Consequences of Disrupting *Salmonella* AI-2 Signaling on Interactions Within Soft Rots

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ABSTRACT

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Within soft rots, *Salmonella* spp. reach population densities 10- to 100-fold higher than within intact plants. The hypothesis that *Salmonella* spp. exchange AI-2 signals with *Pectobacterium carotovorum* to increase its competitive fitness was tested using mutants involved in AI-2 production (*luxS*) or perception (*lsrACDBF* or *lsrG*). Co-infections of a wild-type *Salmonella* sp. and its AI-2 mutants (at ≈3 to 10^4) were established in green or red tomato ('FL 47' or 'Campari' for 3 or 5 days) as well as tomato co-infected with *Pectobacterium* (at 10^9) or its *luxS* mutant. There were no significant differences in the competitive fitness of *Salmonella*, indicating that AI-2 signaling is not a major input in the interactions between these organisms under the tested conditions. A *Salmonella lsrG::tnpR-lacZ* resolvase in vivo expression technology (RIVET) reporter, constructed to monitor AI-2-related gene expression, responded strongly to the *luxS* deletion but only weakly to external sources of AI-2. Growth in soft rots generally decreased RIVET resolution; however, the effect was not correlated to the *luxS* genotype of the *Pectobacterium* sp. The results of this study show that AI-2 signaling offers no significant benefit to *Salmonella* spp. in this model of colonization of tomato or soft rots.

Additional keywords: produce safety, quorum sensing.

Salmonella spp. contamination of produce is responsible for an increasing number of foodborne outbreaks (19). Raw tomato fruit are a prominent produce vector of salmonellosis and have been linked to at least 12 multi-state outbreaks of salmonellosis since 1990 (1,11). Marketing surveys and laboratory studies establish a clear link between the composition of plant-associated microbiota and *Salmonella* spp. persistence in and on plants (2,27,63). *Salmonella* spp. are able to integrate into multicellular consortia formed by epiphytes on leaf surfaces and benefit from damage induced by phytopathogens, reaching higher densities when growing within bacterial lesions on fruit and leaves (2,7,8). Market produce with visual soft-rot symptoms was twice as likely to harbor *Salmonella* spp. and harbored the pathogen at levels more than a log-fold higher than asymptomatic or mechanically damaged produce (63). In a *Salmonella enterica–Pectobacterium carotovorum* co-infection model, a soft rot caused by *P. carotovorum* increases *S. enterica* proliferation up to three-log-fold in green market tomato (36). Although the beneficial association between human enteric pathogens and phytobacteria is well established and may ultimately determine the safety of fresh produce, the mechanisms governing these interactions are currently unknown.

It is hypothesized that human enteric pathogens specifically benefit from the plant polymer-degradative abilities of pectinolytic bacteria, such as *P. carotovorum*, which are normal members of plant-associated microbial communities, to gain access to protected environments or increased availability of nutrients (6,20,21, 45,61,63). On lettuce, *Escherichia coli* O157:H7 preferentially colonizes damaged surfaces and reaches a 27-fold higher population size when grown on leaves infected with *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) 3937 (7,66). However, when grown on lettuce leaves infected with *D. dadantii outC* mutant, which is completely deficient in the export of cell-wall-degrading enzymes, including pectate lyases (PELs), the ultimate population size of *Escherichia coli* O157:H7 is indistinguishable from that reached when grown on non-*D. dadantii*-infected leaves (66).

P. carotovorum is a broad-host-range pectinolytic phytopathogen, which utilizes quorum sensing (QS) systems to coordinate expression of the genes involved in the production of hydrolytic exoenzymes required for virulence in plants (12,15,28,41). There are two well-characterized population-density-dependent gene regulatory QS systems in these bacteria: one based on the production and perception of N-acyl homoserine lactone (AHL) signals and a second which utilizes the LuxS-dependent autoinducer (AI)-2 signal. The role of these QS systems in mediating interactions within plant-associated microbial communities is well established (5,9,38,58,62,65). Because many enteric and phytobacteria share components of QS signaling pathways, it has been commonly hypothesized that signal exchange plays a major role in mediating interactions in planta and could specifically empower beneficial interactions between *Salmonella* spp. and soft-rot-causing organisms in plant tissues.

By exploiting these QS signals, *S. enterica* may be able to increase the suitability of the produce environment and induce its own favorable genetic regulatory changes. *P. carotovorum* possess a fully functional AHL system which is required for plant virulence. *Salmonella* spp. lack an AHL synthase but possess an orphan, yet functional AHL receptor, encoded by *sdiA*, which allow them to perceive AHL signals and differentially regulate expression of several genes with yet-unknown functions (49). Although *S. enterica* can respond to AHL signals produced by *P. carotovorum* as well as their chemical homologs in rich laboratory media, it is unable to recognize AHLs in planta because expression of *sdiA* is "off" within tomato soft rots (36,47). Furthermore, the fitness of a *Salmonella sdiA* mutant was not affected within soft rots, indicating that the ability of *Salmonella* spp. to interact with phytobacteria under normal environmental condi-

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tions does not depend on AHL- or SdiA-mediated signal exchange under the experimental conditions used in the study (36).

Given the wide distribution of the *luxS* synthase gene, AI-2-mediated QS was originally believed to be a common method for interspecies communication (46). This view has been somewhat tempered by evidence showing a relative rarity of AI-2 receptors or response regulators although, among enterobacteriaceae, receptors are more common among pathogenic and endosymbiotic species (17,43,44,50). Both *Salmonella* spp. and *P. carotovorum* are known to possess functional AI-2 signaling systems, making interspecies communication feasible. Therefore, with this study, we tested the hypothesis that AI-2-mediated signaling contributes to the fitness of *S. enterica* sv. Typhimurium within soft rots caused by *P. carotovorum* on tomato.

The rationale for this hypothesis is provided by reports that normal microbiota recovered from several types of fresh produce were positive for AI-2-like activity, and rinses taken from the surface of tomato fruit were able to enhance biofilm formation of an *E. coli luxS* mutant in polystyrene plates, suggestive of AI-2 activity (29). AI-2 activity is known to peak in late exponential phase before declining into stationary phase in both species, which could be an indication of conserved regulatory mechanisms (15,51). Interruption of AI-2 signaling in *P. carotovorum* delays production of virulence factors, including PEL, at low population densities, and *luxS*-deficient strains are less virulent in potato (15,28). *Salmonella luxS* mutants show differential regulation of virulence phenotypes in vitro and in vertebrate hosts (8,14,25,42, 64). However, in *Salmonella* spp., the only known target of AI-2 is the *lsrACDBFG* operon, whose sole known function is AI-2 uptake and processing (56). The *lsrACDBFG* operon and the divergent *lsrR* are controlled by a bidirectional promoter region upstream of *lsrA*. Typically, LsrR acts as a repressor, blocking the *lsrA* promoter until AI-2 reaches a sufficient intracellular concentration to relive repression by LsrR and allow transcription of *lsrACDBFG*.

Little is known about the role of AI-2 signaling in enteric bacteria during colonization of soft rots. A *Salmonella luxS* mutant has no affect on colonization of cilantro leaves and neither the mutant nor wild-type *Salmonella* sp. benefits from co-colonization of cilantro with AI-2-producing epiphytes (8). However, only a signal synthase (*luxS*) mutant was tested in that study, opening the possibility that the production of AI-2 by the plant's native epiphytes complemented the signaling defect in trans. Furthermore, *Salmonella luxS* activity is known to increase in response to the rich environment, acidic pH, and high osmolarity of the human gut (51). These conditions are not present on plant surfaces but are similar to those encountered in tomato fruit, which may represent an environment suitable for *Salmonella* AI-2 activity. The goal of this study was to determine the contribution of AI-2 signaling to the fitness of *Salmonella* spp. in market tomato as well as in tomato with *P. carotovorum* soft rots. For this study, competitive fitness was defined as the relative ability of a particular strain to multiply to a higher population density compared with the isogenic wild-type strain and was evaluated using defined *luxS* and *lsr* operon mutants. Resolvase in vivo expression technology (RIVET) reporters within the corresponding promoters were also used to evaluate the activity of AI-2-related genes within the tomato environment.

