

Contribution of the *Salmonella enterica* KdgR Regulon to Persistence of the Pathogen in Vegetable Soft Rots

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During their colonization of plants, human enteric pathogens, such as *Salmonella enterica*, are known to benefit from interactions with phytopathogens. At least in part, benefits derived by *Salmonella* from the association with a soft rot caused by *Pectobacterium carotovorum* were shown to be dependent on *Salmonella* KdgR, a regulator of genes involved in the uptake and utilization of carbon sources derived from the degradation of plant polymers. A *Salmonella* *kdgR* mutant was more fit in soft rots but not in the lesions caused by *Xanthomonas* spp. and *Pseudomonas* spp. Bioinformatic, phenotypic, and gene expression analyses demonstrated that the KdgR regulon included genes involved in uptake and metabolism of molecules resulting from pectin degradation as well as those central to the utilization of a number of other carbon sources. Mutant analyses indicated that the Entner-Doudoroff pathway, in part controlled by KdgR, was critical for the persistence within soft rots and likely was responsible for the *kdgR* phenotype.

Interactions within the phytopathogen-plant host-microbiome triangle are coming to the forefront of scientific research, as it is becoming clear that these interactions are central to crop disease resistance, productivity, and food safety (1–3). Recent studies suggest that the persistence of human enteric pathogens, such as *Salmonella* and pathogenic *Escherichia coli*, in plants, soil, and water represent an important component of their life cycle outside the animal host (4, 5). The ability of human pathogens, such as pathogenic *E. coli* and nontyphoidal strains of *Salmonella enterica*, to persist in the environment is determined, to a great extent, by their interactions with native microbial communities (3, 4, 6–8). In fact, supermarket surveys established that human pathogens are significantly more likely to be found in produce affected by bacterial soft rot than in lesions caused by mechanical damage or fungal pathogens (9). Follow-up laboratory studies supported conclusions of supermarket surveys and demonstrated that within soft rots, *S. enterica* and *E. coli* reach cell numbers that are 10 to 1,000 times higher than those in intact plants (7, 10–13). The effects of phytopathogens on human pathogens occupying the same niche are significant not only in the magnitude of their growth increase but also in the breadth of physiological adaptation to the cohabitation. For example, the growth of *S. enterica* on lettuce leaf macerate caused by another soft-rot pathogen, *Dickeya dadantii*, affected ~18% of the transcriptome (compared to a rich laboratory medium) and included genes involved in growth under oxygen-limiting conditions, carbohydrate, iron, and zinc acquisition, and responses to inhibitory compounds and toxins (12). Nevertheless, mechanisms leading to the increased proliferation of human pathogens in soft rots remain only partially understood.

Grosskopf and Soyer generalized that interactions within microbial communities are driven by either social or metabolic interactions (14). However, there is no evidence that *Salmonella*'s SdiA- and LuxS-mediated quorum-sensing systems are involved in the interactions with phytopathogens *in planta* (11, 15, 16). Therefore, it is reasonable to hypothesize that metabolic interactions between *S. enterica* and phytopathogens are responsible for its increased proliferation within lesions caused by soft rotters.

However, mechanisms of these metabolic interactions currently are not known.

Previously, it was hypothesized that human pathogens benefit from pectinolytic activities of phytopathogens in soft rots (17). Unlike phytopathogens, neither *S. enterica* nor *E. coli* has the ability to degrade pectin; however, they can take up pectin oligomers and monomers. In gammaproteobacteria, all of these functions are mediated by the transcriptional regulator KdgR (18, 19). In phytopathogens, KdgR regulates plant cell wall degradation and the uptake of the degradation products across the *Enterobacteriaceae* (20, 21). Therefore, this study focused on elucidating the role of KdgR and its regulon in the persistence of *S. enterica* within soft rots.

KdgR is an IclR family transcriptional regulator which functions as a repressor, although other members of the family can function both as repressors and activators (20, 22, 23). *In vitro*, *Erwinia chrysanthemi* KdgR binds to different operators, with K_D (equilibrium dissociation constant) values between 0.1 and 10 nM (21). However, *in vitro* binding affinity generally did not correlate with the degree of repression of the corresponding gene. As a dimer, it binds a 17-bp KdgR box, containing two half sites. Binding of 2-keto-3-deoxygluconate (KDG) to KdgR releases this repressor protein from its binding sites (20, 21). The characterization of the DNA binding site for KdgR in *E. chrysanthemi* (20, 21)

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led to comparative genomics studies that outlined KdgR regulons in a number of gammaproteobacteria (18). In *S. enterica*, initial predictions identified *kduI-kduD* (involved in 5-keto-4-deoxyuronate catabolism), *kdgM* (rhamnogalacturonan catabolism), and *kdgK* (2-keto-3-deoxygluconate kinase) among the 11-member putative regulon (18). *E. coli* KdgR was shown to contribute to the regulation of the Entner-Doudoroff (ED) pathway, which catabolizes glucose to pyruvate and then feeds into glycolysis (24, 25). The Entner-Doudoroff pathway also is involved in the utilization of gluconate, idonate, galacturonate, glucuronate, β -glucuronide, fructuronate, tagaturonate, and 2-keto-3-deoxy-gluconate and is hypothesized to contribute to the fitness of *E. coli* in the large intestine (25). In addition to their metabolic functions in the Entner-Doudoroff pathway, *eda* (2-keto-3-deoxy-6-phosphogluconate aldolase) and *edd* (6-phosphogluconate dehydratase) were shown to contribute to phosphate and nitrogen starvation responses as well as stress responses (25).

