1   # Involvement of the *Salmonella* Typhimurium Rcs regulon in the

2   # persistence within tomatoes.

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11

12   ## Abstract

13   It is becoming clear that human enteric pathogens, like *Salmonella*, can efficiently colonize

14   vegetative and reproductive organs of plants. Even though this ability of human pathogens to

15   proliferate within plant tissues has been linked to outbreaks of salmonellosis, little remains

16   known about regulatory and physiological adaptations of human pathogens to their persistence

17   in plants. A screen of *Salmonella* large deletion mutants identified *rcsA* and *rcsB* genes as

18   important for its proliferation within tomatoes. Within tomato fruits, populations of *Salmonella*

19   *rcsB* mutants were up to 2 $\log_{10}$ lower than those of the wild type, competitive fitness of the *rcsA*

20   and *rcsB* mutants was strongly reduced in tomatoes. Bioinformatics predictions identified a

21   putative *Salmonella* RcsAB binding box (TTMGGAWWAABCTYA) and revealed an extensive

22   putative RcsAB regulon, of which at least 70 members were differentially fit within tomatoes.

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24

25   ## INTRODUCTION

26   Outbreaks of human salmonellosis linked to the consumption of fresh fruits and vegetables have

27   increased over the past decade {Jackson, 2013 #2425; Gould, 2013 #2426; Painter, 2013

28   #2427}. Production practices, pre- and post-harvest management, environmental conditions,

29   cultivar selection, broader distribution and an increase in consumption of fresh produce coupled

30   with improved surveillance likely have contributed to the increased incidence (or reporting) of

31   the outbreaks. However, it is also clear that the ability of opportunistic human pathogens (such

32   as non-typhoidal strains of *Salmonella enterica*) to persist outside of their animal hosts, to

33   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 (<u>r</u>ecombinase-based <u>*in vivo* e</u>xpression <u>t</u>echnology) 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.

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195

196   **ACKNOWLEDGEMENTS**

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