MATERIALS AND METHODS

Strains and culture conditions. *Salmonella* and *E. coli* strains were grown at 37°C and *Pectobacterium* strains at 30°C in Luria-Bertani (LB) medium, unless noted, with antibiotics as necessary. Overnight cultures of *Pectobacterium* SCC3193 and SCC6023 for use in tomato infections were grown in LB 0.2% (wt/vol) glucose. Without pre-growth in a glucose-containing medium, these strains induced soft rots unreliably. This step was not necessary for *Pectobacterium* SR38, a strain originally isolated as hypervirulent in tomato (3). Antibiotics were used at the following final concentrations: ampicillin (200 μg/ml), kanamycin (50 μg/ml) tetracycline (10 μg/ml), and chloramphenicol (10 μg/ml). All strains used in this study are listed in Table 1.

Mutant construction. Deletion mutants were constructed using the methods described by Datsenko and Wanner (16) by removing the portion of the gene between the stop and start codons. RIVET reporters were constructed using the previously described methods as adapted for *Salmonella* spp. (13,31,37). *S. enterica* sv. Typhimurium 14028 was used as the host for all deletion mutants and its derivative JS246 as the host for all RIVET reporters. Primers used are listed in Table 1. The *lsrG* RIVET reporter (CEC0015) was constructed via integration of pGOA1193 containing an *lsr* operon fragment starting 234 bp upstream of *lsrG* (located in *lsrF*) and ending 24 bp upstream of the putative location of the *lsrACDBFG* operon transcriptional terminator (primers cec045 and cec046), as inferred by homology to *E. coli*. This recombination results in a merodiploid generating an *lsrG::tnpR-lacZ* fusion along with the duplication of an intact *lsrG*, ≈7,000 bp downstream of the insertion site. The insertion was confirmed with primers cec047 and MT59. As the last gene in the *lsr* operon, the *lsrG::tnpR-lacZ* reporter should record all activity driven via the *lsrACDBFG* operon promoter, P_{lsrA}, with minimal disruption to the operon. Additionally, microarray evidence from *E. coli* shows that *lsrG* is highly activated by *luxS* in stationary phase, suggesting that the locus is an ideal location for a reporter (60). Reporter strain CEC0018 was constructed by transducing *ΔluxS*51::FRT-*kanR*-FRT from MM_019-C10 into CEC0015 via phage P22-mediated generalized transduction. A P_{luxS}-*tnpR-lacZ* reporter (CEC0026) was constructing using λ-red recombination to replace all of *luxS*, including the start and stop codons (primers cec111 and cec112), with FRT-*kanR*-FRT amplified from plasmid pKD4. Primers cec050 and cec051 were used to confirm the insertion or *kanR* as well as its removal via plasmid pCP20. The resulting FRT scar was utilized as an integration site for the suicide plasmids pCE70 or pCE71, creating a fusion of the *luxS* promoter and *tnpR-lacZ*. Following the electroporation of pCE70 or pCE71, transformants were screened with primers cec113 and BA184 to select those in which the pCE7x plasmid integrated in the desired orientation.

Confirmation of AI-2 production via the *Vibrio harveyi* LUX assay. The production of AI-2 by the *S. enterica* and *P. carotovorum* strains used in this study was confirmed using the *Vibrio harveyi* BB170 reporter assay, as previously described (4,54). To prepare *Salmonella* and *Pectobacterium* culture filtrates, overnight cultures were washed three times in phosphate-buffered saline (PBS) and diluted 1/100 into fresh LB without antibiotics. Subcultures were harvested at an optical density at 600 nm (OD_{600}) of ≈1.00 by centrifuging 1 ml of culture at 13,000 × *g* for 1 min to pellet cells. The resulting supernatant was sterilized by passing through a 0.22-μm filter. Recovered culture filtrates were stored at –20°C until use.

Competitive fitness assays. The competitive fitness of defined deletion mutants compared with wild-type *S. enterica* sv. Typhimurium 14028 was determined using a competitive index, as described previously (36). Briefly, unwaxed red ripe market tomato fruit ('Campari', grown hydroponically and purchased at local grocery stores) or unwaxed mature green tomato fruit ('Florida 47' [FL47], grown conventionally and obtained directly from processors or harvested in the field) were wounded three times by piercing the skin with flame-sterilized paper clips. Care was taken to wound the tomato between the seed sacks for a more uniform infection. For soft-rot treatments, 3 μl of a *Pectobacterium* suspension (10^9 CFU/ml) was introduced into each wound and allowed to infiltrate the tomato tissue. Next, 3 μl of a roughly 50:50 mix of the wild-type and mutant *Salmonella* (10^4 CFU/ml) was introduced to each wound. *S. enterica* sv. Typhimurium

14028 and its isogenic tetracycline-resistant derivative JS246 were similarly inoculated as a control. Prior to the infections, all overnight cultures were washed three times and resuspended in PBS. A sample of each inoculum was plated on xylose lysine deoxycholate agar (XLD) and 50 individual colonies were patched to LB kanamycin or tetracycline plates to determine the initial mutant/wild-type ratio. All tomato fruit were incubated at 22°C for 3 days for green fruit and 5 days for red fruit. These incubation times were sufficient for development of full soft-rot symptoms in fruit inoculated with *Pectobacterium. Salmonella* were recovered directly from the wound using a flame-sterilized wire loop cooled in sterile PBS, quad streaked onto XLD, and then incubated at 42°C to limit the growth of phytobacteria. Severely rotted tomato fruit were harvested by stomaching in a Whirl-Pak bag (Nasco, Fort Atkinson, WI) along with 50 ml of PBS at 260 rpm in a stomacher for 1 min. An aliquot of the resulting homogenate was plated on XLD and then incubated at 42°C. After overnight growth, 50 individual colonies were patched to LB plates with appropriate antibiotics to count the number of antibiotic-resistant mutant colonies. Shifts in the mutant/wild-type ratio between the inoculum and recovered samples were used to calculate a competitive index (CI) according to the following equation: $CI = [M_{out}/WT_{out}]/[M_{in}/WT_{in}]$, where M is the number of mutant cells and WT is the number of the wild-type cells in the inoculum (in) or in the recovered samples (out). The CI values were log transformed to allow even comparison between increases and decreases in competitive fitness.

