfaces and their colonization by *Salmonella* involved aggregative fimbriae, cellulose and O-antigen {Barak, 2007 #135; Barak, 2005 #136; Zaragoza, 2012 #1661; Brankatschk, 2013 #2333}. *Salmonella* regulators of biofilm formation and stress survival (*ycfR, sirA, yigG, rpoS*) also contributed to the ability of this pathogen to colonize plant surfaces {Salazar, 2013 #2420; Barak, 2005 #136}. Once on plant surfaces, *Salmonella* can enter the plants through natural openings such as hydathodes, stomata, wounds at the sites of emergence of secondary roots or lesions caused by phytopathogens {Brandl, 2002 #231;Cooley, 2003 #299;Guo, 2001 #1719; Kroupitski, 2011 #1733; Kroupitski, 2009 #1735; Gu, 2013 #2123}. The ability of human pathogens, like non-typhoidal *Salmonella* and pathogenic *E. coli* to spread endophytically or via the vascular system has been demonstrated under laboratory or greenhouse conditions, but not in the field {Gu, 2011 #2029; Golberg, 2011 #1734; Lopez-Velasco, 2012 #2091}. The ability of *Salmonella* to colonize internal plant tissues appears to be plant species- and bacterial strain-dependent {Golberg, 2011 #1734; Dong, 2003 #358; Kroupitski, 2009 #1735}, although metabolic and regulatory changes associated with proliferation of human pathogens within plant tissues are not yet fully understood.

Several high throughput studies focused on defining patterns of *Salmonella* gene expression during its multiplication within plant tissues. Within leaves, *Salmonella* down-regulates glycolysis and upregulates genes involved in ascorbate metabolism, amino acid, lipid and nucleotide synthesis {Zhang, 2014 #2423}. Expression of pathogen-associated molecular patterns (PAMPs) (flagellin, elongation factor Tu) was downregulated {Zhang, 2014 #2423}, however, plants still appeared capable of detecting a major PAMP, flagellin’s flg22 {Meng, 2013 #2060}. Inside fruits, *Salmonella* upregulates genes involved in amino acid synthesis, sugar and dipeptide transport and cell envelope synthesis {Noel, 2010 #2001}. These high throughput studies, however, did not identify major regulators with potential functions in persistence within plants, likely because gene expression of regulatory genes does not typically change dramatically, however, even subtle changes in their gene expression are consequential and result in a phenotype. Therefore, with this study, we screened a library of *Salmonella* deletion mutants for those with defects in persistence within tomatoes aiming to identify regulators of behaviors involved in persistence within tomatoes. The rationale for this approach was that the identification of regulators will facilitate identification of the members of the regulon controlled by a regulatory protein, and subsequent studies can focus on identifying cues perceived by a regulatory system of interest and leading to the changes in gene expression.

**RESULTS AND DISCUSSION**

***Salmonella* *rcsA* and *rcsB* genes are involved in persistence within tomatoes.** Because individually testing all *Salmonella* mutants for their fitness phenotype within tomatoes is not technically feasible, we screened a library containing large (4-20kB) deletions. The screen revealed that strains carrying deletions of *rcsA* and *rcsB* genes were significantly reduced in their ability to multiply within red ripe tomatoes. In *Salmonella* and *E. coli,* RcsB is a response regulator, which upon phosphorylation by RcsD, induces genes involved in capsular polysaccharide synthesis, resistance to antimicrobial peptides and systemic colonization of mice {Erickson, 2006 #2431; Gottesman, 1985 #2430}. RcsB represses flagellar motility and virulence genes on the *Salmonella* Pathogenicity Island II {Wang, 2007 #2429; Gottesman, 1985 #2430}. *Salmonella* RcsA is an auxiliary protein, which sometimes acts in concert with RcsB, to regulate a distinct subset of the RcsB regulon {Erickson, 2006 #2431; Mouslim, 2003 #2432}. To better characterize contribution of RcsA and RcsB to persistence within tomatoes, *rcsA* and *rcsB* genes were deleted and the abilities of the corresponding mutants to multiply in red and green tomatoes were tested (Strains and primers used to construct them are listed in Tables S1 and S2). As shown in Fig. 1A, in green tomatoes, deletion of *rcsA* did not strongly reduce multiplication of the mutant, however, deletion of *rcsB* had a more severe phenotype, reducing its growth by ~100 fold. This phenotype was restored by the wild type copy of *rcsB* driven by a semi-synthetic promoter. The phenotype of the double *rcsA rcsB* mutant was similar to that of the *rcsB* mutant, consistent with the auxiliary function of RcsA in the RcsB-mediated gene expression (REF). In red ripe tomatoes, proliferation of both *rcsA* and *rcsB* mutants was only modestly reduced (Fig. 1B).

