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 $\log_{10}$ 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

34 salmonellosis from the consumption of fruits and vegetables {Martinez-Vaz, 2014 #2418; Brandl,
35 2013 #2016}. *Salmonella* can persist in manure-amended soils for over 7 months, and the
36 pathogen was detected on above-ground parts of leafy greens, and root vegetables grown in
37 these amended soils for 2-3 months {Islam, 2004 #1712;Islam, 2004 #1713; Hofmann, 2014
38 #2428}. These field reports are consistent with the laboratory studies that demonstrated that
39 plant-associated *Salmonella* and pathogenic *E. coli* ingested by vertebrate and invertebrate
40 herbivores were shed by the animals with feces and were capable of re-colonizing plants once
41 excreted into the environment {Semenov, 2010 #1714}.
42
43 Under laboratory conditions and in the field, *Salmonella* can colonize plant surfaces {Berger,
44 2009 #1756; Kroupitski, 2011 #1733; Cevallos-Cevallos, 2012 #2026; Klerks, 2007 #969}.
45 Attachment to plant surfaces and their colonization by *Salmonella* involved aggregative fimbriae,
46 cellulose and O-antigen {Barak, 2007 #135; Barak, 2005 #136; Zaragoza, 2012 #1661;
47 Brankatschk, 2013 #2333}. *Salmonella* regulators of biofilm formation and stress survival (*ycfR,*
48 *sirA, yigG, rpoS*) also contributed to the ability of this pathogen to colonize plant surfaces
49 {Salazar, 2013 #2420; Barak, 2005 #136}. Once on plant surfaces, *Salmonella* can enter the
50 plants through natural openings such as hydathodes, stomata, wounds at the sites of
51 emergence of secondary roots or lesions caused by phytopathogens {Brandl, 2002 #231;Cooley,
52 2003 #299;Guo, 2001 #1719; Kroupitski, 2011 #1733; Kroupitski, 2009 #1735; Gu, 2013 #2123}.
53 The ability of human pathogens, like non-typhoidal *Salmonella* and pathogenic *E. coli* to spread
54 endophytically or via the vascular system has been demonstrated under laboratory or
55 greenhouse conditions, but not in the field {Gu, 2011 #2029; Golberg, 2011 #1734; Lopez-
56 Velasco, 2012 #2091}. The ability of *Salmonella* to colonize internal plant tissues appears to be
57 plant species- and bacterial strain-dependent {Golberg, 2011 #1734; Dong, 2003 #358;
58 Kroupitski, 2009 #1735}, although metabolic and regulatory changes associated with
59 proliferation of human pathogens within plant tissues are not yet fully understood.
60
61 Several high throughput studies focused on defining patterns of *Salmonella* gene expression
62 during its multiplication within plant tissues. Within leaves, *Salmonella* down-regulates
63 glycolysis and upregulates genes involved in ascorbate metabolism, amino acid, lipid and
64 nucleotide synthesis {Zhang, 2014 #2423}. Expression of pathogen-associated molecular
65 patterns (PAMPs) (flagellin, elongation factor Tu) was downregulated {Zhang, 2014 #2423},
66 however, plants still appeared capable of detecting a major PAMP, flagellin's flg22 {Meng, 2013
67 #2060}. Inside fruits, *Salmonella* upregulates genes involved in amino acid synthesis, sugar

68 and dipeptide transport and cell envelope synthesis {Noel, 2010 #2001}. These high throughput
69 studies, however, did not identify major regulators with potential functions in persistence within
70 plants, likely because gene expression of regulatory genes does not typically change
71 dramatically, however, even subtle changes in their gene expression are consequential and
72 result in a phenotype. Therefore, with this study, we screened a library of *Salmonella* deletion
73 mutants for those with defects in persistence within tomatoes aiming to identify regulators of
74 behaviors involved in persistence within tomatoes. The rationale for this approach was that the
75 identification of regulators will facilitate identification of the members of the regulon controlled by
76 a regulatory protein, and subsequent studies can focus on identifying cues perceived by a
77 regulatory system of interest and leading to the changes in gene expression.