Response of the *lsrG-tnpR* reporter to exogenous AI-2. Both LacZ activity and RIVET resolution of strain CEC0018 (*luxS*$^{-}$) were used to test for perception of exogenous AI-2 by the *lsrG::tnpR-lacZ* reporter. Strain CEC0015 was used as a positive control for *luxS*$^{+}$ expression levels. Synthetic (S)-4,5-dihydroxy-2,3-pentandione (DPD), the universal AI-2 precursor, was supplied as a 3.7-mM stock solution from OMM Scientific (Dallas, TX) and stored at –20°C until use. Prior to assays, overnight cultures of the reporters were started from glycerol stocks in LB with antibiotics. Cells were washed to remove tetracycline and diluted 1/100 into fresh LB without antibiotics. DPD was used at a final concentration of 10 μM and added to the medium just prior to inoculation of the assay or after 2 h of growth. Other authors have indicated that incubation with 10 μM DPD is sufficient to generate strong activation of previously reported *lsr* operon reporters (18,33,55). Assays were performed at 37°C in 10 ml of LB. Samples were taken at 0, 2, 4, and 8 h for analysis of LacZ activity and at 24 h for analysis of TnpR resolution. LacZ activity was determined using a modified Miller assay based on the adapted 96-well protocol of Griffith and Wolf (22,32). Plates were analyzed using a multimode microtiter plate reader (Victor3; Perkin Elmer, Fremont, CA), equipped with Wallac1420 Manager Work-station software. Modified Miller units were calculated as follows: modified Miller units = $[1{,}000 \times \text{absorbance}_{405}]/[t_{\min} \times \text{sample volume}_{ml} \times OD_{600}]$. At 24 h, an aliquot of the culture was streaked onto XLD at 37°C using a sterile wire loop. After overnight growth, 50 colonies were patched to LB tetracycline plates which were incubated overnight at 37°C. The resolution was determined as the number of the tetracycline-sensitive colonies divided by the total number of patched colonies.

In vitro reception of *Pectobacterium* AI-2 signaling by *Salmonella* spp. The *Salmonella lsrG::tnpR-lacZ* RIVET reporter strains CEC0015 and CEC0018 were used to test whether the

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmids, and primers	Characteristics or sequence	Source
Salmonella enterica serovar Typhimurium		
14028	Wild-type strain	ATCC[a]
JS246	14028 *yje*P8103::*res*1-*tetAR-res*1	31
MM_019-C10	14028 Δ*luxS*51::FRT-*kanR*-FRT	This study
MM_074-D12	14028 Δ*lsrACDBF*52::FRT-*kanR*-FRT	This study
MM_015-G07	14028 Δ*lsrG*53::FRT-*kanR*-FRT	This study
CEC0015	JS246 *lsrG*901::*tnpR-lacZ* ampR	This study
CEC0018	JS246 *lsrG*901*::tnpR-lacZ* Δ*luxS*51::FRT-*kanR*-FRT ampR	This study
CEC0026	P_{luxS}-*tnpR-lacZ* Δ*luxS*902 kanR	This study
CEC0035	14028 Δ*lsrACDBF*52 Δ*luxS*51::FRT-*kanR*-FRT	This study
Pectobacterium carotovorum subsp. *carotovorum*		
SR38	Wild-type isolated from soft-rotted Florida tomato	3
SCC3193	Wild-type isolated from soft-rotted Finnish potato	28
SCC6023	SCC3193 *luxS*::CmR	28
Vibrio harveyi		
BB152	BB120 *luxLM*::Tn5	4
BB170	BB120 *luxN*::Tn5	4
Plasmids		
pCR2.1-TOPO	General cloning vector *lacZ*α (kanR, ampR)	Invitrogen
pGOA1193	pIVET5n with promoterless *tnpR-lacZY*α (ampR)	37
pKD4	*oriR6K bla rgnB* FRT-*kanR*-FRT (kanR)	16
pKD46	*repA101ts oriR101 araC* P_{araB}- λ*Red(γ-β-exo)-tL3*) (ampR)	16
pCP20	*repA101ts* λ_{pR}-Flp *ci857 (*ampR, kanR*)*	13
pCE70	*oriR6K* FRT-*promoterless tnpR-lacZY*α (kanR)	31
pCE71	*oriR6K* FRT-*promoterless tnpR-lacZY*α (kanR)	31
Primers		
MT59	CAAAAAGTCGCATAAAAATTTATCC	…
cec045	CTCGAGAGGCGATTGACCAGGGGGCT	…
cec046	CTCTGAGGGTTCAAGCTGCTCCACGCA	…
cec047	TGCTGCTGCCGCACAGGTTT	…
cec050	TGCTAAAAACCCCATCGACCGGC	…
cec051	ATTGGCGGCACCGGGAAAGC	…
cec111	GCCATAAACCGGGGTTAATTTAAATACTGGAACCGCTTACAAATAAGAt gtaggctggagctgcttcg	…
cec112	GGAACAAAGAGTTCAGTTTATTTTTAAAAAATTATCGGAGGTGACTAAc atatgaatatcctccttag	…
cec113	GAAGGCATTGGCGGCACC	…
BA184	GATGTGCTGCAAGGCGATTAAGTTG	…

[a] American Type Culture Collection.

perception of AI-2 produced by the *P. carotovorum* strains SR38, SCC3193, or SCC6023 was at sufficient levels to drive *Salmonella* AI-2-dependent gene expression during co-cultures in soft agar. Both *S. enterica* CEC0018 and *P. carotovorum* SCC6023 lack the *luxS* synthase and are unable to produce their own AI-2. Overnight reporter cultures were washed three times in sterile PBS to remove antibiotics and diluted 1/10,000. The inoculum was mixed 1/100 into 7 ml of warm LB 0.3% soft agar, poured over the previously prepared 1.5% agar plates, and allowed to set in a sterile flow hood. Plates were incubated at 22°C and sampled at 24 h. Samples were recovered with a sterile wire loop directly from the soft agar and streaked to XLD plates incubated at 37°C overnight to select for *Salmonella* spp. RIVET resolution was then quantified as above.

In vivo promoter expression measured via RIVET assays. The RIVET reporters CEC0015, CEC0018, and CEC0026 were used to examine the activity of the *luxS* promoter and the *lsr* operon during the colonization of intact and soft-rotted green tomato fruit. Tomato infections were prepared in the same manner as for the competition assays, except each wound was inoculated with 3 µl of 10^4 CFU/ml dilutions of the RIVET reporter. The tomato fruit were incubated at 22°C for 2 days, which was sufficient for ≥20-mm soft-rot lesions to appear. *Salmonella* spp. were recovered directly from the wound using a flame-sterilized wire loop cooled in sterile PBS and quad streaked on XLD antibiotic plates incubated at 37°C.

Pectate lyase assay. The pectate lyase (PEL) activity of *Pectobacterium* SR38 was measured according to a modified version of the assay described by Matsumoto et al. (30). Briefly, SR38 was grown overnight in M9 with 0.4% glycerol and diluted 1/100 into M9 with 1.0% citrus pectin (Sigma-Aldrich, St. Louis) at pH 6.8. The subculture was grown for 8 h at 30°C and the OD_{600} recorded with a spectrophotometer (BioSPEC-mini, Shimadzu, Japan). A 1-ml aliquot was centrifuged at 16,000 × *g* for 3 min and 10 µl of the supernatant removed. The supernatants were mixed with 990 µl of PEL assay buffer (0.1 M Tris-HCl [pH 8.5], 0.1 mM $CaCl_2$, and 0.05% [wt/vol] sodium polypectate [M. Burger Enterprises, Madison, WI]) which was prewarmed to 30°C. The suspension was incubated at 30°C for 15 min before the OD_{230} was recorded with a spectrophotometer. The OD_{230} was rerecorded after an additional 10-min incubation at 30°C. One unit of PEL activity was defined as a 0.001 increase in OD_{230} per minute. *Salmonella* 14028 culture filtrates (1% vol/vol), DPD (10 µM), and 1/100 dilutions of *Salmonella* spp. were added to the SR38 culture 4 h into the incubation. The *Salmonella* strains were grown overnight in LB at 37°C and washed 3× in PBS to remove antibiotics prior to use.

