***Salmonella* SdiA Recognizes *N*-acyl Homoserine Lactone Signals from *Pectobacterium carotovorum* in Vitro, but Not in a Bacterial Soft Rot**

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Genomes of *Salmonella enterica* isolates, including those linked to outbreaks of produce-associated gastroenteritis, contain *sdiA*, which encodes a receptor of *N*-acyl homoserine lactones (AHL). AHL are the quorum-sensing signals used by bacteria to coordinately regulate gene expression within their populations. Because *S. enterica* does not produce its own AHL, SdiA is hypothesized to function in the interspecies cross-talk with AHL-producing bacteria. Under laboratory conditions, *S. enterica* responded to AHL from phytopathogens by upregulating expression of *srgE*. AHL-dependent expression of *srgE* required a functional *sdiA*. Essentially, no *sdiA*-dependent resolution of the *srgE* recombinase-based (RIVET) reporter was observed inside a soft rot formed on a tomato by an AHL-producing strain of *Pectobacterium carotovorum*. The results of the control experiments suggest that *sdiA* is not expressed inside tomato, pepper, green onion, or carrot affected by the soft rot, and the lack of *sdiA* expression in planta prevents *Salmonella* spp. from responding to AHL. Despite its inability to detect and respond to AHL during colonization of soft rots, *S. enterica* reached higher final cell numbers inside a tomato soft rot compared with its growth in intact tomato fruit. The synergistic effect was the strongest under the conditions that are typical for the Florida fall/winter production season.

Recent outbreaks of gastrointestinal illness caused by *Salmonella enterica* were linked to the consumption of fresh produce (mostly tomato, cucurbits, and sprouts) (Lynch et al. 2009). These outbreaks raise important questions about the ecology of human enteric pathogens outside of their mammalian hosts, and the mechanisms by which they persist within alternate hosts, such as plants (Winfield and Groisman 2003; Brandl 2006; Lynch et al. 2009; Teplitski et al. 2009). It appears that *S. enterica* colonizes plants similarly to the dedicated plant epi- and endophytes (Dong et al. 2003; Iniguez et al. 2005; Brandl 2006). However, published genomes of *Salmonella* do not contain recognizable genes associated with plant-associated growth (e.g., pectinases, hemicellulases, *hrp*, or *hrc*) (Rodionov et al. 2004; Brandl 2006; Abbott and Boraston 2008). It is possible that it has unknown classes of cell-wall-degrading enzymes or it may rely on enzymes from plant

pathogens such as pectobacteria that cause soft rot of vegetables (Brandl 2006).

The hypothesis that *Salmonella* spp. benefit from the association with plant pathogens was supported by supermarket surveys in which salmonellae were isolated from produce affected by bacterial soft rot at least twice as frequently as from vegetables that were intact, mechanically injured, or damaged by fungal rots (Wells and Butterfield 1997, 1999). A coinoculation with *Pectobacterium carotovorum* or *Pseudomonas viridiflava* onto disks cut from tomato fruit increased growth of *S. enterica* sv. Typhimurium ATCC14028 by 7- to 10-fold (Wells and Butterfield 1997). Interestingly, spoilage fungi promoted growth of *Salmonella* spp. by only two- to threefold under similar conditions (Wells and Butterfield 1999). How and why *Salmonella* spp. benefit from the association with bacterial soft rot more than from fungal decay is not yet clear.

The mechanisms of the establishment and maintenance of the associations between *S. enterica* and plant pathogens are not yet clear. It is possible that *Salmonella* spp. recognize signals produced by plant-pathogenic bacteria (Brandl 2006), although the identity of this interspecies signal is not known. It has been hypothesized that *Salmonella* spp. may detect *N*-acyl homoserine lactone (AHL) signals, which many bacteria use for quorum sensing (Brandl 2006; Teplitski et al. 2009).

Salmonella spp. do not produce their own AHL; however, they have a functional AHL receptor encoded by *sdiA* (Ahmer et al. 1998; Smith and Ahmer 2003; Ahmer 2004; Smith et al. 2008). In vitro, *Salmonella* spp. respond to AHL from other bacteria in a *sdiA*-dependent manner (Michael et al. 2001; Smith and Ahmer 2003; Ahmer et al. 2007). In response to AHL under laboratory conditions, *Salmonella* SdiA activates the expression of the chromosomally encoded *srgE* gene and the *rck* operon, which is borne on the *S. enterica* sv. Typhimurium virulence plasmid pSLT (Ahmer et al. 1998; Michael et al. 2001). All of these *sdiA*-regulated genes (*srgs*) are horizontally acquired and are not present in the published genomes of *Escherichia coli* (Ahmer et al. 1998). The *rck* operon contains six genes: *pefI*, *srgD*, *srgA*, *srgB*, *rck*, and *srgC* (Friedrich et al. 1993; Ahmer 2004). *PefI* is a regulator of the upstream *pef* operon, which encodes fimbriae that bind to blood antigens (Nicholson and Low 2000; Chessa et al. 2008). *Rck* is an outer membrane protein with functions in resistance to complement killing and adhesion to fibronectin and laminin (Cirillo et al. 1996; Crago and Koronakis 1999). *SrgD* and *SrgC* are putative transcription factors, the targets of which are not known (Friedrich et al. 1993). Despite the regulation of putative virulence genes by SdiA, a *sdiA* mutant of *S. enterica* sv. Typhimurium 14028 is not attenuated in mouse, chicken, or cow models of infection (Smith et al.

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2008; Ahmer 2004). The *sdiA* gene is differentially regulated in swarm colonies (Wang et al. 2004; Kim and Surette 2006), although neither a *sdiA* nor a *rck* mutant is defective in swarming on laboratory media (Toguchi et al. 2000). Even though gene expression mediated by *sdiA* and AHL has been well characterized under various laboratory conditions, a function for *sdiA* in host-associated mixed microbial communities has been reported only during the passage of *S. enterica* through the gut of turtles that also harbored AHL-producing strains of *Aeromonas hydrophila* (Smith et al. 2008). Therefore, the role of the *sdiA*-dependent detection of the AHL signals from other bacteria in natural environments currently remains largely unclear.

Salmonella SdiA responds most strongly to 3-oxo-C₆-, C₆- and 3-oxo-C₈-HSL (Michael et al. 2001; Smith and Ahmer 2003; Janssens et al. 2007). The same AHL are produced by many bacteria, including soft rot plant pathogens, including *Pectobacterium carotovorum* (Cha et al. 1998; Von Bodman et al. 2003; Charkowski 2009). In pectobacteria, AHL-mediated regulatory cascades control bacterial genes required for virulence and production of plant-tissue-degrading enzymes (Von Bodman et al. 2003; Charkowski 2009). Therefore, the goal of this study was to test the hypothesis that, within tomato, *Salmonella* spp. detect AHL from *P. carotovorum*, and that the detection of AHL from the soft rot bacterium contributes to the fitness of this human pathogen within tomato fruit. The development of the *tnpR-lacZY*-based recombinase-based in vivo expression technology (RIVET) reporters in *srgE* (Ahmer et al. 2007; Smith et al. 2008) now offers an opportunity to test, visualize, and quantify *sdiA*-mediated responses to AHL during interactions of *Salmonella* spp. with phytopathogens in vitro and in planta.