To follow up on this observation and to quantify expression of the *rcsA*, *rcsB* genes within tomatoes, RIVET (recombinase-based *in vivo* expression technology) reporters were constructed and their activation was documented. In soft LB (0.3% agar), *rcsA::tnpR* reporter was not expressed (0% resolution), and the *rcsB::tnpR* reporter was partially activated (48 ± 0.03% resolution). In immature tomatoes, *rcsA::tnpR* was expressed fully 24 hrs after the infection (96 ± 0.08%), and remained fully resolved for the duration of the experiment (Fig. 2). Interestingly, *rcsA* was also expressed during colonization of alfalfa seedlings {Brankatschk, 2013 #2333}, suggesting that while it is not strongly expressed during growth in a rich laboratory medium, this regulator may have an important function during the adaptation to the plant-associated lifestyle of *Salmonella.* The *rcsB::tnpR* reporter was partially expressed on day 0 (48 ± 0.03%), was fully activated 24 hrs after the infection (97 ± 0.07%), and remained fully resolved throughout the duration of the experiment (Fig. S1).

**Deletion of *rcsA, rcsB* reduces fitness in tomatoes.** To better characterize the contribution of *rcsA* and *rcsB* to interactions with tomatoes, competitive fitness experiments were carried out. If the decreased ability to multiply in tomatoes is related to the Rcs-dependent synthesis or release of an extracellular factor, co-infections with the wild type will restore fitness of the mutants. As shown in Fig. 2A, fitness of *rcsA* and *rcsB* mutants was reduced by approximately 5 and 100 fold (respectively) in green tomatoes. Complementation of the mutations with plasmid-borne copies of *rcsA* and *rcsB* expressed from heterologous promoters at least partially restored fitness of the mutants. In red tomatoes, phenotype of the *rcsA* mutant was similar to that of the wild type, although providing additional copies of *rcsA* on a plasmid significantly increased fitness of the mutant. Fitness of the *rcsB* mutant in red tomatoes was reduced by 50-100 fold, similar to its phenotype in green tomatoes. The phenotype of the double *rcsA rcsB* mutant was similar to that of the *rcsB* mutant (Fig. 2B). These results suggest that the phenotype of the *rcsB,* and *rcsA* mutants are not due to the production of an extracellular factor or a surface-associated molecule that could be recognized by plant. Therefore, subsequent experiments focused on delineating contributions of the RcsBA regulon to fitness within tomatoes.

**Known and previously uncharacterized members of the Rcs regulon contribute to persistence within tomatoes.** RcsBA control extensive regulons in *Salmonella* and in other enterobacteriaceae, and these regulons are reasonably well characterized. Microarray analyses of *rcsB-* and/or *rcsB rcsA-*dependent changes in genes expression in *S. enterica, E. coli, Erwinia amylovora* identified dozens of genes that are directly or indirectly controlled by these regulators (Ref). To determine which members of the Rcs regulon contribute to persistence within tomatoes, we chose a two-pronged approach: we first defined a set of likely direct RcsBA targets, we then queried results of a recently completed high-throughput screen of transposon-tagged *Salmonella* mutants (de Moraes *et al*., in review) to determine which of the RcsBA-regulated genes contribute to the reduced fitness of the mutants.