With this study, we tested the overarching hypothesis that the *S. enterica* KdgR regulon contributes to the fitness of this human pathogen in tomato soft rots caused by an aggressive strain of *Pectobacterium carotovorum*. This study sought to better define the *Salmonella* KdgR regulon and further investigated potential mechanisms by which KdgR contributes to the fitness of *Salmonella* within soft rots.

MATERIALS AND METHODS

Bacterial culture. *Salmonella* mutants were grown overnight in a shaker at 37°C in Luria-Bertani (LB) medium supplemented with 50 μ g/ml kanamycin or 10 μ g/ml tetracycline as needed. Wild-type *S. enterica* serovar Typhimurium 14028 was grown without selection at 37°C. *P. carotovorum* SR38 was grown overnight in a shaker at 30°C in LB.

The ability of *Salmonella* to grow on specific carbon sources was tested using M9 minimal medium supplemented with 0.2% (wt/vol) of a carbon source. Prior to dilution in M9, cultures were grown overnight in LB. They then were washed in phosphate-buffered saline, pH 7.0 (PBS). Suspensions were dilution plated onto LB agar.

Strain construction. Deletion mutants were constructed using Datsenko-Wanner mutagenesis (26), and entire open reading frames were replaced with *frt-kan-frt* cassettes (Table 1). Deletions were confirmed by PCR with primers listed in Table S1 in the supplemental material. RIVET (recombinase-based *in vivo* expression technology) reporters were constructed by first removing a kanamycin resistance marker (26) and then mating the plasmid pCE70 or pCE71, containing a *tnpR-lacZ* fusion, to the mutant strain (27). The orientation of the reporter fusion was confirmed by PCR. Phage P22 then was used to transduce a *res1-tetRA-res1* cassette into the strain. Resulting constructs then were purified on LB EGTA (10 mM) with kanamycin and tetracycline and then screened on Evans-blue uranium (EBU) agar.

Vegetable inoculations. For competitive fitness experiments, overnight cultures of mutant and wild-type strains were washed in PBS and diluted to a concentration of $\sim 10^4$ CFU/ml. These suspensions were combined in an approximately 1:1 ratio. Three shallow wounds were made on the surface of each green tomato. Three microliters of the inoculum was spotted per wound. For the assays in soft rots, an additional 3 μ l of an undiluted (10^9 CFU/ml) but washed *P. carotovorum* overnight culture was inoculated into the tomatoes concurrently with *Salmonella*. Tomatoes were sampled 3 days later, which corresponds to the development of a soft-rot lesion covering at least three-quarters of the fruit. Samples were plated on xylose-lysine deoxycholate agar (XLD; Oxoid) for the quantification of *Salmonella*. XLD plates were incubated at 42°C to prevent growth of *Pectobacterium* essentially as described previously (11). *Salmonella* colonies then were patched onto selective media with kanamycin, and the ratio of the wild type (kanamycin sensitive) to the mutant (kana-

mycin resistant) was calculated to determine how much it changed from 1:1. Competitive fitness indices were calculated as described before using the formula $(M_{out}/WT_{out})/(M_{in}/WT_{in})$, where M is the proportion of mutant cells and WT_{in} or WT_{out} is the proportion of the wild-type cells in the inocula or in the recovered samples, respectively (11). Competitive fitness of the *kdgR* mutant in green bell peppers and romaine lettuce was assayed similarly. Vegetables were purchased from a local grocery store.

To determine the growth of *Salmonella* in tomatoes, washed, diluted bacterial cultures were seeded into tomatoes as monocultures starting with approximately 100 CFU per wound. Following a 3-day incubation at 22°C, tomatoes were stomached in a Whirl-Pak bag (with an equal volume of PBS) using a Stomacher 400 circulator (Seward). Aliquots of the homogenate then were plated onto XLD plates and incubated at 42°C.

RIVET assays were conducted in a similar fashion. After the homogenate was plated on XLD, individual colonies were patched onto an LB plate with tetracycline. Percent resolution was taken as the ratio of patched colonies that grew (tetracycline resistant, in which the reporter was not activated) to those that did not (and lost the tetracycline resistance due to the activation of the reporter). Percent resolution was used as a measure of gene expression.

Biolog phenotype microarray. Phenotype microarrays were performed using Biolog phenotype microarrays (PM1 and PM2). Cells were prepared according to the manufacturer's protocols with the following modification: cultures were grown overnight in LB and then starved in M9 minimal medium overnight. The plates then were read using a Victor³ plate reader (PerkinElmer). The function heatmap.2 from the R package *gplots* (<https://cran.r-project.org/web/packages/gplots/index.html>) was used to plot the data using the mean absorbance values (two replicates) per growth condition for each bacterium following 24 h of incubation. The dendrogram imposed over the rows and columns was constructed with a complete-linkage hierarchical clustering using the Euclidean distance between the mean absorbance values.