RESULTS AND DISCUSSION

Characterization of the *srgE* RIVET reporter.

TnpR resolvase-based reporter systems were designed for the quantification of bacterial gene expression in the environmental niches where conventional reporters have limited value (Angelichio and Camilli 2002; Ahmer et al. 2007; Gao and Teplitski 2008; Smith et al. 2008). The RIVET reporters used in this study contain a promoterless cassette containing *tnpR*-

lacZY genes with the wild-type ribosome-binding site for *tnpR* (Merighi et al. 2005; Ahmer et al. 2007). The cassette is cloned downstream from the stop codon but upstream of the transcriptional terminator of the target gene (Smith et al. 2008) (discussed below). The expression of the gene of interest effects transcription of the bicistronic *tnpR-lacZY* reporter. The activity of LacZY β -galactosidase could be measured with a chromogenic substrate (Miller 1972), and the activity of the TnpR recombinase could be quantified based on the loss of the tetracycline resistance gene, which is encoded within the *res* sites (a substrate for the TnpR recombinase) (Angelichio and Camilli 2002; Ahmer et al. 2007).

To compare the LacZ and TnpR reporter activities resulting from the activation of the same *srgE* promoter, responses of the *srgE-tnpR-lacZY* reporter to 3-oxo-C₆-HSL were measured. In order to measure β -galactosidase activity of the reporter, assays were carried out in shake cultures. These conditions, however, are suboptimal for the activity of SdiA, which is known to be stronger in bacteria that actively chemotax within semisolid matrices (Smith and Ahmer 2003). LacZ and TnpR activities were detectable in response to 6 nM 3-oxo-C₆-HSL, and full activity was observed at 60 nM 3-oxo-C₆-HSL (Fig. 1). One resolution percentage of the *srgE-tnpR* reporter roughly corresponds to 17 Miller Units of β -galactosidase activity. Even though the absolute activity of this single-copy chromosomal reporter is several orders of magnitude lower than the activity of a SdiA-dependent plasmid-borne *rck-luxCDABE* reporter (Michael et al. 2001), both the *srgE-tnpR-lacZY* and *rck-luxCDABE* are similarly sensitive to AHL in the nanomolar range. These observations suggest that the RIVET reporter is useful for tracking and quantifying gene expression under the in vitro and in vivo conditions where the use of other conventional reporters (like *lacZY* or *luxCDABE*) is technically difficult.

Salmonella SdiA recognizes AHL from phyto bacteria.

To test whether *Salmonella* spp. respond to AHL from plant-associated bacteria under laboratory conditions, expression of the *srgE-tnpR* fusion in the wild-type and *sdiA* backgrounds was tested in mixed microbial cultures. Expression of *srgE* in cocultures with *Serratia marcescens*, *Pantoea stewartii*, and *Chromobacterium violaceum* depended on the presence of *sdiA* in the *Salmonella* reporter (Table 1). Because *P. stewartii*, *S. marcescens*, and *C. violaceum* are known to produce AHL with 3-oxo-C₆-, C₆-, and C₄- side chains, *sdiA*-dependent expression of *srgE* is consistent with previous reports, which demonstrated that SdiA recognizes and responds to AHL with C₄-C₁₀ acyl side chains (Michael et al. 2001; Smith and Ahmer 2003). In cocultures with the AHL-deficient mutants of these bacteria, expression of the *srgE* gene was eliminated (Table 1), further supporting the hypothesis that the expression of the *srgE* reporter depends on AHL in a *sdiA*-dependent manner. Expression of *srgE* strongly increased in the presence of a synthetic 3-oxo-C₆-HSL, and this activation required the presence of a functional *sdiA* (Table 1), also consistent with previous studies (Smith et al. 2008). Within a mixed culture formed by *Salmonella enterica* and an isolate of *Pectobacterium carotovorum* SR38, which was previously identified as responsible for soft rot of a shipment of tomato fruit (Bender et al. 1992), expression of *srgE* was also increased, suggesting that the field isolate *P. carotovorum* SR38 produces quorum-sensing (QS) signals recognized by SdiA (Table 1).

P. carotovorum SR38 produces AHL in vitro and inside the soft rot.

To more rigorously test the hypothesis that *P. carotovorum* SR38 produces QS signals capable of stimulating *sdiA*-depend-

Fig. 1. Comparison of β -galactosidase and TnpR resolvase reporter activities of *srgE-tnpR-lacZY*. Cultures of the *srgE-tnpR-lacZY* reporter (*Salmonella enterica* sv. Typhimurium JNS3206) were incubated for 6 h at 37°C in Luria-Bertani medium with serial dilutions of 3-oxo-C₆-HSL. TnpR resolvase and β -galactosidase activities were measured in the same cultures. Error bars indicate standard errors of three independent experiments.

ent gene expression, the *S. enterica srgE-tnpR* reporter in the wild-type and *sdiA* backgrounds was seeded into a soft Luria-Bertani (LB) agar essentially as described earlier (Smith and Ahmer 2003). Consistent with Smith and Ahmer (2003), expression of *srgE* required *sdiA* (Table 1). The TnpR activity of the reporters in the wild-type (*sdiA*+) background were stronger (approximately 2-fold over background at 22°C and approximately 40 fold over background at 30°C) in the presence of *P. carotovorum* SR38. These results support the observation that *P. carotovorum* SR38 produces compounds capable of activating *srgE* in a *sdiA*-dependent manner.

The hypothesis that *P. carotovorum* SR38 produces QS signals was then tested using bioassay-coupled thin-layer chromatography (TLC). The *Agrobacterium tumefaciens* NTL4 pZLR4 reporter used in this study responds most strongly to AHL with medium side chains (Cha et al. 1998). Hydrophobic extracts of culture filtrates of *P. carotovorum* SR38 contained an activity capable of stimulating the *A. tumefaciens* AHL reporter (Fig. 2). On the reverse-phase (C_{18}) Si TLC plates, the signal migrates with a R_f similar to that of 3-oxo- C_6 -HSL (Fig. 2). The same activities are produced by *P. carotovorum* SR38 during growth in LB broth and also inside soft, rotted tomato fruit (Fig. 2). The production of 3-oxo- C_6 -HSL is well documented in *P. carotovorum* (Cha et al. 1998; Von Bodman et al. 2003; Charkowski 2009), and this field isolate appears to be similar to other *P. carotovorum* strains in its ability to produce 3-oxo- C_6 -HSL or other QS signals capable of stimulating the *A. tumefaciens* AHL reporter.