78
79 **RESULTS AND DISCUSSION**

80 ***Salmonella rcsA* and *rcsB* genes are involved in persistence within tomatoes.** Because
81 individually testing all *Salmonella* mutants for their fitness phenotype within tomatoes is not
82 technically feasible, we screened a library containing large (4-20kB) deletions. The screen
83 revealed that strains carrying deletions of *rcsA* and *rcsB* genes were significantly reduced in
84 their ability to multiply within red ripe tomatoes. In *Salmonella* and *E. coli,* RcsB is a response
85 regulator, which upon phosphorylation by RcsD, induces genes involved in capsular
86 polysaccharide synthesis, resistance to antimicrobial peptides and systemic colonization of mice
87 {Erickson, 2006 #2431; Gottesman, 1985 #2430}. RcsB represses flagellar motility and
88 virulence genes on the *Salmonella* Pathogenicity Island II {Wang, 2007 #2429; Gottesman,
89 1985 #2430}. *Salmonella* RcsA is an auxiliary protein, which sometimes acts in concert with
90 RcsB, to regulate a distinct subset of the RcsB regulon {Erickson, 2006 #2431; Mouslim, 2003
91 #2432}. To better characterize contribution of RcsA and RcsB to persistence within tomatoes,
92 *rcsA* and *rcsB* genes were deleted and the abilities of the corresponding mutants to multiply in
93 red and green tomatoes were tested (Strains and primers used to construct them are listed in
94 Tables S1 and S2). As shown in Fig. 1A, in green tomatoes, deletion of *rcsA* did not strongly
95 reduce multiplication of the mutant, however, deletion of *rcsB* had a more severe phenotype,
96 reducing its growth by ~100 fold. This phenotype was restored by the wild type copy of *rcsB*
97 driven by a semi-synthetic promoter. The phenotype of the double *rcsA rcsB* mutant was similar
98 to that of the *rcsB* mutant, consistent with the auxiliary function of RcsA in the RcsB-mediated
99 gene expression (REF). In red ripe tomatoes, proliferation of both *rcsA* and *rcsB* mutants was
100 only modestly reduced (Fig. 1B).
101

102 To follow up on this observation and to quantify expression of the *rcsA*, *rcsB* genes within
103 tomatoes, RIVET (recombinase-based *in vivo* expression technology) reporters were
104 constructed and their activation was documented. In soft LB (0.3% agar), *rcsA::tnpR* reporter
105 was not expressed (0% resolution), and the *rcsB::tnpR* reporter was partially activated (48 ±
106 0.03% resolution). In immature tomatoes, *rcsA::tnpR* was expressed fully 24 hrs after the
107 infection (96 ± 0.08%), and remained fully resolved for the duration of the experiment (Fig. 2).
108 Interestingly, *rcsA* was also expressed during colonization of alfalfa seedlings {Brankatschk,
109 2013 #2333}, suggesting that while it is not strongly expressed during growth in a rich laboratory
110 medium, this regulator may have an important function during the adaptation to the plant-
111 associated lifestyle of *Salmonella.* The *rcsB::tnpR* reporter was partially expressed on day 0 (48
112 ± 0.03%), was fully activated 24 hrs after the infection (97 ± 0.07%), and remained fully
113 resolved throughout the duration of the experiment (Fig. S1).
114
115 **Deletion of *rcsA, rcsB* reduces fitness in tomatoes.** To better characterize the contribution
116 of *rcsA* and *rcsB* to interactions with tomatoes, competitive fitness experiments were carried out.
