Previously uncharacterized *Salmonella enterica* genes required for swarming play a role in seedling colonization

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Incidences of bacterial foodborne illness caused by ingestion of fresh produce are rising. Instead of this being due to incidental contamination, the animal pathogen *Salmonella enterica* utilizes specific molecular mechanisms to attach to and colonize plants. This work characterizes two *S. enterica* genes of unknown function: a putative periplasmic protein, STM0278, and a putative protein with a hydrolase in the C-terminus, STM0650. STM0278 and STM0650 are important for seedling colonization but appear to have different roles during the process of colonization. Mutants of either STM0278 or STM0650 showed reduced colonization of alfalfa seedlings at 24 h, and the STM0278 mutant also showed reduced colonization at 48 h. Both genes were expressed *in planta* at 4 h following inoculation of 3-day-old seedlings and at 72 h after seed inoculation. This suggests that the role of STM0650 in seedling colonization is less important later in the process or is duplicated by other mechanisms. Mutants of STM0278 and STM0650 were defective in swarming. The STM0278 mutant failed to swarm in 24 h, while swarming of the STM0650 mutant was delayed. Addition of surfactant restored swarming of the STM0278 mutant, suggesting that STM0278 is involved in surfactant or osmotic agent production or deployment. Alfalfa seed exudates as the sole nutrient source were capable of perpetuating *S. enterica* swarming. Sequence analysis revealed sequences homologous to STM0278 and STM0650 in plant-associated bacteria, but none in *Escherichia coli*. Phylogenetic analysis of STM0650 showed similar sequences from diverse classes of plant-associated bacteria. Bacteria that preferentially colonize roots, including *S. enterica*, may use a similar hydrolase for swarming or biofilm production on plants. Multicellular behaviours by *S. enterica* appear central to plant colonization. *S. enterica* genes involved in plant colonization and survival outside of a host are most likely among the 'function unknown' genes of this bacterium.

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INTRODUCTION

Incidences of bacterial foodborne illness caused by ingestion of fresh produce are rising. Salmonellosis accounts for more than half of foodborne disease outbreaks, and the most common food commodity to which outbreak-related cases were attributed was produce (CDC, 2009). Instead of this being due to incidental contamination, the animal pathogens *Salmonella enterica*, *Escherichia coli* and *Listeria monocytogenes* utilize specific molecular mechanisms to attach to and then colonize plant seedlings and tissues (Gorski *et al.*, 2003; Jeter & Matthysse, 2005). *S. enterica*, in particular, appears to employ an arsenal of surface molecules for attachment and colonization. During seedling colonization, *S. enterica* can reach large populations, similar to plant-associated bacteria (Barak *et al.*, 2002).

S. enterica uses several surface molecules regulated by AgfD to attach to and colonize seedlings (Barak *et al.*, 2005, 2007). AgfD regulates a multicellular behaviour that produces a matrix composed of thin aggregative fimbriae (Tafi) and cellulose. Mutations in the Tafi cell-bound minor subunit, cellulose biosynthesis, or a double mutant of both reduced *S. enterica* colonization of seedlings. However, genetic analysis of *S. enterica* to date has not revealed a single mechanism that abolishes plant attachment or colonization. We hypothesize that *S. enterica* employs many mechanisms either in unison or in parallel and they may include those produced by genes not characterized for other systems, animal pathogenicity or laboratory growth.

Previously, we created a transposon library of attachment-deficient *S. enterica* strains (Barak *et al.*, 2005). Of the

unique insertions, 65 % were in uncharacterized genes; this work further characterizes two insertions. A BLAST search of the affected genes against *S. enterica* serovar Typhimurium strain LT2 identified a putative periplasmic protein, STM0278, and a putative protein with a hydrolase in the C-terminus, STM0650.