RT-qPCR. Cultures were grown, washed, and inoculated into tomatoes as described above, only without the dilution. RNA was extracted following a 24-h incubation. To extract RNA, 4 ml of diethyl pyrocarbonate (DEPC)-treated water and 1 ml of 95:5 absolute ethanol-phenol was added to a 15-ml Falcon tube. Approximately 0.5 g of RNase-free 0.1-mm glass beads then were added to the tubes. A 1.5-g aliquot of inoculated tomato tissue was added to the tube and ground using a glass rod. The tubes then were vortexed, and 500 μ l of the resulting liquid was aliquoted into microcentrifuge tubes. The Ambion RNAqueous kit was used for the remainder of the extraction by following the manufacturer's instructions. Samples were treated using an Ambion Turbo DNasefree kit. cDNA was synthesized using the OriGene cDNA synthesis kit. Quantitative PCR (qPCR) was performed using Applied Biosystems SYBR green reaction mix and measured using an Applied Biosystems thermal cycler. Primers were designed to amplify small (~ 200 -bp) fragments and subjected to a BLAST search against the *P. carotovorum* and *S. enterica* serovar Typhimurium genomes to check for nonspecific binding. To ascertain that nonspecific binding of primer to the *P. carotovorum* template did not occur, controls of soft rot (without *S. enterica*) were tested at least once for each primer pair. qPCR statistics were performed using the Livak method (28). As a control, the housekeeping gene *rpoD* was used after establishing that its expression did not differ regardless of the presence of *kdgR* or the growth in the soft rot. The expression of a particular gene in the wild-type background in intact tomatoes was used as the calibrator. Error was determined using the propagation of errors from the standard deviations.

Bioinformatics prediction of KdgR binding sites. The program Retrieve-Sequence from Regulatory Sequence Analysis Tools (RSAT) was used to obtain the upstream regions (-400 ; $+100$ in relation to the start codon) of all coding sequences (CDS) in 28 RefSeq *Salmonella* genomes and their plasmids (29). Using 40 experimentally validated KdgR binding sites, a position-specific scoring matrix (PSSM) was constructed using the program Consensus with default values (29). For each of the 28 genomes utilized, an oligonucleotide frequency background file was created with

TABLE 1 Strains used in this study^a

Strain or plasmid	Genotype or description	Source, construction, or reference
Strain		
<i>Escherichia coli</i>		
DH5 α	F ⁻ Δ (<i>lacZY-argF</i>)U169 <i>recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA2</i>	Life Technologies
<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>		
SR38	Aggressive soft-rot pathogen isolated from a shipment of Florida tomatoes	Laboratory collection, from J. Bartz
<i>Salmonella enterica</i> serovar Typhimurium		
14028	Wild type	American Type Culture Collection
JS246	14028 <i>yjeP::res1-tetRA-res1</i>	26
JTN215	14028 <i>kdgR27::kan</i>	Made by Datsenko-Wanner (26) mutagenesis using primers JTN177 and JTN178; verified with primers JTN179 and JTN180
AG32	14028 <i>kdgR27::frit res1-tetRA-res1</i>	AG1 \times P22/JS246
AG1	14028 <i>kdgR27::frit</i>	Made by electroporating pCP20 into JTN215
AG2	14028 <i>kdgR27::tnpR-lacZY</i>	Made by electroporating pCE70 into AG1
AG3	14028 <i>kdgR2::tnpR-lacZY res1-tetRA-res1</i>	AG2 \times P22/JS246
AG15	14028 <i>kdgK15::frit-kan-frit</i>	Made by Datsenko-Wanner (26) mutagenesis using primers JTN163 and JTN164; verified with primers JTN165 and JTN166
AG19	14028 <i>kdgK15::frit</i>	Made by electroporating pCP20 into AG15
AG27	14028 <i>kdgK15-tnpR-lacZY</i>	Made by electroporating pCE70 into AG19
AG28	14028 <i>kdgK15-tnpR-lacZY res1-tetRA-res1</i>	AG27 \times P22/JS246
AG38	14028 <i>kdgR27::frit kdgK15::tnpR-lacZY res1-tetRA-res1</i>	AG32 \times P22/AG27
AG25	14028 <i>kduID45::frit-kan-frit</i>	Made by Datsenko-Wanner (26) mutagenesis using primers JTN167 and JTN168; verified with primers JTN169 and JTN170
AG31	14028 <i>kduID45::frit</i>	Made by electroporating pCP20 into AG25
AG33	14028 <i>kduID45::tnpR-lacZY</i>	Made by electroporating pCE70 into AG31
AG36	14028 <i>kduID45::tnpR-lacZY res1-tetRA-res1</i>	AG33 \times P22/JS246
AG39	14028 <i>kdgR27::frit kduID45::tnpR-lacZY res1-tetRA-res1</i>	AG32 \times P22/AG33
AG26	14028 <i>kdgM32::frit-kan-frit</i>	Made by Datsenko-Wanner (26) mutagenesis using primers AG7 and AG8; verified with primers AG33 and AG34
AG30	14028 <i>kdgM32::frit</i>	Made by electroporating pCP20 into AG26
AG34	14028 <i>kdgM32::tnpR-lacZY</i>	Made by electroporating pCE70 into AG31
AG35	14028 <i>kdgM32::tnpR-lacZY res1-tetRA-res1</i>	AG34 \times P22/JS246
AG41	14028 <i>kdgR27::frit kdgM::tnpR-lacZY res1-tetRA-res1</i>	AG32 \times P22/AG34
KI1	14028 <i>eda42::frit-kan-frit</i>	Made by Datsenko-Wanner (26) mutagenesis using primers AG92 and AG93; verified with primers AG94 and AG95
AG42	14028 <i>eda42::frit</i>	Made by electroporating pCP20 into KI1
AG45	14028 <i>eda42::tnpR-lacZY</i>	Made by electroporating pCE71 into AG42
AG46	14028 <i>eda42::tnpR-lacZY res1-tetRA-res1</i>	AG45 \times P22/JS246
AG47	14028 <i>kdgR27::frit eda42::tnpR-lacZY res1-tetRA-res1</i>	AG32 \times P22/AG45
AG44	14028 <i>kdgT44::frit-kan-frit</i>	Made by Datsenko-Wanner (26) mutagenesis using primers AG64 and AG65; verified with primers AG66 and AG67
AG47	14028 <i>kdgK15::frit kduID::frit-kan-frit</i>	AG19 \times P22/AG25
AG48	14028 <i>kdgK15::frit kduID::frit</i>	Made by electroporating pCP20 into AG47
AG49	14028 <i>kdgK15::frit kduID45::frit kdgM32::frit-kan-frit</i>	AG48 \times P22/AG26
Plasmid		
pKD4	<i>frit-kan-frit</i> template	26
pCP20	FLP ⁺ 1 cI857 ⁺ 1 P _R Rep ^{ts} Amp ^r Cm ^r	26
pCE70	FRT- <i>tnpR-lacZY oriR6K</i> (<i>kan</i>); contains wild-type <i>tnpR</i> promoter, FRT orientation A	27
pCE71	FRT- <i>tnpR-lacZY oriR6K</i> (<i>kan</i>); contains wild-type <i>tnpR</i> promoter, FRT orientation B	27
pKD46	λ Red ⁺ (Amp ^r pSC101 <i>oriTS</i>)	26