***Salmonella* spp. do not respond to AHL from *P. carotovorum* in planta.**

Because *Salmonella* spp. recognize and respond to AHL produced by soft rot bacteria under laboratory conditions, we tested the hypothesis that this AHL/*SdiA*-mediated cross-talk takes place inside tomato fruit colonized by *P. carotovorum* SR38. Surprisingly, we observed little or no resolution of the reporter under these conditions (Table 1). Essentially, no *sdiA*-dependent expression of the *srgE* reporter was observed in mixed infections during early stages of the soft rot or in fruit

that were completely degraded by *Pectobacterium* spp. (Table 1). Low levels of *sdiA*-dependent *srgE* expression on surfaces of tomato fruit in the presence or absence of *P. carotovorum* were statistically insignificant (Table 1). These results were unexpected, and several hypotheses were tested in order to explain this observation.

First, we tested the hypothesis that the enzymatic activity of the TnpR resolvase was inhibited in *Salmonella enterica* during growth in tomato. Two *tnpR* reporters were constructed using regions overlapping promoters of *dgt* (STM0208) and *hns* (STM1751). These two *Salmonella* promoters were previously identified as strongly induced inside red, ripe tomato fruit (J. T. Noel, McClelland, and M. Teplitski, *unpublished*). The *dgt-tnpR* and *hns-tnpR* reporters were fully expressed inside a red, ripe

Fig. 2. Production of quorum-sensing (QS) signals by *Pectobacterium carotovorum* SR38. *Agrobacterium tumefaciens* NTL4 pZLR4 bioassay-coupled thin-layer chromatography of QS signals produced by *P. carotovorum* SR38 inside tomato fruit (lane P_T) and in a shake Luria-Bertani culture (lane P_{LB}). No QS signals detectable by the bioassay were detected in extract of a tomato fruit (lane T). *N*-acyl homoserine lactone standards 3-oxo- C_6 -HSL, C_6 -HSL, 3-oxo- C_8 -HSL, and 3-oxo- C_{12} -HSL (lane S) were from Sigma-Aldrich or Cayman Biochemicals.

Table 1. Expression of the *Salmonella* *N*-acyl homoserine lactone-responsive gene *srgE* in vitro and in planta^a

Experimental conditions	TnpR activity (mean percent resolution $\pm$ standard error) in	
	Wild-type (<i>sdiA</i> +) <i>S. enterica</i>	<i>S. enterica</i> <i>sdiA</i> :: <i>Tn3</i>
In vitro—LB broth co-cultures with phyto bacteria, at 30°C		
<i>Salmonella</i> monoculture	0	0
with 3-oxo- C_6 -HSL	30.4 $\pm$ 0.7	0
with <i>Chromobacterium violaceum</i> Bergonzini ATCC 12472, produces 3-oxo- C_6 -HSL	30 $\pm$ 5.6	0
with <i>Agrobacterium tumefaciens</i> NT1 pTiC58 Δ <i>accR</i> , produces 3-oxo- C_8 -HSL	34.29 $\pm$ 1.4	0
with <i>Serratia marcescens</i> MG1, produces C_4 -HSL, C_6 -HSL	16 $\pm$ 4.2	0
with <i>S. marcescens</i> MG44, AHL deficient	4 $\pm$ 1	0
with <i>Pantoea stewartii</i> DC283, produces 3-oxo- C_6 -HSL	30 $\pm$ 2.8	0
with <i>P. stewartii</i> DC283 <i>esal</i> :: <i>Tn5</i> , AHL-deficient	0	0
with <i>Pectobacterium carotovorum</i> SR38	38 $\pm$ 2	0
In vitro—0.3% LB agar		
<i>Salmonella</i> monoculture at 22°C	33 $\pm$ 2	1 $\pm$ 1
with 3-oxo- C_6 -HSL at 22°C	41 $\pm$ 5	0
<i>Salmonella</i> monoculture at 30°C	1 $\pm$ 1	0
with 3-oxo- C_6 -HSL at 30°C	22 $\pm$ 2	0
with <i>P. carotovorum</i> SR38 at 22°C	77 $\pm$ 1	0
with <i>P. carotovorum</i> SR38 at 30°C	38 $\pm$ 6	0
In planta—cv. Campari (store-bought)		
Tomato wound	0*	0*
Lesion** on a tomato fruit caused by <i>P. carotovorum</i> SR38	0	0
Tomato soft rot caused by <i>P. carotovorum</i> SR38	4 $\pm$ 2	1 $\pm$ 1
Tomato wound spiked with 3-oxo- C_6 -HSL	0	0
Intact tomato surface	0***	0***
Intact tomato surface, co-inoculated with <i>P. carotovorum</i> SR38	2 $\pm$ 2	0

^a Asterisks: * indicates the data are based on three inoculations; ** indicates early stage in the soft rot, as described in Materials and Methods; *** the data are based on two inoculations. LB = Luria-Bertani.

tomato, reaching 98 and 100% resolution, respectively. This suggests that the TnpR resolvase is functional in *Salmonella enterica* during growth inside tomato fruit. We also tested the hypothesis that, despite the fact that AHL are produced by *P. carotovorum* inside the soft rot, they may be quickly degraded within the fruit tissues. AHL were injected into tomato fruit and recovered at various time intervals. Based on the results of the *A. tumefaciens* bioassay-coupled TLC experiment, roughly the same amounts of 3-oxo-C₆-HSL were recovered after 1 min, 1 h, and 1 day of incubation inside the fruit, indicating that AHL are stable inside the fruit and that degradation or inactivation of AHL cannot explain the observed lack of expression of *srgE* (data not shown). Last, as a spiked control, 20 nmol of 3-oxo-C₆-HSL were injected into a wound that was preinfected with the *srgE*-*tnpR* reporter. No expression of *srgE*-*tnpR* was observed (Table 1), indicating that, even in the presence of AHL, no *srgE* expression took place within tomato fruit.

Expression of *sdiA* and competitiveness of the *Salmonella sdiA* mutant during growth in tomato fruit.

Because no expression of *srgE* was observed even when fruit were spiked with synthetic AHL, we next tested the hy-

pothesis that *sdiA* is not expressed during the interactions of *Salmonella* spp. with tomato, and that the lack of *sdiA* expression results in the lack of *srgE* regulation. To test this hypothesis, a *sdiA*-*tnpR* reporter was constructed. Under the conditions tested, *sdiA* was not expressed to a significant extent in *S. enterica* growing in or on tomato fruit with or without *P. carotovorum* (Table 2). In contrast, the same reporter was strongly expressed under some laboratory conditions at 22°C (Table 2).

To further test the hypothesis that the lack of the expression of *sdiA* explains lack of *srgE* resolution inside a tomato, pJVR2 (a high-copy-number plasmid which carries *sdiA* expressed from an arabinose-inducible *P_{BAD}* promoter) was electroporated into *S. enterica* sv. Typhimurium JNS3226. The resulting construct was preinduced by growth in a laboratory medium containing arabinose and tetracycline, washed with phosphate-buffered saline (PBS), and then seeded into a tomato wound. The overexpression of *sdiA* from *P_{BAD}* led to $89 \pm 3\%$ resolution of the *srgE*-*tnpR* reporter in a tomato wound and $93 \pm 1\%$ resolution in the tomato wound spiked with 3-oxo-C₆-HSL. These results are consistent with the earlier report that overexpression of *sdiA* from pJVR2 bypasses the need for AHL with regard to SdiA activity (Smith and Ahmer 2003). These results further suggest that the lack of *sdiA* expression (rather than the inhibition of *sdiA* function) is responsible for the inability of *Salmonella* spp. to respond to pectobacterial AHL in planta.