METHODS

Bacterial strains, media and culture conditions. The strains used in this study are listed in Table 1. Complemented strains were constructed by amplifying wild-type copies of the affected genes (primers listed in Table 2) from *S. enterica* Newport, cloning into pCR2.1 (Invitrogen), and electroporating the resulting plasmids into the appropriate mutant strain. A 1149 bp fragment was amplified and cloned for STM0278 (pJDB3) and a 1235 bp fragment for STM0650 (pJDB4). Each plasmid was transformed into its respective mutant and used for phenotype analysis.

Bacteria were grown in, or plated on, Luria–Bertani (LB) medium. When necessary, antibiotics were used at the following concentrations: ampicillin, 100 µg ml⁻¹; kanamycin, 50 µg ml⁻¹. *Salmonella-Shigella* (SS) agar, a *Salmonella* semi-selective indicator medium, was used to determine *S. enterica* populations. Alfalfa medium (AE) was made by sprouting alfalfa seeds in sterile water at a ratio of 1 g per 10 ml. Exudates were removed daily for 3 days, filter-sterilized (0.2 µm), and stored at 4 °C until used, but for no longer than 1 week. Sterile water was added to the seeds following removal of exudates.

We tested the ability of each strain to swarm or swim using swarm plates (LBG) composed of LB with 0.5 % (w/v) glucose and 0.5 % (w/v) agar, and swim plates composed of LB with 0.3 % (w/v) agar. Overnight bacterial cultures grown on LB agar were suspended in sterile water at a concentration of ~10⁹ c.f.u. ml⁻¹. Twenty microlitres of the bacterial suspension was spotted in the middle of a swarm or swim plate, and the spot allowed to dry for 1 h at room temperature. All plates were incubated at 28 °C for 24 h or as noted otherwise. The swarming or swimming distance was measured from the centre of the original spot to the furthest distance that the bacteria travelled. To determine if swarming deficiencies were related to a lack of surfactant,

Bacillus subtilis surfactin (2.5 mg ml⁻¹) was added to LBG plates and the rate of travel was measured over time as described above.

Flagella were counted from swarm cells grown on LBG plates for 24 h at 37 °C. Sterile water was placed on a glass slide, swarm cells lifted from the agar surface with a plastic loop, and the loop touched to the water drop to transfer the cells to the slide. Slides were left in a laminar flow biosafety hood to dry. A drop of flagella stain (Becton Dickinson) was then added to the bacteria, which were incubated at room temperature for 3.5 min, rinsed with sterile water, and left to dry. Fifty bacterial cells were examined and flagella per cell were counted. Fifty flagella were chosen randomly and their lengths were recorded.

To test if alfalfa exudates could trigger swarming, a second type of swarm plate was made, composed of alfalfa exudates at a ratio of 2 : 1 (alfalfa exudates:water agar). Water agar was made to a final concentration of 0.5 % (w/v) agar. Hybrid LBG-AE swarm plates were prepared by a slight modification of the method of Kim & Surette (2004). Briefly, a small Petri dish was inverted in the middle of a large Petri dish and the inverted plate was sealed to the bottom of the large plate with Vaseline. Semi-solid AE was poured around the inverted dish. When the medium had solidified, the small dish was removed and the space was filled with LBG to the height of the AE. The hybrid plates were allowed to dry overnight and inoculated and incubated as described above.

Biofilm assays. Biofilm assays were conducted using 5 ml LB made without salt (LBNS) in 13 × 100 mm test tubes. A bacterial suspension was added to the medium to give a starting concentration of approximately 10⁵ c.f.u. ml⁻¹. Tubes were incubated stationary at 28 °C. After 3 days, media and pellicles were poured from the tubes, and the tubes were rinsed three times with sterile water. A solution of 30 µl crystal violet (0.5 g per 100 ml in 20 % methanol) was added to each tube with 5 ml sterile water and the tubes were left standing for 15 min. The solution was poured out and the tubes rinsed with sterile water until no remaining crystal violet was rinsed from the biofilms. Thirty per cent acetic acid was added to each tube, and the tubes placed in a spectrophotometer, where transmission was read at 540 nm.