To identify potential targets of RcsAB, PSSM (Position Specific Scoring Matrix, {Thomas-Chollier, 2011 #2435}) was constructed with Regulon DB criteria as described in {Salgado, 2013 #2433} using sixteen experimentally validated *rcsAB* binding sites {Wehland, 2000 #2434}. The program “Retrieve-Sequence” from the RSA–tools (Thomas-Chollier et al., 2011) was used to obtain the upstream regions (-400, +100 in relation to the start codon) of all the coding sequences (CDS) of 25 RefSeq *Salmonella* genomes and their plasmids (Table S3). Then, the upstream regions of each *Salmonella* coding sequences were scanned with the constructed PSSM using the program “Matrix-Scan” with an upper threshold value of 1e-5 and using a Markov model order of four constructed for each genome with the software ”Convert-background” {Turatsinze, 2008 #2436}. This led to the identification of 742 putative *rcsAB* boxes in *S.* Typhimurium genome. In order to reduce bias generated by utilizing the original inter-species *rcsAB* PSSM matrix, we decided to construct a second PSSM using *rcsAB* sites present in the orthologous sequences of the 16 experimentally validated *rcsAB* binding sites {Salgado, 2013 #2433; Wehland, 2000 #2434}. To that end, 73 orthologous sequences were retrieved from Biocyc database {Caspi, 2010 #2437} and were used to construct the final fitted *Salmonella* consensus PSSM matrix (Fig. 4). Based on the “Matrix-Scan” analysis with the revised PSSM (Fig. 4), 228 *S. enterica* sv. Typhimurium genes can be under RcsAB control (Table S4).

Even though RcsB was initially characterized as a regulator of capsular polysaccharide in *E. coli* {Gottesman, 1985 #2430}, our bioinformatics analysis revealed a diversity of functions likely to be under RcsAB control. For example, genes predicted to be under RcsAB control include those involved in septation regulation (*serC*), purine metabolism (STM1097), solute-binding proteins (STM1128, STM1633, STM1635), biofilm/motility switch *ybaJ* (Barrios 2006) and at least two putative transcriptional regulators *yhjB* and *yjjQ* (Table S4). Of the genes in Table S4, five (*pagO, spvR,* STM0346, STM2797 and STM2800) were previously shown to be under RcsAB control in *Salmonella* cultures {Mariscotti, 2009 #2438}. Orthologs of *ompX, osmB* and *rfbB* were subject to RcsB and/or RcsAB control in *E. amylovora* in LB shake cultures or during the infections of pears {Wang, 2012 #2440}. In our high throughput identification and sequencing of the *Salmonella* mutants defective in tomatoes (de Moraes *et al.* in review), at least 70 of these genes were more or less fit inside red tomatoes (Table S4).

To test the bioinformatics prediction, regulation of a subset of the genes encoded downstream from putative RcsAB binding sites was tested by qRT-PCR. As shown in Table S5, the expression of *yjbE, yhhA* and *ygdI* was strongly reduced in the *rcsAB* mutant, whether it was grown in LB or in tomatoes. Mutants in *yjbE* and *yhhA* had reduced fitness in red tomatoes, while the phenotype of the *ygdI*  mutant was less pronounced (Table S4, de Moraes *et al.*, in review).

In trying to understand mechanisms *Salmonella* fitness within plants, there is a lack of scientific consensus on whether this pathogen repurposes its impressive animal virulence arsenal to colonize plants, or whether it behaves more as a typical phytobacterium during plant colonization. RcsAB have well-characterized roles in controlling regulatory pathways leading to virulence in a murine model of infection (Erickson & Detweiler 2006; García-Calderón et al., 2007). Plant pathogens from the *Enterobacteriaceae* family (*E. amylovora* Ancona et al., 2015, Wang et al., 2012, Wang et al., 2009; and *D. dadantii,* Wu et al., 2014) also rely on the Rcs-mediated regulatory cascades for controlling virulence in plants. The *Salmonella* Rcs cascade is activated in response to the membrane disorder caused by animal cationic antimicrobial peptides (Farris et al., 2010). Given the sensitivity of the *E. amylovora rcs* mutants to polymyxin (Wang et al., 2009), a similar mechanism is likely at play in phytopathogens. It is intriguing to speculate that the Rcs-mediated signal perception, transduction and regulation represents one of the mechanisms of virulence that is used universally by animal and phytopathogens, even when they colonize alternate hosts.