117 If the decreased ability to multiply in tomatoes is related to the Rcs-dependent synthesis or
118 release of an extracellular factor, co-infections with the wild type will restore fitness of the
119 mutants. As shown in Fig. 2A, fitness of *rcsA* and *rcsB* mutants was reduced by approximately 5
120 and 100 fold (respectively) in green tomatoes. Complementation of the mutations with plasmid-
121 borne copies of *rcsA* and *rcsB* expressed from heterologous promoters at least partially restored
122 fitness of the mutants. In red tomatoes, phenotype of the *rcsA* mutant was similar to that of the
123 wild type, although providing additional copies of *rcsA* on a plasmid significantly increased
124 fitness of the mutant. Fitness of the *rcsB* mutant in red tomatoes was reduced by 50-100 fold,
125 similar to its phenotype in green tomatoes. The phenotype of the double *rcsA rcsB* mutant was
126 similar to that of the *rcsB* mutant (Fig. 2B). These results suggest that the phenotype of the
127 *rcsB,* and *rcsA* mutants are not due to the production of an extracellular factor or a surface-
128 associated molecule that could be recognized by plant. Therefore, subsequent experiments
129 focused on delineating contributions of the RcsBA regulon to fitness within tomatoes.
130
131 **Known and previously uncharacterized members of the Rcs regulon contribute to**
132 **persistence within tomatoes.** RcsBA control extensive regulons in *Salmonella* and in other
133 enterobacteriaceae, and these regulons are reasonably well characterized. Microarray
134 analyses of *rcsB*- and/or *rcsB rcsA*-dependent changes in genes expression in *S. enterica, E.*
135 *coli, Erwinia amylovora* identified dozens of genes that are directly or indirectly controlled by

136 these regulators (Ref). To determine which members of the Rcs regulon contribute to
137 persistence within tomatoes, we chose a two-pronged approach: we first defined a set of likely
138 direct RcsBA targets, we then queried results of a recently completed high-throughput screen of
139 transposon-tagged *Salmonella* mutants (de Moraes *et al*., in review) to determine which of the
140 RcsBA-regulated genes contribute to the reduced fitness of the mutants.
141
142 To identify potential targets of RcsAB, PSSM (Position Specific Scoring Matrix, {Thomas-
143 Chollier, 2011 #2435}) was constructed with Regulon DB criteria as described in {Salgado, 2013
144 #2433} using sixteen experimentally validated *rcsAB* binding sites {Wehland, 2000 #2434}. The
145 program "Retrieve-Sequence" from the RSA–tools (Thomas-Chollier et al., 2011) was used to
146 obtain the upstream regions (-400, +100 in relation to the start codon) of all the coding
147 sequences (CDS) of 25 RefSeq *Salmonella* genomes and their plasmids (Table S3). Then, the
148 upstream regions of each *Salmonella* coding sequences were scanned with the constructed
149 PSSM using the program "Matrix-Scan" with an upper threshold value of $1e^{-5}$ and using a
150 Markov model order of four constructed for each genome with the software "Convert-
151 background" {Turatsinze, 2008 #2436}. This led to the identification of 742 putative *rcsAB*
152 boxes in *S.* Typhimurium genome. In order to reduce bias generated by utilizing the original
153 inter-species *rcsAB* PSSM matrix, we decided to construct a second PSSM using *rcsAB* sites
154 present in the orthologous sequences of the 16 experimentally validated *rcsAB* binding sites
155 {Salgado, 2013 #2433; Wehland, 2000 #2434}. To that end, 73 orthologous sequences were
156 retrieved from Biocyc database {Caspi, 2010 #2437} and were used to construct the final fitted
157 *Salmonella* consensus PSSM matrix (Fig. 4). Based on the "Matrix-Scan" analysis with the
158 revised PSSM (Fig. 4), 228 *S. enterica* sv. Typhimurium genes can be under RcsAB control
159 (Table S4).
160
161 Even though RcsB was initially characterized as a regulator of capsular polysaccharide in *E. coli*
162 {Gottesman, 1985 #2430}, our bioinformatics analysis revealed a diversity of functions likely to
163 be under RcsAB control. For example, genes predicted to be under RcsAB control include
164 those involved in septation regulation (*serC*), purine metabolism (STM1097), solute-binding
165 proteins (STM1128, STM1633, STM1635), biofilm/motility switch *ybaJ* (Barrios 2006) and at
166 least two putative transcriptional regulators *yhjB* and *yjjQ* (Table S4). Of the genes in Table S4,
167 five (*pagO, spvR,* STM0346, STM2797 and STM2800) were previously shown to be under
168 RcsAB control in *Salmonella* cultures {Mariscotti, 2009 #2438}. Orthologs of *ompX, osmB* and
169 *rfbB* were subject to RcsB and/or RcsAB control in *E. amylovora* in LB shake cultures or during

170 the infections of pears {Wang, 2012 #2440}. In our high throughput identification and
171 sequencing of the *Salmonella* mutants defective in tomatoes (de Moraes *et al.* in review), at
172 least 70 of these genes were more or less fit inside red tomatoes (Table S4).