^a FRT, FLP recombination target.

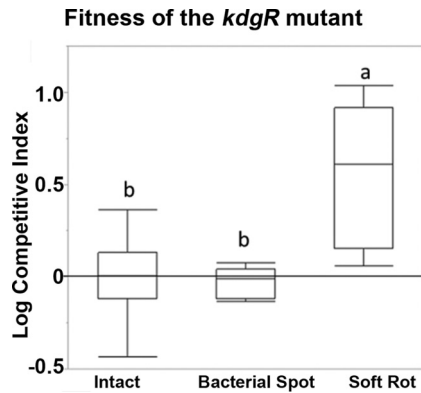


FIG 1 Fitness of the *kdgR* mutant in soft rots. *S. enterica* serovar Typhimurium 14028 and the isogenic *kdgR* mutant were coinoculated at a 1:1 ratio into intact green tomatoes or into tomatoes with *P. carotovorum* SR38 or *Xanthomonas* species. The ratio of the wild-type *Salmonella* to the mutant was assessed by plating and patching onto selective media after a 3-day incubation. Box plots indicate 25 to 75% quantiles, and lines within box plots are medians. Whiskers indicate the first or third quantile plus 1.5 multiplied by the inner quantile range. The zero line indicates no difference in competitive fitness. Competitive indices were compared using Student's *t* test, and different letters indicate statistically significant differences ($P < 0.05$).

the program Convert-Background from RSAT using Markov Model Order 4 (30). The upstream regions of each *Salmonella* CDS then were scanned with the constructed PSSM using Matrix-Scan with an upper threshold value of $1e-5$ and inputting a previously generated genome-specific background file (30).

Salmonella orthologous genes from 40 experimentally validated KdgR regulons were downloaded from the Biocyc database. An *ad hoc* Perl script recovered 211 sequences corresponding to the predicted binding sites that matched the upstream regions of the orthologous genes previously retrieved. The *Salmonella*-fitted PSSM was constructed with an input of 211 sequences to the program consensus. The genome of *S. enterica* serovar Typhimurium LT2 was rescanned using this final PSSM with the program Matrix Scan with an upper threshold value of $1e-5$ and inputting a previously generated genome-specific background file (30). The PSSM *kdgR* logo was created using Weblogo interface (31).

RESULTS AND DISCUSSION

***kdgR* contributes to fitness within soft rots.** To determine whether *kdgR* contributes to the persistence of *Salmonella* within soft rots, we first tested the expression of the *kdgR* RIVET reporter in intact tomatoes and those with the soft rot caused by *Pectobacterium carotovorum*. After 24 h of incubation within intact tomatoes and within soft rots, the activation of the reporter reached ~90% with no significant differences in the expression of the reporter within soft rots and intact tomatoes (see Fig. S1 in the supplemental material). Within 3 days, the reporter was fully activated inside tomatoes, regardless of the presence of the soft rot (see Fig. S1 in the supplemental material). It is important to note, however, that RIVET reporters, once resolved, are not useful for the detection of downregulation of gene expression.