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**Figure Legends.**

**Fig. 1. *Salmonella* mutants proliferation in post-harvested unripe and ripe tomatoes.**

Proliferation of the *rcsA, rcsB Salmonella* mutants (Table S1) was tested in red (**A**) and green (**B**) tomatoes cv. Ailsa Craig, which were grown in the rooftop greenhouse. To track developmental stages of the fruit, they were tagged 7 days post anthesis (d.p.a.) as before {Alba, 2005 #1691}). For the inoculations, *S. enterica* sv Typhimurium ATCC14028 or mutants were grown overnight at 37°C in LB with 200 rpm shake cultures. They were then washed twice in Phosphate-Buffered Saline (PBS) (Fisher Scientific, Hampton, NH) and 3 µl of the suspension (containing between 100 and 1,000 CFU) were spotted onto shallow (~ 1 mm) wounds in the fruit epidermis. There were three wounds in each fruit. For each inoculation, the dose was calculated based on the results of dilution plating. Infected fruits were incubated at room temperature for a week. Upon completion of the incubation, tomatoes were macerated in an equal volume of PBS using a stomacher (Sevard, West Sussex, UK) (260 rpm for 1 minute) and the suspension was plated onto a xylose-lysine deoxycholate (XLD) agar (BD, East Rutherford, NJ) agar and incubated at 37°C over night. Proliferation was calculated by dividing the CFU/ml harvested by the CFU/ml inoculated. The ratios were further subjected to the log10 transformation. ANOVA and Tukey means separation were inferred in order to determine significant differences of the means (JMP software, SAS). (\*) Complemented *Salmonella* Δ*rcsA3*::kan harboring pWSK29-*rcsA*. Tukey means separation was inferred to determine significant differences. Lower case letters indicate groupings (p=0.05) representing significant different means. In box plots, boxes include the lower and upper quartiles, lines within the box are the medians and whiskers indicate the degree of dispersion of the data.

**Fig. 2. Competitive fitness of *Salmonella* mutants in unripe and ripe tomatoes.** Competitive Index of *S. enterica* Typhimurium mutants during post-harvest proliferation from mature (panel A) and immature (panel B) tomato of cv. Ailsa Craig. (\*) Complemented *Salmonella* Δ*rcsA3*::kan harboing pWSK29-*rcsA*. To calculate competitive index, wild type *S. enterica* sv. Typhimurium 14028 and isogenic mutants were seeded at 104 cfu/infection, roughly at a 1:1 ratio into tomatoes. In parallel, *S. enterica* sv. Typhimurium 14028 and its isogenic kanamycin-resistant strain CEC1000 were similarly inoculated into tomato fruits, three wounds per fruit. All samples were incubated for a week at 22°C in vented chambers. To harvest samples, 15 mm x 0.5 mm cores were removed from fruits, homogenized in PBS and plated onto XLD. The relative ratios of the strains in the inocula and in the recovered samples were calculated by dilution plating and patching on antibiotic-containing media. Competitive indices were calculated for each treatment using the formula (Mout/WTout)/(Min/WTin), where M is the proportion of mutant cells and WT is the proportion of the wild type cells in the inocula (in) or in the recovered samples (out). Log-transformed values of competitive index are presented. The statistical and biological significance of each competitive index was established by comparing log values of the competitive indices of each pair to the log of competitive index similarly calculated for ATCC14028 vs CEC1000, using the ANOVA test (p <0.05) and Tukey means separation analysis (JMP software, SAS). At least three technical (individual infections on the same tomato) and three biological (different tomatoes) replications were carried out for each experiment. In box plots, boxes include the lower and upper quartiles, lines within the box are the medians and whiskers indicate the degree of dispersion of the data. Lower case letters indicate groupings (p=0.05) representing significant different means.

**Fig. 3. *rcsAB* box logo.** Sequence logo for *rcsAB* box responsive genes (located at -400+100 in relation to the start codon). The bit score, or overall height, represents sequence conservation at a given position, while the height of each residue within each stack represents the frequency of that residue.

**Fig. S1. Resolution of the *rcsA* and *rcsB* RIVET reporters in unripe tomato cv. Ailsa Craig.** Resolution of the *rcsA* and *rcsB* RIVET reporters were tested in unripe (34 d.p.a.) fruit of cv. Ailsa Craig. Three biological replications and three technical replicas were carried out, and averages of all experiments are shown; error bars are standard errors. As a control (0 days), the reporters were tested in soft LB agar (0.3% agar). For the RIVET assays in tomatoes, *Salmonella* cultures were grown at 37°C overnight in LB supplemented with tetracycline. Bacterial cultures were then pelleted, washed three times in an equal volume of sterile PBS. Approximately 104 cfu (in 3 µl of PBS) were inoculated onto superficial 1 mm-deep wounds on surfaces of unwaxed fruits. Infected tomatoes were incubated at 22°C in vented chambers. All RIVET assays were incubated for three days. To harvest samples, 15 mm x 0.5 mm cores were removed from fruits, homogenized in PBS and plated onto XLD agar with appropriate antibiotics. Individual colonies were then patched onto LB agar with tetracycline to detect constructs in which TnpR recombinase was active.