Consistent with the observation that *sdiA* was not expressed inside tomato fruit or on tomato surfaces, deletion of *sdiA* conferred no competitive phenotype. When coinoculated with *P. carotovorum* inside tomato fruit and on tomato surfaces at a 1:1 ratio, both the wild-type *Salmonella* sp. and its isogenic *sdiA* mutant were recovered at roughly the same ratio. The competitive index calculated based on the recovery ratio was not statistically significant from the competitive index for a wild type and its antibiotic-marked derivative (Table 3).

We attempted to further explain this behavior of *sdiA* and tested the expression of the *sdiA* reporters under various conditions. We first tested the hypothesis that lack of *sdiA* expression inside a tomato is due to the low pH of the tomato fruit. *sdiA* was expressed in soft LB agar (0.3%) and in laboratory shake cultures at neutral pH (Table 2). There was essentially no expression of *sdiA* in LB broth and in soft LB agar at pH 4.2 (pH of a ripe tomato); however, buffering tomato pulp to neutral pH did not restore expression of *sdiA* to the levels observed in LB at the same pH (Table 2). Furthermore, even though the lack of *sdiA* expression at low pH may explain why we did not observe any *sdiA*-dependent expression of *srgE* inside tomato fruit, this does not explain why *sdiA* was not expressed on surfaces of tomato fruit or in soft rots (pH 6.8). The presence of an AHL did not affect expression of *sdiA* (Table 2). Providing additional copies of *sdiA* on a high-copy-number arabinose-inducible vector, pJVR2, did not affect expression of *sdiA*, indicative of the lack of autoinduction (Fig. 3). This observation is consistent with an earlier report of Kim and Surette (2006).

Table 2. Expression of the *Salmonella* N-acyl homoserine lactone receptor gene *sdiA* under different conditions at 22°C

Incubation conditions ^a	Resolution (%) ^b
In vitro	
0.3% LB agar (pH 6.8)	69 ± 6
0.3% LB agar, 0.1 M MES (pH 5.4)	50 ± 4
0.3% LB agar, 0.1 M MES (pH 4.2)	0
LB liquid (pH 6.8)	9 ± 2
LB liquid, 0.1 M MES (pH 5.4)	2 ± 1
LB liquid, 0.1 M MES (pH 4.2)	0
Tomato juice (pH 4.3)	0
Tomato juice, 0.1 M MES (pH 5.4)	0
Tomato juice, 0.1 M MES (pH 6.8)	0
In planta	
cv. Campari (store-bought)	
Tomato wound (pH 4.2 to 4.4)	1 ± 1
Tomato wound (pH 4.2 to 4.4) with <i>Pectobacterium carotovorum</i> SR38	0
Tomato soft rot caused by <i>P. carotovorum</i> SR38 (pH 5.4 to 6.0)	2 ± 1
Tomato surface	0
Tomato surface with <i>P. carotovorum</i> SR38	0
cv. Hawaii 7997 (greenhouse-grown)	
Tomato wound	0
Tomato surface	0
Tomato surface with <i>P. carotovorum</i> SR38	0
cv. Bonny Best (greenhouse-grown)	
Tomato wound	4 ± 1
Tomato soft rot caused by <i>P. carotovorum</i> SR38	4 ± 1
Tomato surface	0
Tomato surface with <i>P. carotovorum</i> SR38	0

^a LB = Luria-Bertani; MES = morpholineethanesulfonic acid.

^b TnpR activity (mean percent resolution ± standard error).

Table 3. Competitive indices for *Salmonella enterica sdiA*⁺/*sdiA*::*Tn3* co-infections of tomato cv. Campari

Incubation conditions	Competitive index ^a
Wound on a tomato fruit	0.97 ± 0.34
Lesion on a tomato fruit caused by <i>Pectobacterium carotovorum</i> SR38	1.62 ± 0.24
Tomato soft rot caused by <i>P. carotovorum</i> SR38	0.93 ± 0.30
Tomato wound spiked with 3-oxo-C ₆ -HSL	1.36 ± 0.18
Intact tomato surface	1.48 ± 0.21
Intact tomato surface, co-inoculated with <i>P. carotovorum</i> SR38	1.48 ± 0.20

^a For statistical analyses, competitive indices were compared individually with the competitive index calculated for two near-isogenic wild-type strains, 14028 and JS246 (1.32 ± 0.05). None of the competitive indices were statistically different from the control.

sdiA is not expressed in produce.

To test whether *sdiA* is expressed in soft rots formed by *P. carotovorum* SR38 on other fruit and vegetables, *S. enterica* sv. Typhimurium JSN3216 was coinoculated onto field- and greenhouse-grown tomato, bell pepper (red and green), carrot, and green onion grown with conventional or organic-like practices. All produce was inoculated with the *sdiA*-*tnpR* reporter and *P. carotovorum* SR38 within 24 h of harvest, and maintained at 60 to 100% relative humidity (RH) at 22°C. Very low levels of *sdiA* expression were observed in some tomato samples inoculated with *P. carotovorum* SR38 (Table 2); resolution of the reporter (at 4%) was observed in one of the six samples of soft, rotted red and green bell peppers. These low levels of *sdiA* expression are likely to be biologically irrelevant because essentially no *sdiA*-dependent resolution of *srgE* took place under the same conditions (Table 1).

No resolution of the *sdiA* reporter was observed in soft rots on green onion or carrot. These results indicate that the lack of *sdiA* expression during colonization of a soft rot may not be limited to tomato or the members of the Nightshade family.

Growth of *Salmonella* outbreak isolates in soft, rotted tomato.

The rationale for this investigation was based on a market survey in which incidents of *Salmonella* spp. were observed to be higher in soft, rotted produce compared with uninjured fruit and vegetables (Wells and Butterfield 1997). To test the hypothesis that the association with a soft rot promotes growth of *Salmonella* spp., a cocktail of five *S. enterica* serovars (originally traced to produce-borne outbreaks of salmonellosis) were coinoculated with *P. carotovorum* SR38 onto tomato. The presence of the *sdiA* and the genomic arrangement in which it occurs in each of the outbreak isolates was confirmed by polymerase chain reaction (PCR) as described below.