However, when the fitness of the *kdgR* mutant was compared with that of the wild type in intact tomatoes and those affected by soft rot or bacterial spot (associated with *Xanthomonas* spp. and *Pseudomonas* spp.), a significant increase in the fitness of the *kdgR* mutant was observed in soft-rotted tomatoes only (Fig. 1). While the *kdgR* mutant was more competitive than the wild type in the soft-rotted tomatoes, there were no significant differences in fit-



FIG 2 Sequence logo for KdgR binding sites in *Salmonella enterica*. The position-specific scoring matrix (PSSM) was derived from the KdgR binding sequences experimentally validated in other gammaproteobacteria. A PSSM was generated from the targets identified in the first scan based on 211 sites found in the upstream regions of the *Salmonella* genes that are orthologous to the previously validated KdgR regulon members and 40 experimentally validated targets of KdgR. The PSSM *kdgR* logo was created using the Weblogo interface.

ness between the wild type and mutant in intact tomatoes and those infected with necrotizing pathogens leading to the bacterial spot (Fig. 1). In green bell peppers and romaine lettuce coinoculated with *S. Typhimurium* and *P. carotovorum*, a trend for increased fitness of the *kdgR* mutant also was observed, although the differences in fitness were not statistically significant (data not shown). The increased fitness of the mutant is consistent with the function of KdgR as a repressor of gene expression. With the follow-up experiments, we focused on defining the mechanisms by which *kdgR* affects the fitness of *Salmonella* within tomato soft rots. Because *Salmonella* KdgR is not well characterized, initially we carried out two high-throughput studies to delineate potential members of the KdgR regulon and to identify differences in metabolic capabilities of the mutant and the wild type that allow it to persist better in soft rots.

Bioinformatic predictions identify novel members of the KdgR regulon. A whole-genome scan was performed on 28 complete *Salmonella* genomes (see Table S2 in the supplemental material) using a PSSM derived from the KdgR binding sequences experimentally validated in other gammaproteobacteria (18, 30). A PSSM was generated from the targets identified in the first scan based on 211 sites found in the upstream regions of the *Salmonella*

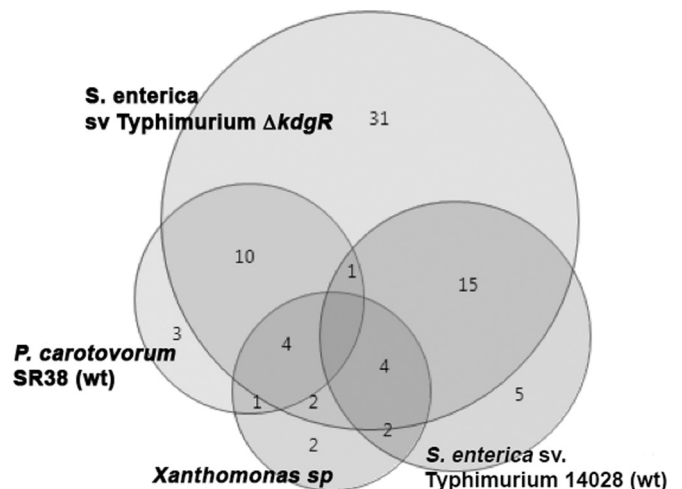


FIG 3 Overlap in utilization of carbon sources by *S. enterica* serovar Typhimurium, *S. enterica* serovar Typhimurium *kdgR*, *P. carotovorum* SR38, and *Xanthomonas* species. The ability of the bacteria to respire on substrates in Biolog plates PM1 and PM2 was documented (see Fig. S2 in the supplemental material).

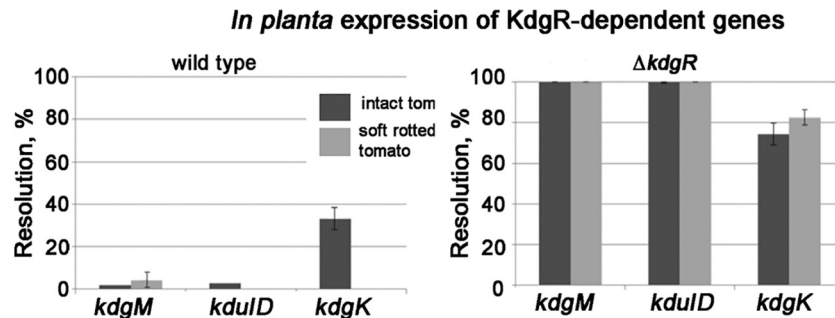


FIG 4 *In planta* expression of KdgR-regulated genes. RIVET reporters in *kdgM*, *kduID*, and *kdgK* were constructed in the wild-type and $\Delta kdgR$ backgrounds. The resolution of the reporters was tested in soft rots and in intact green tomatoes (tom) following a 3-day incubation. Soft rots were initiated by coinoculating *P. carotovorum* SR38 with *S. enterica* RIVET reporters.

genes that are orthologous to the previously validated KdgR regulon members (Fig. 2). This *Salmonella* KdgR PSSM shared some features with those of *Vibrio* spp. and *E. chrysanthemi* (18); however, it was distinct from both.