Alfalfa seedling attachment and colonization assays. Alfalfa seedling colonization assays were performed as described previously (Barak *et al.*, 2005). All experiments were performed at least three

Table 1. Bacterial strains used in this study

Strains	Description	Reference
<i>S. enterica</i> Newport		
96E01152C-TX	Wild-type; alfalfa seed isolate	Inami & Moler (1999)
JDB 249	STM0278::Tn10:lac:kan	Barak <i>et al.</i> (2005)
JDB 634	STM0278::Tn10:lac:kan complemented with pJDB3	This study
JDB 277	STM0650::Tn10:lac:kan	Barak <i>et al.</i> (2005)
JDB 636	STM0650::Tn10:lac:kan complemented with pJDB4	This study
Plasmids		
pJDB3	pDrive::STM0278; 1149 bp fragment containing STM0278 cloned from 96E01152C-TX using primers 0278F and 0278R, inserted into pDrive	This study
pJDB4	pDrive::STM0650; 1235 bp fragment containing 0650 cloned from 96E01152C-TX using primers 0650F1 and 0650R1, inserted into pDrive	This study
pDrive	Cloning vector	Qiagen, Valencia, CA
pProbe-NT	pVS1/p15a replicon, <i>gfp</i> reporter	Miller <i>et al.</i> (2000)
pProbe-NT::STM0278	910 bp fragment containing the STM0278 promoter, inserted into pProbe-NT	This study
pProbe-NT::STM0650	573 bp fragment containing the STM0650 promoter, inserted into pProbe-NT	This study

Table 2. PCR primers used in this study

Complementation	Sequence (5'→3')
0278F	GCA CTG CTC CTG CTT TCC
0278R	AGC CCT GAT TTA TCT GCT TTA T
0650F1	TGA ACG CGG ACG TGG TGA
0650R1	TGT GCT GCC CGA TTT GGT
Promoter probes	
0278_pro-F	ATG GTG AAA TCG CCT CCC
0278_pro-R	GTC CCA ACT TCA GGA ACA AA
0650_pro-F	AGG CGT CCG TGA GGA GGT GA
0650_pro-R	TCG CAC AAA CGC TGG TAG

times. Briefly, alfalfa seeds were surface sanitized, sprouted in Petri plates, and irrigation water was exchanged daily. For initial attachment assays, 3-day-old uninoculated seedlings were used that ranged from 0.01 to 0.03 g (mean 0.0182 ± 0.004 g). Twelve seedlings were transferred to 50 ml conical tubes containing bacterial suspensions ($\sim 10^4$ c.f.u. ml⁻¹) and incubated for 4 h at 25 °C with constant shaking at 40 r.p.m. Individual seedlings were rinsed in 1 ml sterile water for 30 s and then homogenized. The homogenate for each seedling was dilution plated on SS agar; the plates were incubated overnight at 37 °C, and the colonies counted.

For colonization and microscopy assays, seeds were incubated in a bacterial suspension of $\sim 10^6$ c.f.u. ml⁻¹. After 1 h, the suspension was replaced with sterile water and the seeds were sprouted for 3 days at room temperature. To determine bacterial populations, seedlings were removed after the daily water change at 24 and 48 h and sampled as described above.

Microscopy. Putative promoter regions of the STM0278 and STM0650 genes were PCR amplified (PCR primers listed in Table 2) from *S. enterica* Newport and cloned into pCR2.1. All constructs were confirmed and promoter orientation determined by sequencing. Putative promoter regions were excised with *HindIII/XbaI* and cloned into pProbe NT (Miller *et al.*, 2000). Each pProbe NT construct was electroporated into *S. enterica* Newport wild-type. These strains were used to determine whether STM0278 or STM0650 was expressed on alfalfa seedlings.