The infections were carried out for 3 days under storage conditions typical of i) standard ripening room conditions (90% RH, 20°C), ii) Florida fall/winter production line conditions (60% RH, 27°C), and iii) optimum conditions for the

Fig. 3. Expression of *sdiA* is not autoregulated. β-Galactosidase activity of an *sdiA-lacZ* reporter was measured in the wild-type background (*Salmonella enterica* sv. Typhimurium 14028), BA612 (*sdiA::Tn3*), BA612 pJVR2, and a vector control BA612 pBAD33. Bacteria were grown in Luria-Bertani medium (LB) to an optical density at 600 nm = 0.8 to 0.9, diluted 50-fold in fresh LB, and incubated with 1 μM 3-oxo-C₆-HSL. Strains carrying pJVR2 or pBAD33 were diluted in LB buffered to pH 7 with 0.1 M morpholinepropanesulfonic acid (MOPS) with 0.2% arabinose and 1 μM 3-oxo-C₆-HSL, and incubated for 2 h. Experiments were carried out at 37°C. Averages of three independent cultures are shown; error bars indicate standard errors.

survival and growth of *P. carotovorum* (90% RH, 27°C). Inoculated fruit were sampled daily to determine the survival and growth of the bacteria.

Under all storage conditions, 50- to 100-fold more CFU of *P. carotovorum* than *S. enterica* were recovered from tomato

Fig. 4. Growth of the outbreak serovars of *Salmonella* and *Pectobacterium* spp. in tomato following laboratory co-infections. A cocktail of *Salmonella enterica* isolates traced to produce-borne salmonellosis outbreaks was seeded into tomato fruit in the presence (or absence) of *Pectobacterium carotovorum* SR38. *Salmonella* spp. recovered from tomato fruit are indicated by a triangle (*Salmonella* outbreak serovars = empty triangle, recovered *Salmonella* spp. from a mixed inoculation with *Pectobacterium* = filled triangle); recovered *P. carotovorum* SR38 is indicated by a square (empty square = *P. carotovorum* recovered from a single infection; *P. carotovorum* SR38 recovered from mixed infections with mixed inoculum of the outbreak strains of *Salmonella* = filled square). An increase in CFU counts is shown. **A**, Inoculated tomato fruit were incubated at 60% relative humidity (RH), 27°C, conditions that are typical for the fall/winter production season in Florida; **B**, inoculated tomato fruit were stored at 90% RH, 27°C, conditions that are favored by *P. carotovorum*; **C**, inoculated tomato fruit were stored at 90% RH, 20°C, standard ripening room conditions.

tissues (Supplementary Table S1; Fig. 4). Depending on the experimental conditions, *Salmonella* populations reached 10^2 to 10^5 CFU/ml of the tissue sample, indicating that, even though inside tomato fruit, *S. enterica* grows slower and to lower final populations compared with the dedicated plant pathogens, the *Salmonella* populations within a fruit easily reach doses infectious to humans.

When fruit were stored at 60% RH and 27°C, counts for both the *Salmonella* and *Pectobacterium* spp. increased throughout the three-day study, resulting in a $3.99 \log_{10}$ CFU/ml increase in *Pectobacterium* spp. counts and a $2.72 \log_{10}$ increase in *Salmonella* CFU/ml (Fig. 4A). The most significant increase occurred between day 1 and day 2 of the incubation, and no such increase was observed when the pathogens were inoculated individually. When the same experiments were carried out under the conditions that are considered favorable for *P. carotovorum* (90% RH, 27°C), both *Salmonella* and *Pectobacterium* spp. grew substantially over the period of 3 days, resulting in a $4.94 \log_{10}$ CFU/ml increase of *Pectobacterium* spp. and a $4.81 \log_{10}$ CFU/ml increase of *Salmonella* spp. (Supplementary Table S2; Fig. 4B). The differences observed in growth of *Salmonella* spp. when comparing the individual counts and those recovered in combination with *Pectobacterium* spp. on days 1, 2, or 3 were not statistically significant, even though a plot of trend lines indicates a beneficial effect of the association with the soft rot bacterium. Under standard ripening conditions (90% RH, 20°C), *Salmonella* and *Pectobacterium* spp. grew daily, with the biggest increase seen between day 0 and day 1 (Supplementary Table S3; Fig. 4C). *Pectobacterium* spp. had a total increase of $6.26 \log_{10}$ CFU/ml while the *Salmonella* spp. had a $4.18 \log_{10}$ CFU/ml increase over 3 days. Growth of *Salmonella* spp. combined with *P. carotovorum* was not significantly different from *Salmonella* spp. individually inoculated onto shave-wounded tomato fruit except on day 3, where growth was significantly less.

These results indicate that, under 60% RH and 27°C, which are typical of Florida fall/winter production line conditions, the presence of soft rot bacteria may favor growth of *S. enterica*. However, under standard ripening room conditions, the statistical significance of such effect was less pronounced. Interestingly, even though growth of *Salmonella* spp. in soft rots was promoted in the presence of pectobacteria, *P. carotovorum* SR38 reached lower final densities in soft rots coinoculated with *Salmonella* spp. (Fig. 4). It is possible that *Salmonella* spp. produce antibiotic or bacteriostatic compounds that inhibit growth of pectobacteria.

Conclusions.

Members of the genera *Salmonella*, *Escherichia*, and *Klebsiella* encode an AHL receptor, SdiA, even though they do not produce their own AHL signals (Ahmer 2004). In vitro, SdiA responds most strongly to AHL with medium-length acyl side chains but has a detection range that is broader than most LuxR homologs in that it also responds to 3-oxo-homocysteine thiolactones and 3-oxo-trans-2-aminocyclohexanols (Janssens et al. 2007). SdiA was observed to become active during the transit of *S. enterica* through turtles but not during transit through pathogen-free chicks, pigs, mice, guinea pigs, calves, or rabbits (Smith et al. 2008). The only AHL-producing organism recovered from turtles was *Aeromonas hydrophila*, suggesting that it is the likely source of the AHL. A deletion of *sdiA*, however, did not strongly affect the ability of *Salmonella* spp. to survive within the gastrointestinal tract of turtles (Smith et al. 2008).

Because SdiA recognizes and binds to AHL similar to those produced by soft rot *Pectobacterium* spp. (Smith and Ahmer 2003; Janssens et al. 2007), we tested the hypothesis that *Sal-*

monella SdiA may respond to AHL produced by *P. carotovorum* during colonization of a tomato soft rot. This study demonstrated that, even though *Salmonella* spp. respond to AHL from *P. carotovorum* in vitro, there was essentially no *sdiA*-dependent gene expression on or in tomato fruit, regardless of the presence of the AHL-producing soft rot pectobacterium. The lack of response to AHL during growth inside tomato is due to the lack of *sdiA* expression during plant-associated growth. It is likely that, in addition to low pH, the expression of *sdiA* is repressed by compounds produced by plants. Although the chemical nature of the *sdiA* inhibitor from plants is not yet known, there are examples of *sdiA* inhibitors in other systems. A heat- and pH-stable compound inhibiting expression of *sdiA* was previously identified in spent cultures of *S. enterica*, although its chemical identity is not yet known (Volf et al. 2002). A plant sugar, xylose, was shown capable of binding to SdiA_{*E. coli*} (Yao et al. 2007), although it is not yet clear whether this adventitious binding outside of the AHL-binding pocket observed in vitro can alter SdiA-mediated gene expression. Because our experiments (Fig. 3) and those of Kim and Surette (2006) demonstrate that the expression of *sdiA* is not autoregulated, reduction in the activity or stability of SdiA will not affect expression of the *sdiA* gene. Therefore, binding of plant compounds to SdiA cannot explain the lack of *sdiA* expression in planta.