Data analysis. Box plots, analysis of variance (ANOVA), pairwise Student's *t* test, Dunnett's *t* test, and the Tukey-Kramer honestly significant difference (HSD) method were generated using JMP 9.0 (SAS Institute, Cary, NC). Dunnett's *t* test was used to compare competitive co-infections in intact tomato fruit with the JS246 versus 14028 control. A two-tailed Student's *t* test with unequal variances was performed in Excel to compare similar competitive co-infections between tomato treatments; $\alpha = 0.05$ for all tests.

RESULTS

AI-2 activity of wild-type and mutant strains. Analysis of AI-2 activity using the *V. harveyi* BB170 reporter assay showed high levels of activation from 0.4 µM DPD, culture filtrates collected from the *luxS*$^+$ strains of *Pectobacterium*, and *luxS*$^+$ strains of *Salmonella* grown at 22°C (Fig. 1). Interestingly, the culture filtrates of the same strains of *Salmonella* grown at 37°C only activated the *V. harveyi* reporter ≈10-fold (at least 10-fold less than the culture filtrates harvested from the cultures grown at 22°C). In both species, an *luxS* mutation essentially eliminated detectable AI-2 activities in the supernatant, consistent with the function of LuxS in AI-2 synthesis (52). The supernatant of the hypervirulent *P. carotovorum* SR38 produced approximately double the AI-2 activity of the laboratory strain SCC3193 (Fig. 1). Whether or not this elevated production of AI-2 is linked to the hypervirulence of SR38 is not known and was not tested with this study.

Fitness of *Salmonella luxS* and *lsr* operon mutants in soft rots. The fitness of defined AI-2 *Salmonella* mutants was tested under several conditions in order to examine the effect that AI-2 signal production or perception, growth in two common market cultivars, or the presence of soft rot has on the proliferation of *Salmonella* spp. in tomato (Fig. 2). None of the mutations tested under any of the conditions, intact green tomato ($F = 1.206$, $P = 0.321$), green tomato with soft rots caused by *P. carotovorum* SR38 ($F = 1.537$, $P = 0.203$), intact red tomato ($F = 1.537$, $P = 0.203$), or red tomato with SR38 soft rots ($F = 1.234$, $P = 0.309$) were determined to be significant by ANOVA, indicating that

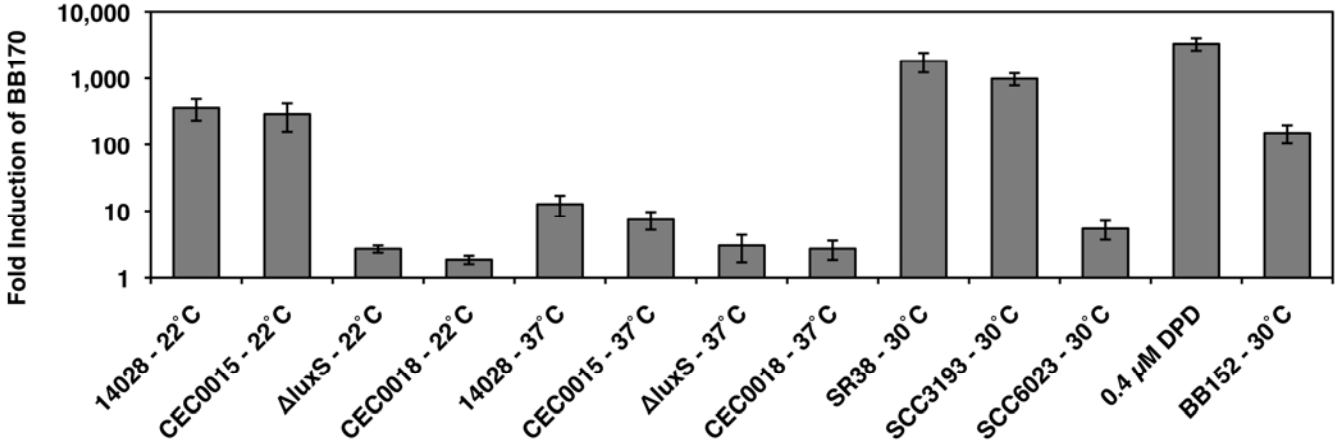

Fig. 1. AI-2 activity in culture filtrates of *Salmonella* and *Pectobacterium* strains as measured with the *Vibrio harveyi* BB170 reporter. The *luxS*+ *Pectobacterium* strains (SR38 and SCC3193) produced strong AI-2 activity as expected. Incubation temperature strongly affects the AI-2 activity of *luxS*+ *Salmonella* strains (CEC0015 and 14028), with an approximately 30× increase in AI-2 activity at 22°C compared with activity at 37°C. As expected, an *luxS* mutation nearly eliminated AI-2 activity in *Salmonella* CEC0018 and *Pectobacterium* SCC6023. Culture filtrate of *V. harveyi* BB152 (N-acyl homoserine lactone$^-$ and AI-2$^+$) was used as a positive control. Synthetic AI-2 ((S)-4,5-dihydroxy-2,3-pentandione) was diluted in AB medium and added at a final concentration of 0.4 µM as an additional control. AI-2 activity was measured for three independent samples. Each independent sample consisted of four technical replicates. Bars represent standard error.

none of the *Salmonella* mutations significantly altered the competitive fitness compared with the wild type. Overall, the majority of the mean logCI values fell between –0.25 and 0.25. Previous studies have considered only a CI of ≥3 to be biologically relevant (log 3 = |0.477|) (48). Only two assays, *luxS* versus 14028 in SR38 soft-rotted green tomato (logCI = 0.491) and *luxS lsrACDBF* versus 14028 in SR38 soft-rotted red tomato (logCI = 0.695) exceed this arbitrary threshold. However, the Tukey-Kramer HSD confirmed that these increases were not significant.

In comparing the mean logCI results for each mutant between treatments, growth in SR 38 soft rots noticeably increased the competitive fitness of the *luxS* mutant (ΔlogCI = 0.624, P = 0.070) in green tomato and both the *luxS* mutant (ΔlogCI = 0.431, P = 0.223) and *luxS lsrACDBF* double mutant (ΔlogCI = 0.386, P = 0.274) in red tomato. However, the effects due to SR38 soft rots were not significant as determined by Student's t test.

Interestingly, the *luxS* mutant showed a trend of decreased fitness while the *luxS lsrACDBF* double mutant showed a trend of increased fitness in red Campari tomato compared with green FL47 for both intact and soft-rotted samples. However, due to differences in cultivar and incubation times, the effect of ripeness stage could not be analyzed.

Effect of *Pectobacterium luxS* genotype on the fitness of *Salmonella luxS* and *lsr* operon mutants in soft rots. Competitive fitness assays in green tomato fruit soft rotted by either wild-type (SCC3193) or *luxS*⁻ (SCC6023) *Pectobacterium* strains showed that both the *Salmonella luxS* and *luxS lsrACDBF* mutants were more competitive than the wild type only in the soft rots caused by *Pectobacterium* SCC6023 (Fig. 3). The increase in fitness for the *Salmonella luxS* mutant was significant by ANOVA (F = 3.706, P = 0.038); however, no pairs were significant when analyzed with the more conservative Tukey-Kramer HSD test (P = 0.054, intact green tomato versus SCC6023 soft rot and P = 0.070, SCC3193 soft rot versus SCC6023). The increase in fitness for the *luxS lsrACDBF* double mutant was not significant by ANOVA (F = 2.925, P = 0.069) and, similarly, no pairs were significant according to the Tukey-Kramer HSD test (P = 0.266, intact green tomato versus SCC6023 soft rot and P = 0.060, SCC3193 soft rot versus SCC6023).