The *Salmonella enterica* serovar Typhimurium LT2 genome then was rescanned with this new PSSM, recovering a total of 38 putative KdgR binding sites (see Table S3 in the supplemental material). This two-step methodology was chosen to reduce bias generated by utilizing the original interspecies KdgR PSSM (32). The list of putative KdgR targets (see Table S3) included 8 out of 12 putative KdgR targets previously suggested (18), as well as over 30 additional targets, including those with predicted functions in iron acquisition, uptake and/or utilization of arabinose, ketoglutarate, rhamnose, fructose, ribose, myoinositol, and lyxose, aspartate and pyruvate metabolism, as well as several regulators with unknown functions (see Table S3). This significantly expands the putative *Salmonella* KdgR regulon beyond controlling functions involved in the uptake and utilization of dimers and monomers resulting from pectin degradation (18). Some of these predicted functions of the KdgR regulon members were tested in subsequent experiments to determine to what extent they contribute to the fitness of *Salmonella* in soft rots.

Biolog profiling defines metabolic contributions of KdgR. In parallel with the comparative genomics analyses to identify targets of KdgR, we carried out Biolog profiling of the *S. enterica* serovar Typhimurium wild type, its isogenic *kdgR* mutant, and the strains of *Pectobacterium* and *Xanthomonas* that were used in the competitive fitness experiments presented in Fig. 1. The goal of this experiment was 3-fold: (i) to define metabolic consequences of the disruption of *kdgR*, (ii) to determine the level of concordance between predicted targets of KdgR and metabolic consequences of the *kdgR* mutation, and (iii) to characterize metabolic differences between the wild-type *Salmonella* strain, its *kdgR* mutant, and the two phytopathogens used in this study.

Based on the results of the Biolog test, there were over 40 substrates that the *kdgR* mutant was able to utilize better than the wild type (Fig. 3; also see Fig. S2 in the supplemental material). Compared to the wild type, the *kdgR* mutant was better able to utilize L-arabinose, D-ribose, L- and D-aspartic acids, α -ketoglutarate, myoinositol, L-rhamnose, L-lyxose, and pyruvate, which is consistent with the bioinformatic identification of the KdgR binding sites upstream of *araH* (arabinose permease), STM1933 (ribose 5-phosphate isomerase), STM4510 (aspartate racemase), *kdgK* (ketodeoxygluconokinase), *yifZ*

(ketodeoxyglutarate permease), STM4435 (2-keto-myoinositol isomerase), STM1911 (rhamnolacturonidase), *yiaK* (2,3-diketo-L-gulonate reductase), and *pykF* (pyruvate kinase). Biolog tests were followed up with retesting the ability of the wild type and the *kdgR* mutant to utilize carbon sources such as rhamnose, fructose, and pyruvate. The results of the retesting (see Table S4) are consistent with the high-throughput Biolog test and indicated an increased ability of the *kdgR* mutant to utilize these carbon sources.

In general, the *kdgR* mutant was able to respire better than the isogenic wild type on more than 40 substrates. Of them, 31 substrates were more efficiently utilized by the *kdgR* mutant than by the parental wild type, *P. carotovorum* or *Xanthomonas* species (Fig. 3). Of the substrates that were differentially utilized by *S. enterica* serovar Typhimurium and its *kdgR* mutant, the mutant also was more proficient in the utilization of nutrients than *Pectobacterium* (with the exception of *N*-acetylglucosamine, asparagine, serine, rhamnose, arabinose, ribose, fructose, and glucose). Compared with the *kdgR* mutant, *Xanthomonas* species appear to be able to better utilize only *N*-acetylglucosamine. Even though the *kdgR* mutant was better than the wild type at the utilization of the common plant sugars (rhamnose, arabinose, glucose, and fructose), *P. carotovorum* was significantly more efficient at respiring on these substrates (Fig. 3; also see Fig. S2 in the supplemental material). Nevertheless, to more robustly test this hypothesis, fitness of the mutants in the KdgR regulon members, which represent a diversity of KdgR-controlled functions, was tested in subsequent experiments.

TABLE 2 RT-qPCR analysis of KdgR-regulated genes

Gene	Expression level			
	<i>kdgR</i> mutant vs wild type in green tomatoes	Wild type in soft rot vs wild type in tomatoes	<i>kdgR</i> mutant in soft rot vs <i>kdgR</i> mutant in green tomatoes	<i>kdgR</i> mutant in soft rot vs wild type in soft rot
<i>kdgK</i>	26.9 \pm 0.7	59.6 \pm 3.3	4.3 \pm 2.2	1.9 \pm 3.9
<i>kduI</i>	342.5 \pm 2.0	148.1 \pm 2.1	2.1 \pm 0.3	4.86 \pm 3.1
<i>araH</i>	9.4 \pm 0.8	19.3 \pm 1.6	6.8 \pm 2.2	3.3 \pm 2.7
<i>fruF</i>	2.1 \pm 0.6	15.8 \pm 1.5	9.8 \pm 2.1	1.3 \pm 2.5
<i>edd</i>	4.2 \pm 1.0	26.6 \pm 1.7	5.4 \pm 2.1	0.8 \pm 2.5
<i>eda</i>	6.5 \pm 3.0	8.1 \pm 3.4	1.2 \pm 2.1	0.9 \pm 2.6
<i>sodA</i>	0.86 \pm 1.7	0.97 \pm 0.2	8.5 \pm 0.6	7.5 \pm 0.6
<i>iroN</i>	0.59 \pm 0.9	1.1 \pm 1.4	5.4 \pm 0.4	2.9 \pm 1.2