Even though *Salmonella* spp. did not respond to AHL from *P. carotovorum* within a tomato soft rot under these conditions, an association with the soft rot promoted growth of *Salmonella* spp. under the conditions found during the cool-season production period. This increase in *Salmonella* spp. growth in association with soft rot is consistent with the earlier supermarket surveys (Wells and Butterfield 1997) and has direct implications for microbiological safety of produce. The mechanisms by which this interaction is established and the benefits (if any) derived by *Salmonella* spp. and the soft rot bacteria from this association are still unclear.

MATERIALS AND METHODS

Strains.

All strains are listed in Table 4. *P. carotovorum* SR38 was isolated from a shipment of Florida tomato fruit that was discarded due to a soft rot. The identity of the isolate was further confirmed by PCR amplification of its 16S ribosomal RNA gene with primers 8F and 1489R (Bruneel et al. 2006), cloning and sequencing the PCR product at the University of Florida Biotech Core facility.

S. enterica sv. Typhimurium JNS3216 *sdiA*-*tnpR*-*lacZY* transcriptional reporter was constructed in three steps. First, a FRT-kan-FRT cassette was integrated after the stop codon of *sdiA* by the Datsenko and Wanner mutagenesis protocol (Datsenko and Wanner 2000) using primers TGCTACGCTGCGGACAGGTCTGATATGAgtgtagctggagctgcttc and CAGCCGGTTTCGCGTCTGGCGCGAAGCATcatatgaatctcctt ag (sequences in CAPS bind to the *Salmonella* chromosome, *sdiA* stop codon is shown in bold, and priming sites for pKD4 are indicated by lowercase letters). This resulted in *S. enterica* sv. Typhimurium JNS3004. The kanamycin cassette was then removed from JNS3004 as in Datsenko and Wanner (2000), resulting in JNS3104. Last, pCE70, a suicide plasmid that carries a *tnpR*-*lacZY* downstream from a FRT "scar" and a wild-type ribosome binding site (Merighi et al. 2005), was integrated into *S. enterica* sv. Typhimurium JNS3104. The integration was verified by PCR. The resulting strain (JNS3216) has *tnpR*-*lacZY* integrated immediately after the *sdiA* stop codon, yielding a *sdiA*-*tnpR*-*lacZY* fusion in which *sdiA* is fully functional and located in its natural position in the chromosome.

S. enterica sv. Typhimurium JTN26 and JTN72 RIVET reporters containing promoters strongly expressed inside tomato fruit were constructed as controls. The promoters for *dgt* and *hns* were PCR amplified using primer pairs CTCGAGGT TCAACTGGCCAGTGTAAATTTTC and CTCGAGGCGAATA TCGTTTCAGATTTTCTTC, CTCGAGCGGGTGATGATAAG CATTGTTGAT and CTCGAGAAACCGATACGAGAGCTG ACTTTG (underlined sequences indicate *Xho*I sites introduced for cloning into pGOA1193). The PCR fragments were cloned into pCR2.1, and then subcloned into pGOA1193 (Osorio et al. 2005). The constructs were sequenced with MT59 (CAAA AAGTCGCATAAAAATTTATCC), which binds within the pGOA1193 *tnpR*. The resulting plasmids were mated into *S. enterica* sv. Typhimurium JS246. The integration was verified using primers TAATTGCCCGTATTCATAGCCGAA and AAGTCTGTTCGTAGCCCATAGCAA (for the reporters in *dgt* and *hns*, respectively), each paired with MT59.

The presence of *sdia* in the outbreak isolates of *S. enterica* svs. Poona, Montevideo, Agona, Gaminarum, and Michigan (Table 4) was verified by *Taq* PCR with TGGTCATCAACAA GAAGAACGTTG (binds nucleotide 2,038,875 of NC_003197) and GCCAGCACGTCCGGCTTTGTCTTCCAG (binds nucleotide 2,041,114 of NC_003197).

Construction of *sdia*::mTn3-*lacZY*.

Mutagenesis of *sdia* with mTn3-*lacZY* was performed as described previously (Seifert et al. 1986). Briefly, *E. coli* strain RDP146 carrying an F':mTn3-*lacZY* and plasmid pLB101 encoding the Tn3 transposase was transformed with pBA302 (Ahmer et al. 1998). Transformants, now carrying cointegrates between pBA302 and the F':mTn3 mTn3-*lacZY*, were mated

with NS2114Sm. In this strain, the cointegrates are resolved by Cre recombinase. Transpositions within the *sdia* gene were identified by restriction mapping. One pBA302::mTn3-*lacZY* isolate, pBD333, contained an insertion after nucleotide 641 of GenBank accession number U88651, and was saved for further analysis.

Culture conditions and reporter assays.

All strains were maintained as frozen glycerol stocks, and were subcultured into LB with appropriate antibiotics (kanamycin at 50 µg/ml, ampicillin at 200 µg/ml, and tetracycline at 10 µg/ml) prior to the experiments. Outbreak isolates of *S. enterica* were maintained on PROTECTBacterial Preservers (Scientific Device Laboratories, Des Plaines, IL, U.S.A.) and stored at -70°C. Arabinose was added to 0.2% (wt/vol) as indicated in the text to bacterial cultures grown in LB buffered to pH 7 with 0.1 M morpholinepropanesulfonic acid (MOPS).

For plate assays, bacteria were seeded into soft LB agar (0.3%) with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (40 µg/ml) as described previously (Smith and Ahmer 2003). As indicated in the text, buffered LB and tomato juice were prepared by first buffering them (Fisher Scientific, Pittsburgh) with 0.1 M morpholineethanesulfonic acid (MES) and then adjusting pH with 1 M HCl or 1 M NaOH. β-Galactosidase activity in bacterial shake cultures was measured as in Miller (1972) using *p*-nitrophenyl-β-galactopyranoside (Sigma Aldrich, St. Louis); Miller units were calculated as in Miller (1972).