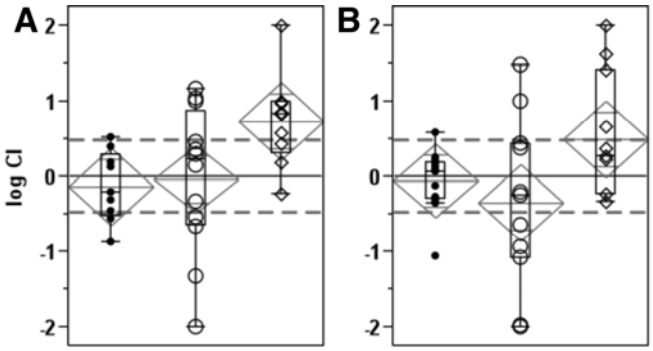

Fig. 3. Effect of *Pectobacterium luxS* genotype on the *Salmonella* AI-2 mutants **A,** MM_074-D12 *(luxS–)* and **B,** CEC0035 (*lsrACDBF-luxS–*). The fitness of the *Salmonella* mutants in non-soft-rotted (●) or *Pectobacterium* SCC3193 (*luxS+*, ○) or *Pectobacterium* SCC6023 (*luxS–*, ◇) soft-rotted green tomato fruit was compared using the Tukey Kramer honestly significant difference test. No results were significant. Box plots (represented by rectangles) show 10, 25, 75, and 90% quantiles. Points outside the whisker lines are treated as outliers. Gray diamonds represent analysis of variance, where the middle line is the mean and the upper and lower lines show the 95% confidence intervals. Dashed gray lines correspond to log competitive index = |0.477|, the threshold for differences in competitive fitness considered to be biologically relevant. The zero line indicates no difference in competitive fitness.

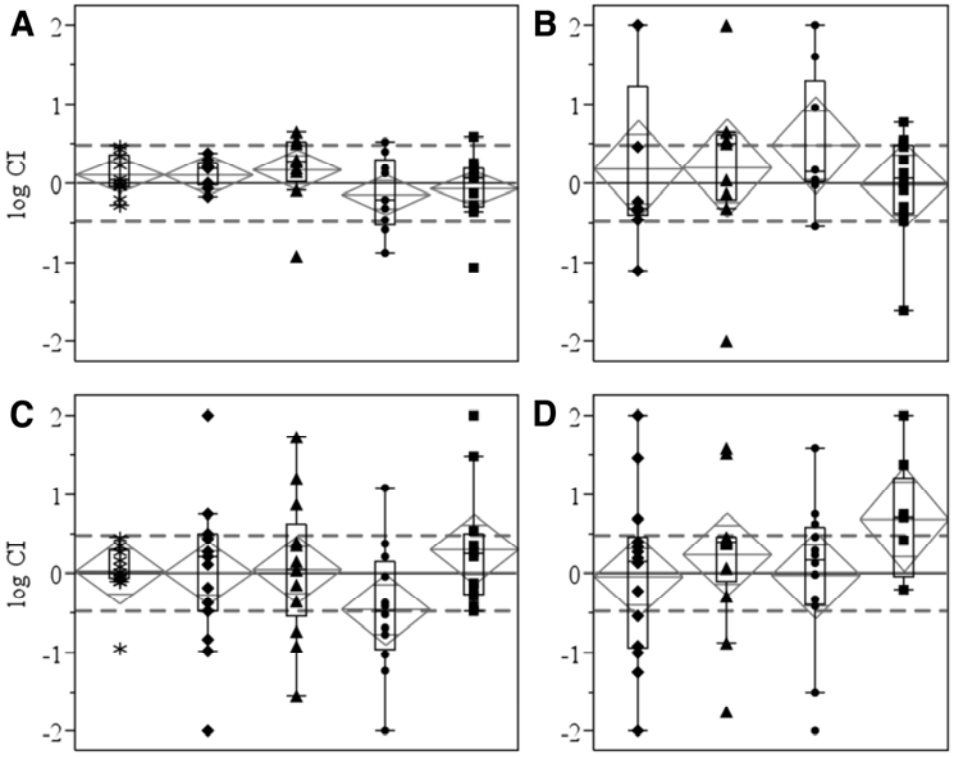

Fig. 2. Competitive fitness of defined *Salmonella* AI-2 mutants versus the isogenic wild type determined by competitive index in **A,** intact green 'FL47' tomato fruit; **B,** *Pectobacterium* SR38 soft-rotted green FL47 tomato fruit; **C,** intact red 'Campari' tomato fruit; and **D,** *Pectobacterium* SR38 soft-rotted red Campari tomato fruit. The fitness of the mutants MM_019-C10 (*lsrACDBF*, ◆), MM_015-G07 (*lsrG*, ▲), MM_074-D12 *(luxS,* ●), and CEC0035 (*lsrACDBF-luxS*, ■) was compared with the Tukey Kramer honestly significant difference test (B and D) or to the JS246 control (*yjeP,* ✱) using Dunnett's t test (A and C). No results were significant. Box plots (represented by rectangles) show 10, 25, 75, and 90% quantiles. Points outside the whisker lines are treated as outliers. Gray diamonds represent analysis of variance, where the middle line is the mean and the upper and lower lines show the 95% confidence intervals. Dashed gray lines correspond to log competitive index = |0.477|, the arbitrary threshold for differences in competitive fitness considered to be biologically relevant. The zero line indicates no difference in competitive fitness.

Response of *lsrG-tnpR* to synthetic AI-2. Because of the lack of effect on fitness observed for the *Salmonella* AI-2 mutants, the ability of the *lsr* operon to respond to exogenous AI-2 was examined using RIVET reporters. The *lsrG::tnpR-lacZ* reporter was constructed in *luxS*$^+$ and *luxS*$^-$ backgrounds to distinguish between responses to the *luxS* genotype and exogenous AI-2 signal. Response to synthetic AI-2 was tested in LB liquid culture at 37°C; however, the addition of 10 μM DPD, the synthetic AI-2 precursor, resulted in only a small increase in activity and failed to rescue the *luxS*$^-$ reporter back to wild-type levels (Fig. 4). Results of the RIVET assay showed that the reporter responded strongly to the *luxS* genotype but only weakly to the presence of exogenous AI-2. The results of the Miller assay showed that a relatively small increase in LacZ activity, <2×, generated a large (40×) difference in TnpR-based resolution. Because it is based on enzymatic activity, the Miller assay allows for a continuous reporter response, whereas RIVET requires a critical threshold concentration of TnpR for resolution. These results show that the responses generated by the chromosomal copy of *luxS* are much stronger than external signal molecules for this reporter system.

In vitro perception of the *Pectobacterium* AI-2 signal by *Salmonella* spp. The ability of AI-2-producing cells to alter gene expression in *Salmonella* spp. was tested by co-inoculating the *Salmonella* RIVET reporter, in both *luxS*$^+$ and *luxS*$^-$ backgrounds, with wild-type *Salmonella* or *Pectobacterium* strains on LB soft-agar plates. The response of the *Salmonella* RIVET reporter-only controls showed no difference in resolution between incubation at 37 or 22°C, indicating that the reporter was active at environmental temperatures (Fig. 5). A significant difference in resolution due to the reporter's *luxS* genotype was observed for the reporter-only controls; however, the presence of AI-2-producing strains of *Salmonella* or *Pectobacterium* was unable to rescue activity of the *Salmonella luxS*$^-$ RIVET reporter back to wild-type *luxS*$^+$ levels. Co-culture with the AI-2-producing *Pectobacterium* strains increased resolution in the *luxS*$^+$ *Salmonella* reporter; however, the increase was not significant and was not duplicated in the *luxS*$^-$ reporter.