173 To test the bioinformatics prediction, regulation of a subset of the genes encoded downstream
174 from putative RcsAB binding sites was tested by qRT-PCR. As shown in Table S5, the
175 expression of *yjbE, yhhA* and *ygdI* was strongly reduced in the *rcsAB* mutant, whether it was
176 grown in LB or in tomatoes. Mutants in *yjbE* and *yhhA* had reduced fitness in red tomatoes,
177 while the phenotype of the *ygdI* mutant was less pronounced (Table S4, de Moraes *et al.*, in
178 review).
179
180 In trying to understand mechanisms *Salmonella* fitness within plants, there is a lack of scientific
181 consensus on whether this pathogen repurposes its impressive animal virulence arsenal to
182 colonize plants, or whether it behaves more as a typical phytobacterium during plant
183 colonization. RcsAB have well-characterized roles in controlling regulatory pathways leading to
184 virulence in a murine model of infection (Erickson & Detweiler 2006; García-Calderón et al.,
185 2007). Plant pathogens from the *Enterobacteriaceae* family (*E. amylovora* Ancona et al., 2015,
186 Wang et al., 2012, Wang et al., 2009; and *D. dadantii,* Wu et al., 2014) also rely on the Rcs-
187 mediated regulatory cascades for controlling virulence in plants. The *Salmonella* Rcs cascade is
188 activated in response to the membrane disorder caused by animal cationic antimicrobial
189 peptides (Farris et al., 2010). Given the sensitivity of the *E. amylovora rcs* mutants to polymyxin
190 (Wang et al., 2009), a similar mechanism is likely at play in phytopathogens. It is intriguing to
191 speculate that the Rcs-mediated signal perception, transduction and regulation represents one
192 of the mechanisms of virulence that is used universally by animal and phytopathogens, even
193 when they colonize alternate hosts.
194
195
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199
200 **Figure Legends.**
201 **Fig. 1. *Salmonella* mutants proliferation in post-harvested unripe and ripe tomatoes.**
202 Proliferation of the *rcsA, rcsB Salmonella* mutants (Table S1) was tested in red (**A**) and green
203 (**B**) tomatoes cv. Ailsa Craig, which were grown in the rooftop greenhouse. To track

204 developmental stages of the fruit, they were tagged 7 days post anthesis (d.p.a.) as before
205 {Alba, 2005 #1691}). For the inoculations, *S. enterica* sv Typhimurium ATCC14028 or mutants
206 were grown overnight at 37°C in LB with 200 rpm shake cultures. They were then washed twice
207 in Phosphate-Buffered Saline (PBS) (Fisher Scientific, Hampton, NH) and 3 μl of the suspension
208 (containing between 100 and 1,000 CFU) were spotted onto shallow (~ 1 mm) wounds in the
209 fruit epidermis. There were three wounds in each fruit. For each inoculation, the dose was
210 calculated based on the results of dilution plating. Infected fruits were incubated at room
211 temperature for a week. Upon completion of the incubation, tomatoes were macerated in an
212 equal volume of PBS using a stomacher (Sevard, West Sussex, UK) (260 rpm for 1 minute) and
213 the suspension was plated onto a xylose-lysine deoxycholate (XLD) agar (BD, East Rutherford,
214 NJ) agar and incubated at 37°C over night. Proliferation was calculated by dividing the CFU/ml
215 harvested by the CFU/ml inoculated. The ratios were further subjected to the $\log_{10}$
216 transformation. ANOVA and Tukey means separation were inferred in order to determine
217 significant differences of the means (JMP software, SAS). (*) Complemented *Salmonella*
218 Δ*rcsA3*::kan harboring pWSK29-*rcsA*. Tukey means separation was inferred to determine
219 significant differences. Lower case letters indicate groupings (p=0.05) representing significant
220 different means. In box plots, boxes include the lower and upper quartiles, lines within the box
221 are the medians and whiskers indicate the degree of dispersion of the data.