Statistics. The mean *S. enterica* populations on seedlings (c.f.u. g⁻¹) were calculated and the 24 and 48 h data were log-transformed prior to statistical analysis. To determine whether the swarming distance or rate differed between wild-type and mutant, 10 plates were used per strain for each experiment. The mean distance was calculated for each time point. The mean swarming rate was calculated for each strain tested and separately for each medium. Determination of the *S. enterica* populations on alfalfa seedlings and swarm experiments was repeated three times. Differences between means for wild-type and mutant, and when appropriate the complemented strains, were analysed by unpaired *t*-tests with Welch correction.

Phylogenetic analysis. For the phylogenetic analysis of STM0278 and STM0650, sequences were downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>). Amino acid sequences from taxa identified in the STM0650 BLAST analysis were aligned using CLUSTAL W in MEGALIGN 5.08 (Lasergen, DNASTAR) in slow-accurate mode with a pairwise alignment gap-opening penalty of 10 and a gap-extension penalty of 0.1, and multiple alignment gap-extension penalty of 0.2. Phylogenetic affiliations based on the alignment matrix were inferred by maximum-parsimony and distance using PAUP v 4.0b10. For parsimony analyses, informative characters were used in a heuristic search with random-stepwise addition of taxa

and tree bisection–reconnection branch swapping (1000 replicates). Tree topology and statistical estimates of branch support were based on 1000 bootstrap replicates, with group retention set at >70 %.

RESULTS

Hypothetical proteins are involved in different phases of plant colonization

Individual *S. enterica* strains were inoculated either on 3-day-old seedlings to ascertain initial attachment capacity or on seeds to determine the ability to colonize alfalfa seedlings over time. In comparison with the wild-type, significantly fewer cells of STM0278 and STM0650 were detected on the 3-day-old seedlings (Fig. 1a). The complemented strain of STM0278 had populations higher than the original strain and similar to those of the wild-type. The complemented strain of STM0650 had higher populations than the original strain but smaller populations than the wild-type. In the continued colonization assays, mutants of STM0278 and STM0650 both had populations smaller than the wild-type at 24 h. At 48 h the STM0278 mutant population was significantly smaller than wild-type (Fig. 1a). The complemented strain of STM0278 had populations higher than the original strain and similar to those of the wild-type at both 24 h and 48 h. The complemented strain of STM0650 had higher populations than the original strain and similar to those of the wild-type at 24 h. At 48 h, the populations of both the STM0650 mutant and its complemented strain were similar to those of the wild-type.

To visualize gene expression *in vivo*, the promoters of STM0278 and STM0650 were cloned upstream of the green fluorescent protein gene in pProbe-NT and the plasmids were transformed into *S. enterica* Newport cells. Green fluorescent cells were not observed when the bacteria were grown on LB or SS plates. The reporter plasmids did not appear to inhibit bacterial colonization since similar numbers of *S. enterica* Newport cells colonized individual seedlings regardless of the plasmid carried (data not shown). When seedlings were examined microscopically, green fluorescent cells were observed on alfalfa roots from both promoter reporters (Fig. 1b). For both constructs green fluorescent cells were observed attached to alfalfa roots at 4 h post-inoculation, and dense aggregates of green fluorescent cells colonizing roots were observed at 72 h post-inoculation.

Plant attachment and colonization genes also play a role in swarming

To characterize mutants of STM0278 and STM0650, their ability to swim and swarm was tested. Mutants of STM0278 and STM0650 swam similarly to wild-type (data not shown). Within 24 h, the wild-type swarmed to the perimeter of the plate while the STM0278 mutant was unable to swarm, and the STM0650 mutant was reduced in

swarming ability. The complemented STM0650 swarmed to wild-type levels, and the STM0278 complement actually swarmed faster than the wild-type (Fig. 2a, b). The mean number of flagella per cell on cells from swarm-inducing medium was similar for all strains (data not shown). Flagellar length was also examined and no difference was found among the strains (data not shown).