In vivo promoter expression measured via RIVET assays. To determine how soft rots affected the activity of *Salmonella luxS*, the activity of CEC0026 (P_{luxS}-*tnpR-lacZ*) was determined in green tomato with *Pectobacterium* soft rots (Fig. 6A). The reporter was strongly expressed in all samples, indicating that the *luxS* promoter was highly active in intact and *Pectobacterium* soft-rotted green tomato fruit. The small reduction of expression in *Pectobacterium* SR38 soft rots was the result of 3 of 11 samples not reaching 100% resolution and was not significant as determined by a *t* test comparison with non-soft-rotted green tomato ($P = 0.193$).

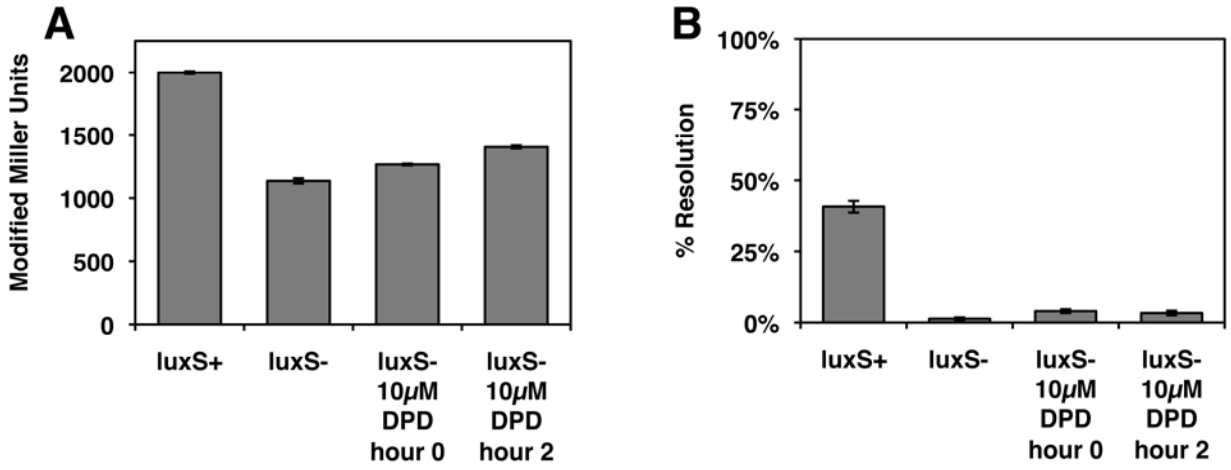

Fig. 4. Activity of the *lsrG::tnpR-lacZ* reporter in response to synthetic AI-2. **A,** Expression of the resolvase in vivo expression technology (RIVET) reporter was determined after 24 h of growth in Luria-Bertani liquid culture at 37°C. Addition of synthetic AI-2 (10 μM (S)-4,5-dihydroxy-2,3-pentandione [DPD]) increased resolution of the *lsrG::tnpR-lacZ luxS*$^-$ reporter (CEC0018) but did not rescue resolution to the level of the *luxS*$^+$ reporter (CEC0015). Bars represent standard error of three technical and three biological replicates. **B,** LacZ activity 8 h after inoculation. Differences between groups are all significant according to the Tukey-Kramer honestly significant difference test; however, addition of DPD does not fully rescue activity of the *luxS*$^-$ background back to the wild-type level. Bars represent standard error of four technical and three biological replicates.

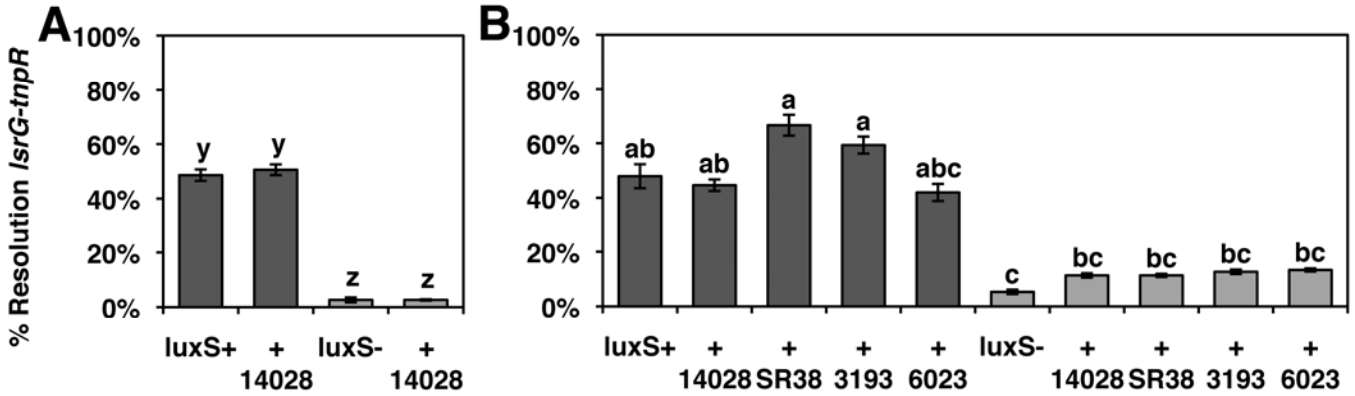

Fig. 5. Resolution of the *lsrG::tnpR-lacZ* reporter in response to co-culture with *Salmonella* or *Pectobacterium* strains SR38 (*luxS*$^+$), SCC3193 (*luxS*$^+$), and SCC6023 (*luxS*$^-$) on Luria-Bertani soft agar at **A,** 37°C and **B,** 22°C. Letters represent significance groups assigned by the Tukey-Kramer honestly significant difference test. Differences are only significant for those groups which share no letters in common. Dark bars represent the *luxS*$^+$ background reporter (CEC0015) and the light bars represent the *luxS*$^-$ background reporter (CEC0018). Co-cultured strains were mixed 1:1 prior to inoculation of the soft agar. Bars represent standard error of three technical and three biological replicates.

Expression of *lsrG::tnpR-lacZ* in response to the soft rots in green tomato was dependent on the *luxS* background of the *Salmonella* host as well as the *Pectobacterium* strain which caused the soft rot (Fig. 6B). In non-soft-rotted green tomato ($P < 0.001$) and *Pectobacterium* SCC3913 (*luxS*$^+$) soft-rotted tomato ($P = 0.026$), there was a significant decrease in RIVET resolution in the *Salmonella luxS*$^-$ reporter (CEC0018) compared with the *luxS*$^+$ reporter (CEC0015). The reduction in resolution was of a magnitude similar to that seen between the same reporters due to their *luxS* backgrounds during the in vitro assays. Soft rots caused by *Pectobacterium* strains SR38 (*luxS*$^+$) ($P = 0.134$) and SCC6023 (*luxS*$^-$) ($P = 0.781$) showed nonsignificant decreases in resolution between the *Salmonella luxS*$^-$ reporter compared with the *luxS*$^+$ reporter. The lack of significance may be due to the reduction in resolution seen with the *luxS*$^+$ reporter in these soft rots compared with intact green tomato, which reduces the reporter's dynamic range. However, *Pectobacterium* SR38 soft rots significantly reduced *Salmonella* RIVET resolution compared with non-soft-rotted tomato regardless of the reporter's *luxS* genotype, ($P < 0.001$ for CEC0015/SR38 and $P = 0.002$ for CEC0018/SR38). SCC3913, which is also *luxS*$^+$, had no affect on resolution ($P = 0.924$ for CEC005/SCC3913 and $P = 0.992$ for CEC0018/SCC3193). Interestingly, the *luxS*$^-$ strain SCC6023 caused a significant reduction in *lsrG::tnpR-lacZ* expression in *luxS*$^+$ *Salmonella* ($P = 0.001$ for green tomato versus SCC6023) and a small, nonsignificant increase in expression in *luxS*$^-$ *Salmonella* ($P = 0.537$ for green tomato versus SCC6023). Taken together, these results show that interactions between *Pectobacterium* and *Salmonella* spp., which alter *Salmonella* gene regulation, are influenced by environmental conditions and strain genotype but not AI-2 signal exchange.