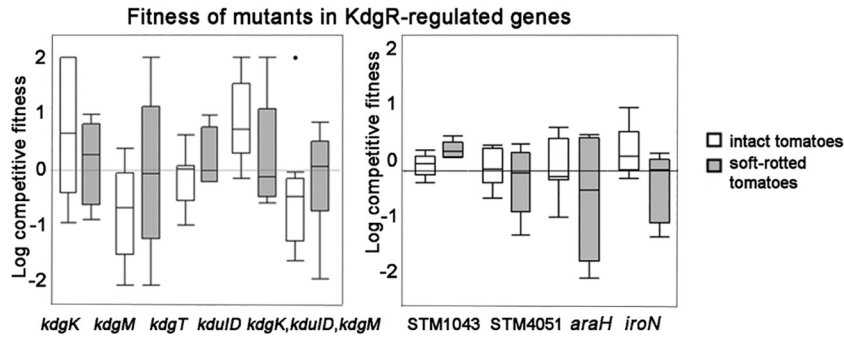


FIG 5 Fitness of the mutants in *kdgR*-regulated genes in soft rots and intact tomatoes. Mutants were coinoculated with the isogenic wild type in a 1:1 ratio in green tomatoes (empty boxes) or in tomatoes that also were seeded with *P. carotovorum* SR38 (shaded boxes). Ratios of the mutant to the wild type were assessed by patching onto selective media, and log competitive indices were determined. Box plots indicate 25 to 75% quantiles, and lines within box plots are medians. Whiskers indicate the first or third quantile plus 1.5 multiplied by the inner quantile range. The fitness of mutants in intact tomatoes and in soft rots was compared using Student's *t* test, and none were significant.

Contribution of members of the KdgR regulon to fitness in soft rots. To test which of the members of the KdgR regulon contribute to fitness within soft rots, their regulation was tested using RT-qPCR and RIVET as well as fitness tests of the corresponding mutants. Results of the RT-qPCR experiments and/or RIVET reporter assays indicate that *kdgK*, *kduI*, *araH*, and *fruF* are subject to *kdgR* regulation in green tomatoes and in soft rots (Fig. 4 and Table 2). *kdgR*-dependent regulation of *sodA* and *ironN* was observed only in the soft rot (Table 2); however, the magnitude of the response was essentially the same when the accumulation of these transcripts was tested in the *kdgR* mutant in intact tomatoes versus that in the *kdgR* mutant in a soft rot (Table 2). Genes *kduI* (encoding 4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase) and *kdgM* (encoding oligogalacturonate-specific porin) appear to be most strongly controlled by KdgR, while *kdgK* (2-keto-3-deoxygluconate kinase), *araH*, and *fruF* likely are controlled by additional regulators (Table 2 and Fig. 4).

The fitness of mutants (*kdgK*, *kdgM*, *kdgT* [2-keto-3-deoxygluconate permease], STM1043 [putative attachment and invasion protein], STM4051 [hypothetical protein], *araH*, and *ironN*) was tested in intact tomatoes and those with soft rot (Fig. 5). Mutations in any of these genes, or a quadruple mutation in *kdgK*, *kduID*, and *kdgM*, did not decrease the competitive fitness of the strains (Fig. 5). These data indicate that by themselves these genes are not responsible for the *kdgR* phenotype in soft rot.

The Entner-Doudoroff pathway is important for growth in soft rots. Because none of the tested genes within the KdgR regulon appeared to be responsible for the observed phenotype of the *kdgR* mutant in soft rots, we focused on an alternative hypothesis. We hypothesized that the increased fitness of the *kdgR* mutant within soft rots is due to the regulatory effects of KdgR on the Entner-Doudoroff pathway. The rationale for this hypothesis is based on the observation that the *kdgR* mutant was better able to utilize carbon sources (gluconic acid, glucuronic acid, and glucose) that feed into the Entner-Doudoroff pathway (see Fig. S2 in the supplemental material) and based on the report that *E. coli* KdgR contributes to the regulation of *eda* (Entner-Doudoroff aldolase), which is central to the metabolism of sugar acids (24). *Eda* is involved in the conversion of the phosphorylated KDG molecule (KDPG) into pyruvate and glyceraldehyde-3-phosphate, which feeds into the tricarboxylic acid (TCA) cycle. It has been reported that *eda* is important for growth of *Salmonella* on D-glu-

conate and D-glucuronate (33). The latter is a monomer which results from the breakdown of pectic polymers (34). Furthermore, Goudeau et al. reported that genes involved in the uptake of gluconate and idonate (metabolized via the Entner-Doudoroff pathway) were upregulated in *Salmonella* grown on lettuce leaf macerate caused by *D. dadantii* (12, 33–35).