For the RIVET assays in tomato, *Salmonella* cultures were grown at 37°C overnight in LB supplemented with tetracycline and *P. carotovorum* SR38 was grown in LB at 30°C without

Table 4. Strains and plasmids used in this study

Strain, plasmid	Description ^a	Reference
Strains		
<i>Agrobacterium tumefaciens</i> NTL4 pZLR4	AHL reporter, <i>traR</i> ⁺ <i>PtraG-lacZ</i> , <i>gent</i> ^r	Cha et al. 1998
<i>Pantoea stewartii</i> DC283	Wild-type isolate, vascular pathogen of corn; produces 3-oxo-C ₆ -HSL	Beck von Bodman and Farrand 1995
<i>P. stewartii</i> DC283 <i>esaI</i> :: <i>Tn5</i>	AHL-deficient mutant	Beck von Bodman and Farrand 1995
<i>Pectobacterium carotovorum</i> SR38	Hypervirulent strain, isolated as responsible for a soft rot of a tomato shipment in Florida	Bender et al. 1992
<i>Salmonella enterica</i> sv. Agona LJH517-rif	Spontaneous rifamycin-resistant derivative of the strain isolated from a salmonellosis outbreak linked to alfalfa sprouts	L. J. Harris
<i>S. enterica</i> sv. Gaminarum LJH518-rif	Spontaneous rifamycin-resistant derivative of the strain isolated from a salmonellosis outbreak linked to orange juice	L. J. Harris
<i>S. enterica</i> sv. Michigan LJH521-rif	Spontaneous rifamycin-resistant derivative of the strain isolated from a salmonellosis outbreak linked to cantaloupes	L. J. Harris
<i>S. enterica</i> sv. Montevideo LJH519-rif	Spontaneous rifamycin-resistant derivative of the strain isolated from a salmonellosis patient in a tomato-linked outbreak	L. J. Harris
<i>S. enterica</i> sv. Poona LJH630-rif	Spontaneous rifamycin-resistant derivative of the strain isolated from a salmonellosis patient in a tomato-linked outbreak	L. J. Harris
<i>S. enterica</i> sv. Typhimurium ATCC14028	Wild-type strain	American Type Culture Collection
<i>S. enterica</i> sv. Typhimurium BA612	14028 <i>sdia</i> ::mTn3	Ahmer et al. 1998
<i>Salmonella enterica</i> sv. Typhimurium JS246	14028 <i>yjeP8103</i> :: <i>resI-tetRA-resI</i>	Merighi et al. 2005
<i>S. enterica</i> sv. Typhimurium JTN6	JS246 <i>dgt</i> ⁺ <i>dgt</i> :: <i>tnpR-lacZY</i>	This study
<i>S. enterica</i> sv. Typhimurium JTN72	JS246 <i>hns</i> ⁺ <i>hns</i> :: <i>tnpR-lacZY</i>	This study
<i>S. enterica</i> sv. Typhimurium JNS3004	JS246 <i>sdia-FRT-kan-FRT</i>	This study
<i>S. enterica</i> sv. Typhimurium JNS3104	JS246 <i>sdia-tnpR-lacZY</i> . Constructed: JNS3004 × pCE70	This study
<i>S. enterica</i> sv. Typhimurium JNS3206	JS246 <i>srgE10-tnpR-lacZY</i> (kan ^r)	Smith et al. 2008
<i>S. enterica</i> sv. Typhimurium JNS3216	JNS3104 <i>sdia-tnpR-lacZY</i> (kan ^r)	This study
<i>S. enterica</i> sv. Typhimurium JNS3226	JS246 <i>srgE10-tnpR-lacZY sdiaI</i> :: <i>Tn3</i> (kan ^r amp ^r)	Smith et al. 2008
<i>Serratia marcescens</i> MG1	Wild-type strain, isolated from a rotten cucumber; produces C ₄ -HSL, C ₆ -HSL	Givskov et al. 1996
<i>S. marcescens</i> MG44	MG1 <i>swrI</i> :: <i>Tn5</i> , AHL deficient	Givskov et al. 1996
Plasmids		
pBD333	Carries <i>sdia-lacZ</i>	This study
pCE71	<i>oriR6K FRT-tnpR</i> ^{wt} - <i>lacZY aph</i> (kan ^r)	Merighi et al. 2005
pJVR2	<i>sdia</i> expressed from an arabinose-inducible promoter on a pACYC plasmid	Ahmer et al. 1998

^a AHL = *N*-acyl homoserine lactone; *gent*^r, *kan*^r, and *amp*^r indicate gentamicin, kanamycin, and ampicillin resistance, respectively.

antibiotic selection. Bacterial cultures were then pelleted, washed three times in an equal volume of sterile PBS, and diluted to 10^8 CFU/ml. Approximately 10^5 CFU (in 1 to 3 μ l of PBS) were inoculated onto superficial 1-mm wounds on surfaces of unwaxed red, ripe, round tomato fruit (cv. Campari) sold on the vine at local supermarkets (batches of tomato fruit originated in Mexico, Canada, or New Jersey, United States as per the box label). Additional experiments were carried out with red, ripe, unwaxed tomato cvs. Bonny Best and Hawaii 7997 grown in a roof-top greenhouse at the Genetics Institute of the University of Florida, red or green tomato cv. Florida 47, and red and green bell pepper cv. Aristotle grown in the field at North Florida Research and Education Center, Marianna, FL; and green onion and carrot grown under organic-like conditions on a private farm in Archer, FL. When appropriate, an approximately equal number of cells of *P. carotovorum* SR38 was coinoculated onto shallow wounds on produce surfaces. Inoculated produce was incubated at room temperature (22°C, RH approximately 40%) or in moist chambers (22°C, RH approximately 60 to 100%). At least three technical and six biological replications were carried out for each experiment, unless otherwise indicated in the text. Samples were obtained first at the appearance of the initial signs of the soft rot (sunken brown lesion) and then after tomato fruit developed a characteristic appearance and strong odor of the soft rot (these were achieved by varying inoculum doses of *P. carotovorum*). All RIVET assays were carried out for a week. Tomato inoculated only with *S. enterica* did not present obvious symptoms of decay or degradation aside from localized dry browning at the site of inoculation. To harvest samples, cores of 15 by 0.5 mm were removed from the fruit or vegetables and homogenized in PBS, and aliquots were then plated on xylose-lysine deoxycholate (XLD) agar (Oxoid, Hampshire, U.K.) supplemented with kanamycin (for JNS3206, JNS3216, and JNS3226) or ampicillin (for JTN26 and JTN72). Samples from soft, rotted tomato fruit were collected with a sterile cotton swab and then streaked on XLD with the appropriate antibiotics. *Salmonella* colonies from each XLD plate were then patched on a plate containing tetracycline or ampicillin, essentially as described by Smith and associates (2008). In several control samples, no *Salmonella* spp. were detected in uninoculated tomato fruit.