PEL activity in response to exogenous AI-2. Although results of the competitive fitness and RIVET assays clearly showed that AI-2 signal exchange does not significantly affect the fitness of *Salmonella* spp., they also show that unknown interactions which influence *Salmonella* fitness and gene expression occur under certain circumstances. To test whether these interactions could impact the PEL activity of *Pectobacterium* spp., synthetic DPD, *Salmonella* strains, and culture filtrates were incubated with *Pectobacterium* SR38 under PEL-inducing conditions (Fig. 7). Only culture filtrates from *Salmonella* spp. grown at 37°C, which showed low AI-2 activity in the *V. harveyi* assay, significantly ($P = 0.003$) decreased the PEL activity of SR38 in regards to the *Pectobacterium* SR38 control. Because the filtrate was generated from cultures grown in LB, their addition also altered the metabolic conditions of the M9 1% pectin induction media. The additional nutrients allowed a slightly faster growth rate and reduced reliance on PEL, which could result in lower PEL activity. These results suggest that nutritive conditions are more relevant to *Pectobacterium* PEL activity than AI-2-based signal exchange under the conditions tested.

DISCUSSION

Because the role of AI-2 signaling in *Salmonella* spp. has primarily been studied in relation to vertebrate hosts, there are few examples of its potential functions in interactions with phytobacteria. A study of fresh produce identified AI-2 activity in

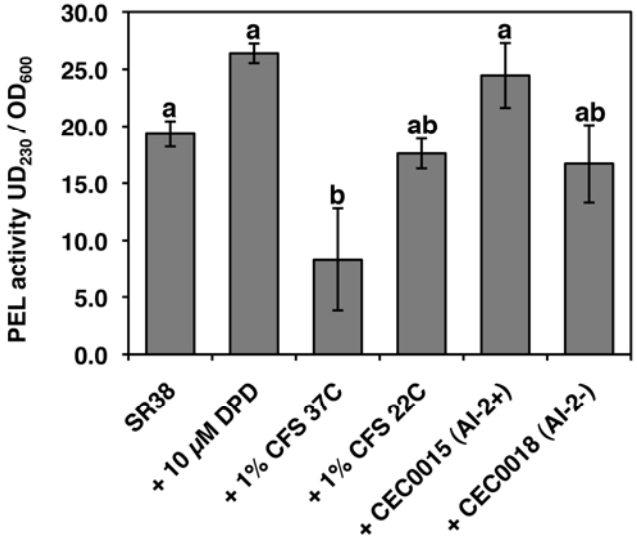

Fig. 7. Pectate lyase activity of *Pectobacterium* SR38 in response to co-culture with *Salmonella* spp., synthetic AI-2 ((S)-4,5-dihydroxy-2,3-pentandione), or culture filtrates from *Salmonella* 14028 cultures grown at 22 or 37°C. Letters represent significance groups assigned by the Tukey-Kramer honestly significant difference test. Bars represent standard error of three technical and three biological replicates.

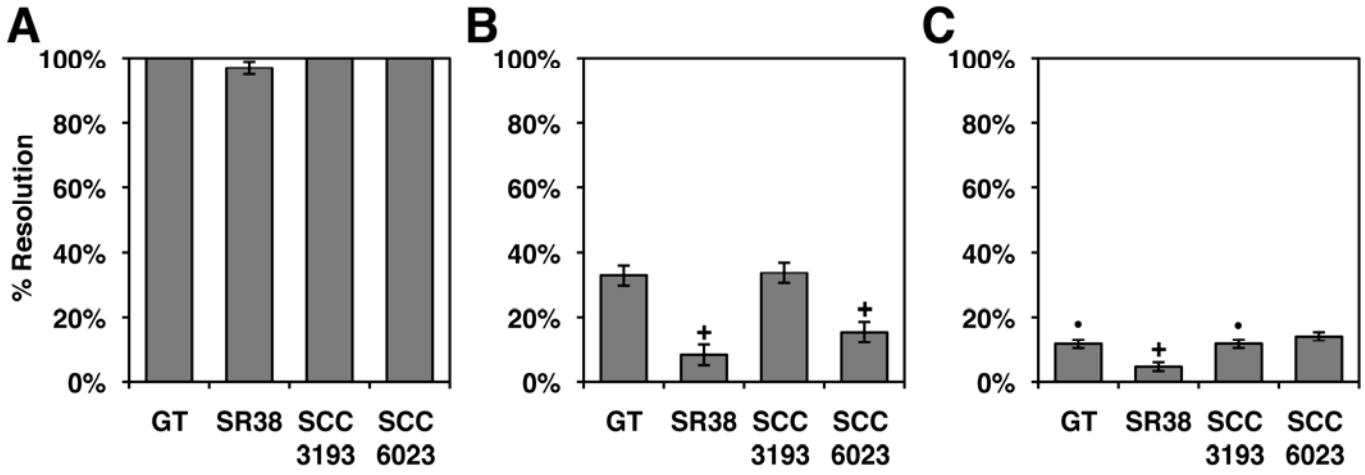

Fig. 6. Resolution of resolvase in vivo expression technology (RIVET) reporters in intact green tomato fruit (GT) and those with soft rots caused by *Pectobacterium* strains SR38 (*luxS*$^+$), SCC3193 (*luxS*$^+$), and SCC6023 (*luxS*$^-$). Resolution of **A,** CEC0026 (P_{luxS}-*tnpR-lacZ*); **B,** CEC0015 (*lsrG::tnpR-lacZ luxS*$^+$); and **C,** CEC0018 (*lsrG::tnpR-lacZ luxS*$^-$) during 48-h infections. Symbols: **+** denotes a significant difference (Student's *t* test) within reporter group due to the strain of *Pectobacterium carotovorum* and **•** indicates a significant difference (Student's *t* test) between the CEC0015 and CEC0018 reporters due to the *Salmonella luxS* mutation. Bars represent standard error, resolution was determined as an average of three technical replicates per sample; $n \geq 11$ samples for each assay. All assays were carried out at 22°C for 2 days.

surface swabs of 11 of the 12 commodities examined, including tomato, indicating that AI-2-based signaling is likely to be widespread in the phyllosphere (29). The AI-2 activity recovered from surface washes during a 9-day study of stored Roma tomato fruit varied independently of the total heterotrophic bacterial count, indicating that the concentration is dynamically controlled by the commensal population or that unculturable members of the phytomicrobiota are responsible for a significant portion of the AI-2 production (29).

The only known previous study to examine competitive fitness phenotypes of AI-2-related mutants of *Salmonella* in produce found no difference in ultimate population density, growth dynamics, or formation of cell aggregates (an important survival phenotype to resist desiccation in the phyllosphere) between an *luxS* mutant and wild-type *Salmonella* spp. during colonization of cilantro leaves (8). The same *luxS* mutation significantly reduced population densities in the intestines, spleen, and feces during infection of live chicks (8). The *luxS* mutant utilizes some carbon sources less efficiently compared with the wild type and these differences in the nutrition available to *Salmonella* spp. in the respective environments are hypothesized to be responsible for the differences in fitness observed for the *luxS* mutant between colonization of chicks and cilantro leaves (8).