222
223
224
225 **Fig. 2. Competitive fitness of *Salmonella* mutants in unripe and ripe tomatoes.**
226 Competitive Index of *S. enterica* Typhimurium mutants during post-harvest proliferation from
227 mature (panel A) and immature (panel B) tomato of cv. Ailsa Craig. (*) Complemented
228 *Salmonella* Δ*rcsA3*::kan harboing pWSK29-*rcsA*. To calculate competitive index, wild type *S.*
229 *enterica* sv. Typhimurium 14028 and isogenic mutants were seeded at 10^4 cfu/infection, roughly
230 at a 1:1 ratio into tomatoes. In parallel, *S. enterica* sv. Typhimurium 14028 and its isogenic
231 kanamycin-resistant strain CEC1000 were similarly inoculated into tomato fruits, three wounds
232 per fruit. All samples were incubated for a week at 22°C in vented chambers. To harvest
233 samples, 15 mm x 0.5 mm cores were removed from fruits, homogenized in PBS and plated
234 onto XLD. The relative ratios of the strains in the inocula and in the recovered samples were
235 calculated by dilution plating and patching on antibiotic-containing media. Competitive indices
236 were calculated for each treatment using the formula $(M_{out}/WT_{out})/(M_{in}/WT_{in})$, where M is the
237 proportion of mutant cells and WT is the proportion of the wild type cells in the inocula ($_{in}$) or in

238 the recovered samples ($_{out}$). Log-transformed values of competitive index are presented. The
239 statistical and biological significance of each competitive index was established by comparing
240 log values of the competitive indices of each pair to the log of competitive index similarly
241 calculated for ATCC14028 vs CEC1000, using the ANOVA test ($p < 0.05$) and Tukey means
242 separation analysis (JMP software, SAS). At least three technical (individual infections on the
243 same tomato) and three biological (different tomatoes) replications were carried out for each
244 experiment. In box plots, boxes include the lower and upper quartiles, lines within the box are
245 the medians and whiskers indicate the degree of dispersion of the data. Lower case letters
246 indicate groupings ($p=0.05$) representing significant different means.
247
248 **Fig. 3. *rcsAB* box logo.** Sequence logo for *rcsAB* box responsive genes (located at -400+100
249 in relation to the start codon). The bit score, or overall height, represents sequence conservation
250 at a given position, while the height of each residue within each stack represents the frequency
251 of that residue.
252
253 **Fig. S1. Resolution of the *rcsA* and *rcsB* RIVET reporters in unripe tomato cv. Ailsa Craig.**
254 Resolution of the *rcsA* and *rcsB* RIVET reporters were tested in unripe (34 d.p.a.) fruit of cv.
255 Ailsa Craig. Three biological replications and three technical replicas were carried out, and
256 averages of all experiments are shown; error bars are standard errors. As a control (0 days), the
257 reporters were tested in soft LB agar (0.3% agar). For the RIVET assays in tomatoes,
258 *Salmonella* cultures were grown at 37°C overnight in LB supplemented with tetracycline.
259 Bacterial cultures were then pelleted, washed three times in an equal volume of sterile PBS.
260 Approximately 10^4 cfu (in 3 µl of PBS) were inoculated onto superficial 1 mm-deep wounds on
261 surfaces of unwaxed fruits. Infected tomatoes were incubated at 22°C in vented chambers. All
262 RIVET assays were incubated for three days. To harvest samples, 15 mm x 0.5 mm cores were
263 removed from fruits, homogenized in PBS and plated onto XLD agar with appropriate antibiotics.
264 Individual colonies were then patched onto LB agar with tetracycline to detect constructs in
265 which TnpR recombinase was active.
266