To quantify the resolution of reporters on surfaces of intact tomato fruit, bacterial cultures were prepared as above except that the reporters were diluted to 10^7 CFU/ml, and 10^6 CFU (in 5 μ l of PBS) of *P. carotovorum* SR38 was spotted onto intact surfaces of unwaxed tomato cvs. Campari, Bonny Best, and Hawaii 7997. The spotted suspensions were briefly air dried in a biosafety hood, and tomato fruit were then incubated at 22°C and RH 90% for 1 day before spotting *Salmonella* RIVET reporters. Suspensions of *S. enterica* sv. Typhimurium JNS3206 and JNS3226 or JNS3216 containing 10^4 CFU (in 5 μ l of sterile PBS) were spotted on top of the film of *P. carotovorum*, and then were briefly air dried prior to being returned to the moist chamber (22°C, RH 90%). Bacteria were recovered from surfaces by rinsing individual spots with 10 ml of sterile PBS and then plating aliquots of PBS rinsates onto XLD plates with kanamycin. Resulting *Salmonella* colonies were patched onto LB agar with tetracycline and, when appropriate, LB agar with ampicillin as described above. RIVET assays were carried out as previously described (Ahmer et al. 2007; Smith et al. 2008). Resolution percentages were calculated as a proportion of cells in which the tetracycline resistance marker (within the *res-tet-resI*) cassette was lost. The resolution of the tetracycline resistance marker was selectively confirmed by PCR using TATTTTCGCTTTATGAATCTAAAGG and TTATCAAGAGG GTCATTATATTTTCGC (primers that bind within the *res-tet-res* cassette).

Fitness and growth of the *Salmonella* isolates in soft rot tomato fruit.

To calculate competitive index, wild-type *S. enterica* sv. Typhimurium 14028 and its derivative carrying a *sdIA::Tn3* were seeded at 10^5 CFU/infection, roughly at a 50:50 ratio, into or on tomato fruit. In parallel, *S. enterica* sv. Typhimurium 14028 and its isogenic tetracycline-resistant derivative JS246 were similarly inoculated onto nine tomato fruit (cv. Campari), three wounds per fruit. All treatments were incubated and harvested using similar protocols. The relative ratios of the strains in the inocula and in the recovered samples were calculated by dilution plating and patching on antibiotic-containing media. Competitive indices were calculated for each treatment using the formula $(M_{out}/WT_{out})/(M_{in}/WT_{in})$, where M is the proportion of mutant cell and WT is the proportion of the wild-type cells in the inocula ($_{in}$) or in the recovered samples ($_{out}$). Statistical and biological significance of each competitive index were established by comparing with the competitive index similarly calculated for 14028 versus JS246, using a two-tailed *t* test with unequal variances ($P < 0.05$).

To determine growth of the outbreak *Salmonella* isolates in tomato, rifampicin-resistant derivatives of *S. enterica* svs. Poona, Montevideo, Agona, Gammarum, and Michigan were first grown individually in shake cultures and inside tomato fruit. No differences in their growth rates were observed under these conditions (data not shown). For the inoculations, bacteria were grown in LB broth with rifampicin from glycerol stocks to the final population densities of approximately 1.0×10^8 CFU/ml. Suspensions were pelleted and washed in PBS. The five *Salmonella* cultures were combined. When mixed cultures (with *P. carotovorum*) were prepared, they consisted of one-half mixed *Salmonella* cultures and one-half *P. carotovorum*. Cell numbers were ascertained by dilution plating.

For the tomato inoculations, unwashed, unwaxed mature green tomato fruit (cv. Florida 47) were supplied by Six L's Packing, Inc. (Immokalee, FL, U.S.A.) and DiMare (Tampa, FL, U.S.A.). Tomato fruit were classified as 6×7 (medium) by the Florida Tomato Committee. When preparing for inoculation, tomato fruit were placed aseptically onto sterile fiberglass trays with the stem scars facing down. The tomato fruit were cleaned with reagent alcohol and wiped with a Kimwipe (Kimberly-Clark, Neenah, WI, U.S.A.) and then 1- to 2-mm surface wounds were made with a sterile sharp knife. Suspensions of bacteria were seeded onto the wounds as described above. Once dry, tomato fruit were placed in a Caron 6030 (Caron, Marietta, OH, U.S.A.) environmental humidity chamber. The temperature and RH inside the chamber were continuously maintained by a Whatlow Series 96 controller (Whatlow, Winoona, MN, U.S.A.). Samples were incubated under three storage conditions: standard tomato ripening room (20°C, 90% RH), conditions that were considered optimal for the development of pectobacterial soft rots (27°C, 90% RH), and conditions in the Florida fall/winter production season (27°C, 60% RH). For each experiment, 3 biological and 10 technical replications were set up.

Samples were collected by excising 1- to 2-mm-thick slices of the fruit tissue and immediately placing them into sterile Stomacher bags containing 100 ml of sterile 0.1% peptone water. The samples were stomached (AES Laboratoire, Combourg, France) for 1 min, then serially diluted in sterile 0.1% peptone water and the dilutions plated on nutrient agar with rifampicin. Uninoculated whole tomato fruit and wounded tomato fruit were used as controls throughout the studies.

QS signal extraction and characterization.

To isolate QS signals produced by *P. carotovorum* SR38, overnight cultures in LB broth were extracted twice with one-

half volume of acidified ethyl acetate, and rotary evaporated. To recover QS signals from soft rots formed on tomato by *P. carotovorum* SR38, approximately 10^6 cells of the bacterium were inoculated onto shallow wounds on tomato surfaces; tomato fruit (including uninfected controls) were incubated at 22°C and RH 40% for 7 days (upon completion of the incubation, tomato fruit infected with *Pectobacterium* spp. developed a characteristic appearance and a strong odor of a soft rot). Tomato fruit were homogenized with a mortar and pestle, transferred to a glass container, and extracted twice with two volumes of acidified ethyl acetate. Samples were rotary evaporated and brought up in 500 µl of ethyl acetate. To remove pigments and other strongly hydrophobic substances that coextracted with QS signals and then confounded the interpretation of the TLC-assisted bioassay, 30 µl of extract were mixed into 450 µl of high-performance liquid chromatography (HPLC)-grade water, and further purified using C18 reverse-phase silica resin. The C18 silica resin slurry (150 µl) was washed in methanol, equilibrated in HPLC-grade water, and mixed for 15 min with the 480 µl of the sample. The mix was pelleted, the supernatant removed, and AHL were eluted with three bed volumes of 100% methanol. The methanol eluent was collected and dried. To standardize the extraction procedure, LB culture filtrates were subject to a similar C18 purification step. Previous studies demonstrated that similar combined protocols are reasonably effective and consistent for extracting AHL with medium and long side chains (Teplitski et al. 2003).

When AHL were added directly into tomato fruit for spiked controls, 20 nmol of 3-oxo-C₆-HSL in HPLC water was injected into red, ripe Campari tomato fruit which were seeded with *S. enterica* sv. Typhimurium JNS3206 24 h earlier. To evaluate the recovery of AHL from tomato, 3-oxo-C₆-HSL was injected into red, ripe tomato fruit (final concentration approximately 1 µM). Fruit were incubated for 1 min, 1 h, and 1 day. Following the incubation, tomato fruit were ground and AHL were extracted with two volumes of acidified ethyl acetate and further purified using C18 resin, as described above.

Extracts were separated by C18 reverse-phase silica TLC (60:40, methanol/water). The TLC plates were dried in a fume hood and then overlaid with a soft agar suspension containing X-gal at 20 µg/ml and *A. tumefaciens* NTL4 pZLR4 AHL reporter, essentially as described before (Cha et al. 1998). Plates were incubated and sealed at 30°C overnight.

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