Studies of AI-2-related phenotypes in *Salmonella* spp. and *E. coli* have typically used *luxS* mutants to eliminate AI-2 production. Because LuxS has a dual role in signal production and the degradation of toxic intermediates in the activated methyl cycle, it is difficult to separate effects due to metabolic regulation from those specifically associated with AI-2 signaling during assays which only use an *luxS* mutant (17,23,40,57). In this study, the use of an *lsrACDBF*, "signal-blind" mutant provided an opportunity to observe phenotypes related specifically to the perception of AI-2 signaling without the metabolic side effects of the *luxS* mutation. However, the competitive fitness of the *lsrACDBF* mutant was essentially unaltered compared with *S. enterica* 14028 in intact or soft-rotted tomato fruit, indicating that the perception of AI-2 did not significantly affect the fitness of *Salmonella* spp. The *luxS* and *luxS lsrACDBF* mutants showed a statistically nonsignificant trend of increased fitness in the presence of *Pectobacterium* SR38 soft rots in both green FL47 and red Campari tomato, indicating that LuxS may induce changes in *Salmonella* spp. which influence cross-species interactions. The use of *Pectobacterium* SCC3192 and its isogenic *luxS* mutant SCC6023 for competitive fitness assays allowed evaluation of how the *Pectobacterium luxS* phenotype affected *Salmonella* survival in soft rots. Both *Salmonella luxS* and *luxS lsrACDBF* mutants showed a trend toward increased competitive fitness in SCC6023 (*luxS*$^-$) soft-rotted green tomato fruit, providing additional evidence that LuxS-mediated interactions may occur in soft rots, although it remains unclear how *luxS* mutations in both bacteria could increase the fitness of *Salmonella* spp.

A mutation in *luxS* did not affect in vitro growth rate of the *Salmonella* or *Pectobacterium* strains in this study. Deletion of *luxS* is linked to a reduction of motility in a number of species, including *Salmonella* spp. and *P. carotovora* (15,23,25). Although motility is linked to plant virulence in *P. atroseptica*, an *luxS* mutation in *P. carotovora* reduces motility but does not substantially attenuate potato virulence (15,28,34). Similarly, no reduction in the tomato virulence due to an *luxS* mutation in *P. carotovorum* was observed in this study. The presence of flagella is known to reduce the competitive fitness of *Salmonella* spp. in planta and a number of motility genes, including those associated with flagella, were identified by microarray as upregulated in wild-type *Salmonella* spp. compared with an *luxS* mutant (24,25,35). However, the hypothesis that the effects of *luxS* mutation on motility are indirectly responsible for the phenotype of the mutants in soft rots is unlikely to be true because motility genes were shown to be downregulated inside tomato soft rots (21).

The metabolic conditions in ripening tomato fruit are complicated, because the concentrations of at least 60 metabolites constantly change during the ripening process (10). *P. carotovorum* soft rots are a result of extensive tissue degradation and represent a dynamic environment where the ripening tomato is rapidly degrading into a nutrient-rich liquid, causing additional nutritive alterations. The drastic difference in the development time of *Pectobacterium* SR38 soft rots between red and green tomato and the inability of *Pectobacterium* SCC3193 and SCC6023 to consistently reproduce soft rots in red tomato indicated the importance of nutritive and environmental conditions to the establishment of bacteria within the tomato fruit. The difference in observed trends of competitive fitness between the *luxS* and *lsrACDBF luxS* mutant of *Salmonella* in green FL47 and red Campari tomato, although inconclusive, suggest regulation of metabolism by LuxS may also impact *Salmonella* fitness in tomato.

In general, there were large variations in the competitive index values for individual samples within each *Salmonella* mutant coinfection group for the intact red tomato and *Pectobacterium* SR38 soft-rot treatments. However, this level of variability was not observed in the intact green tomato treatment, the JS246 versus 14028 control infection of intact red tomato, or previously reported competitive co-infections with defined mutants of genes identified as active by a promoter probe screen in intact red tomato and, therefore, does not appear to be inherent in the assay (35). Thus, the high variability in treatments other than intact green tomato observed during this study likely indicates that survival in these nutritionally dynamic environments is driven to an important extent by stochastic processes or is a result of interactions of multiple factors. Differing nutritive conditions are known to cause large changes in the LuxS regulon which could contribute to the trends in competitive fitness observed for *Salmonella* spp. in soft rots (25,39,59,60).

Microarray studies have shown that a majority of *luxS*-responsive genes are not influenced by the AI-2 signal itself. For example, in *Streptococcus mutans*, only 9.2% (59/644) of the genes differentially regulated between the wild type and an *luxS* mutant responded to exogenous AI-2 (53). In *E. coli* O157:H7, only 1.9% of genes (18/951) responded directly to synthetic AI-2 when metabolic effects of the high concentration of DPD (100 μM) were controlled (26). Of the 547 genes in the *Salmonella* LuxS regulon, only 43 genes (7.9%) were differentially regulated by both the *luxS* mutation and the addition of *Salmonella* culture filtrates with AI-2 activity (25,64). Of those genes, 31 (72.1%) generated a larger response in the *luxS* mutant, indicating that the presence of *luxS*, not AI-2 signal exchange, is primarily responsible for the regulatory changes.

In the current study a P_{luxS}-*tnpR-lacZ* (CEC0026) RIVET reporter was highly active in intact tomato as well as soft rots. Although culture filtrates taken from wild-type *Salmonella* cultures grown in LB at 22°C showed increased AI-2 activity compared with those grown at 37°C, expression of the *lsrG-tnpR* reporter on LB soft agar was similar at 22 and 37°C, indicating that the threshold concentration of AI-2 sufficient for regulatory activity was present at both temperatures. Because *Salmonella* AI-2 production is induced by preferred carbon sources (such as glucose), low pH, and high osmolarity, conditions which should all be present in tomato fruit, it is likely that *luxS* was expressed and AI-2 produced in tomato fruit under the tested conditions (51). The *lsrG::tnpR-lacZ* RIVET reporters had similar activity in vitro and in vivo, indicating that the physical environment of tomato fruit was permissive to perception of AI-2. The reporters CEC0015 (*luxS*$^+$) and CEC0018 (*luxS*$^-$) showed strong differences in resolution in response to their *luxS* genotype in vitro and during infection of intact green tomato fruit. However, activity of the *luxS*$^-$ reporter was not rescued back to wild-type level by incubation with DPD or co-culture with AI-2-producing strains. The inability to rescue an *luxS* mutation in *Salmonella* spp. by

addition of exogenous AI-2 has been frequently reported by others and is believed to be indicative of unknown metabolic factors which affect AI-2-based regulatory changes instead of a problem with reporter function (40).

Interestingly, soft rots caused by *Pectobacterium* strains SR38 (*luxS*$^{+}$) and SCC6023 (*luxS*$^{-}$), but not SCC3193 (*luxS*$^{+}$), repressed resolution of the *Salmonella luxS*+ RIVET reporter (CEC0015) to the level of the *luxS*$^{-}$ reporter (CEC0018). SR38 also significantly repressed resolution of CEC0018. However, because SCC6023 lacks *luxS* it cannot affect resolution via AI-2 signaling. Also, CEC0018 does not supply AI-2 to the surrounding environment, and the perception of AI-2 from SR38 would be expected to increase RIVET resolution. Therefore, the observed the reductions in RIVET resolution do not appear related to AI-2 signaling but may respond to differences in LuxS activity.

The relationship between *Salmonella* spp. and *P. carotovorum* during colonization of produce continues to be an interesting area of research relevant to produce safety. However, this study shows that, under the conditions tested, these interactions are not significantly influenced by AI-2 signaling and are likely controlled by the nutritive environment of the tomato fruit as well as the metabolic capabilities of each strain, which could be influenced by LuxS.

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