To further characterize the role that STM0278 and STM0650 play in the ability of *S. enterica* to swarm, strains were spotted on medium with *B. subtilis* surfactin. Both mutants swarmed more slowly over LBG compared to wild-type. The STM0278 mutant swarmed at the same rate as the wild-type when surfactin was present in the medium, whereas the STM0650 mutant still showed reduced swarming (Fig. 2c).

To determine if *S. enterica* would swarm on alfalfa seedlings grown in a hydroponic environment, alfalfa seedling exudates were used as the sole carbon source to induce or maintain swarming. *S. enterica* could not be induced to swarm on AE when inoculated at high density. However, when the bacteria were induced to swarm on LBG, swarming was perpetuated on AE to the plate edge of some alfalfa exudates (Fig. 3). Alfalfa exudates from 24 h seedling failed to maintain swarming (Fig. 3b) while exudates from older, 48 and 72 h, seedlings did maintain swarming (Fig. 3c, d).

Mutants of STM0278 and STM0650 are altered in biofilm formation, in a temperature-sensitive manner

To further characterize STM0278 and STM0650, the ability of the mutants to form biofilms was tested. Wild-type biofilms were more developed at 30 °C than 37 °C as measured by transmission of light through crystal violet. Both STM0278 and STM0650 mutants developed less biofilm at 30 °C compared to wild-type and more biofilm at 37 °C (Table 3). The mutant phenotype was more pronounced in STM0278. The complemented strain of STM0278 had a staining intensity similar to wild-type; the complemented strain of STM0650 did not.

Homologues of the hypothetical proteins

To compile information related to STM0278 and STM0650, several sequence analyses were performed. STM0278 is the gene following STM0277 in a two-gene operon. STM0277 is also an uncharacterized gene, whose product may be a putative cytoplasmic protein. A protein BLAST analysis of STM0278 identified homologous sequences in *S. enterica*, *Burkholderia ambifaria* and *Enterobacter sakazakii* (Table 4). Sequences homologous to STM0277 were found by protein BLAST in *S. enterica*, *Erwinia tasmaniensis*, *Vibrio* sp., *Acinetobacter baumannii*, *Pseudomonas syringae* pv. *syringae* and *Burkholderia*

Fig. 2. Mutants of STM0278 and STM0650 are defective in swarming. (a, b) Data from representative experiments showing the mean colony radius ($\pm$ SD) of cultures inoculated on Luria–Bertani supplemented with glucose (LBG) semi-solid agar. (c) Mean swarming rate with and without the addition of surfactant (Sur) to LBG semi-solid agar. Different letters in columns signify significant differences between *S. enterica* populations ($P < 0.05$).

cenocepacia. A multi-genome alignment revealed no orthologues in *S. enterica* serovar Typhi or serovar Choleraesuis, or in *Escherichia coli*. Orthologues were identified for both STM0277 and STM0278 in *S. enterica* serovars Typhimurium and Paratyphi, and in *Ps. syringae* pv. *syringae* (data not shown). However, the *Ps. syringae* pv. *syringae* operon has a third gene of unknown function following the STM0278 orthologue. Single-gene orthologues of STM0278 not found in operons were identified in *Yersinia pestis* and *Ps. syringae* pv. *tomato*.

STM0650 is the second gene of a two-gene operon, and the gene product may contain a hydrolase in the C-terminus. STM0649 is also an uncharacterized gene whose product may contain a hydrolase in the N-terminus. A protein BLAST of STM0650 identified homologous sequences in all *S. enterica* serovars available, *Desulfitobacterium hafniense*, *Carboxydotherrmus hydrogenoformans*, *Clostridium botulinum*, *Clostridium difficile*, *Thermosinus carboxydivorans*, *Thermoanaerobacter pseudethanolicus* and *Geobacillus kaustophilus* (Table 4). A protein BLAST of STM0649 identified homologous sequences in the same bacterial species as that of STM0650, except those of *Clostridium* (data not shown).