Consistent with this hypothesis, RT-qPCR and RIVET experiments demonstrated that the expression of *eda* and *edd* (6-phosphogluconate dehydratase) was significantly derepressed when *kdgR* was deleted (Table 1 and Fig. 6). The magnitude of the derepression was higher in the tomatoes with soft rot than in intact tomatoes (Table 2 and Fig. 6). In competition assays, the fitness of the *eda* mutant in the intact tomato did not differ significantly from that of the wild-type parental strain. However, in soft rot the *eda* mutant is almost completely uncompetitive against the wild type, and phenotype of the *edd* mutant is similar although not as severe (Fig. 7). To ensure that the *eda* mutation is not generally deleterious, the growth of the mutant and the wild type in LB broth shake cultures was compared. Both strains had the same growth rate and reached the same final population densities (data not shown). Collectively, these observations indicate that the Entner-Doudoroff pathway is important for the fitness of *Salmonella* within soft rots. The increased fitness of the *kdgR* mutant is consistent with the reduced fitness of the *eda* mutant in this model.

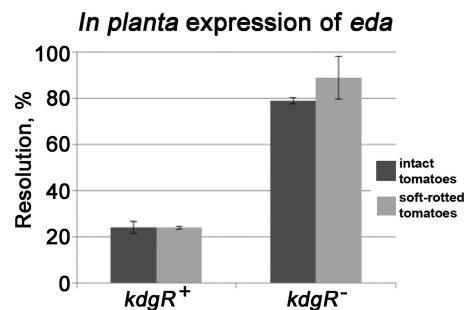


FIG 6 *In planta* expression of *eda*. Expression of *eda::tmpR-lacZY* reporters in *S. enterica* serovar Typhimurium 14028 was determined in wild-type and *kdgR* backgrounds. Reporters were inoculated into tomatoes with or without *Pectobacterium carotovorum* SR38. Samples were plated following 3 days of incubation and scored for the loss of the tetracycline marker.

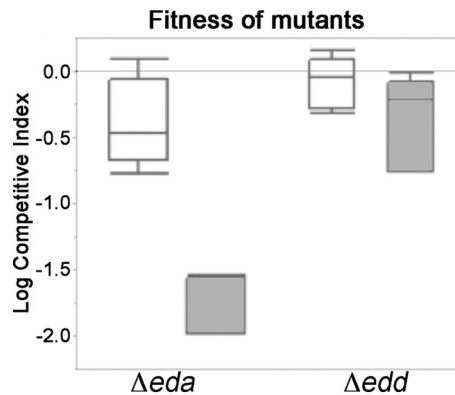


FIG 7 Fitness of the *eda* and *edd* mutants in tomatoes. The mutants were coinoculated with the isogenic wild type in a 1:1 ratio in green tomatoes or into tomatoes that also were seeded with *P. carotovorum* SR38. Ratios of the mutants to the wild type were assessed by patching onto selective media, and log competitive indices were determined. Box plots indicate 25 to 75% quantiles, and lines within box plots are medians. Whiskers indicate the first or third quartile plus 1.5 multiplied by the inner quartile range.

Conclusions. Interactions of pathogens with native host-associated microbiota are complex, and their mechanisms only now are beginning to be understood (7, 11, 12, 36–39). *Salmonella* appears to be able to repurpose the strategies it uses to obtain nutrients in the intestine in order to gain 1,2-propanediol and ethanolamine from soft rots (12). Nevertheless, mechanisms by which these enteric pathogens take advantage of the presence of phytopathogens only now are beginning to be understood (7, 12, 38).

Several models could be tested to define mechanisms behind these interactions. Six basic motifs (from commensalism to amensalism) generally are recognized in microbe-microbe interactions (14). Under the conditions that were optimal for soft-rot development, the presence of *Salmonella* reduced growth rates and final population densities reached by *P. carotovorum* in planta while *S. enterica* benefited from this relationship (11, 13), suggesting that these interactions are either predatory or amensalistic (14). At least in part, this could be due to the ability of *S. enterica* to manipulate the pH of the soft rot and inhibit growth of *P. carotovorum* in microaerophilic, but not aerobic, conditions (13).

None of the KdgR-regulated genes directly involved in the uptake or metabolism of 2-keto-3-deoxygluconate (KDG) or pectin oligomers seem to play a role in this interaction. However, the decreased fitness of *eda* in soft rots points to a role of this *Salmonella* gene during interactions with *P. carotovorum*. The mechanism by which *Salmonella* KdgR-regulated genes are derepressed within the soft rot remains unclear. In soft-rot bacteria, binding of KdgR to KDG releases the protein from its operator sites (20); however, in our experiments the addition of KDG to *lacZ* reporter fusions in *kdgR*-regulated genes did not relieve the repression (data not shown). Metabolic interactions clearly play a role in the interactions of enterics with the soft-rot phytopathogens. The full complement and the exact mechanisms governing these interactions have yet to be fully understood, although it seems likely that an alternative glycolytic pathway, as well as pathways involved in 1,2-propanediol and ethanolamine utilization, are critical to the persistence of enterics within soft rots.

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