Fig. 3. Swarming maintenance of cells induced on Luria–Bertani supplemented with glucose (LBG) semi-solid agar. Hybrid medium consists of LBG in the inner circle, with the outer ring containing LBG (a), 24 h alfalfa exudates (b), 48 h alfalfa exudates (c), or 72 h alfalfa exudates (d).

A multi-genome alignment identified orthologues for STM0649 and STM0650 in *S. enterica* serovars Typhimurium, Paratyphi A and Typhi, but not in other *Enterobacteriaceae*, e.g. *Escherichia coli* and *Yersinia pestis*. STM0649 and STM0650 are within a 4 kb region found in *S. enterica* and not in *E. coli*.

Numerous plant-associated taxa were identified in the protein BLAST analysis of STM0650, and a subset were used in a phylogenetic analysis of the *S. enterica* serovar Newport STM0650 homologue. The subset was chosen to include both plant pathogens and symbionts and at least one sequence to represent each species identified in the original BLAST analysis. The *S. enterica* serovar Newport amino acid sequence was identical to other *S. enterica* sequences and most closely related to sequences from the enteric plant pathogens *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* subsp. *carotovorum* (Fig. 4). Orthologues of STM0650 were identified in plant-associated bacterial

Table 3. Light transmission (540 nm; mean $\pm$ SD) through crystal violet suspensions from *S. enterica* biofilms grown in LBNS

Strain	30 °C	37 °C
Wild-type	22.1 $\pm$ 7.9 ^a	60.4 $\pm$ 5.7 ^a
STM0278 (J249)	76.4 $\pm$ 2.8 ^b	34.1 $\pm$ 6.4 ^b
STM0278 complement (J634)	26.2 $\pm$ 7.6 ^a	69.3 $\pm$ 5.1 ^a
STM0650 (J277)	58.7 $\pm$ 20.1 ^b	38.6 $\pm$ 13.9 ^b
STM0650 complement (J636)	61.6 $\pm$ 12.6 ^b	37.6 $\pm$ 3.4 ^b

Significant differences are shown as in Figs 1(a) and 2(c).

Table 4. Top five results from BLASTP analysis

Gene	Function	Taxon	E value
STM 0278	Putative periplasmic protein	Newport*	0.0
		Saintpaul*	
		Kentucky*	
		Schwarzengrund*	
		Heidelberg*	
STM0650	Hypothetical protein	Hadar*	1e-21
		<i>Burkholderia ambifaria</i>	
		<i>Enterobacter sakazakii</i>	
		<i>Erwinia tasmaniensis</i>	
		<i>Yersinia pseudotuberculosis</i>	
	Putative hydrolase	Typhi*	0.0
		Paratyphi B*	
		Paratyphi A*	
		Saintpaul*	
		Kentucky*	
	Putative hydrolase	Dublin*	1e-175
		Newport*	
		Virchow*	
		Javiana*	
		Heidelberg*	
		<i>Desulfotobacterium hafniense</i>	
		<i>Carboxydotherrmus hydrogenoformans</i>	
	Hydrolase, UxaA family	<i>Clostridium botulinum</i> C	1e-131
	D-Galactarate dehydratase		1e-130

**Salmonella enterica* serovar.

genera, both pathogenic and symbiotic, from various taxonomic classes, including *Agrobacterium tumefaciens*, *Ralstonia solanacearum* and *Bradyrhizobium japonicum*.

DISCUSSION

The uncharacterized genes of *S. enterica* examined in this study were both important for seedling colonization but may have different roles over time. Both the STM0278 and STM0650 mutants showed a reduction in initial attachment and colonization of alfalfa seedlings at 24 h, but the STM0278 mutant also showed reduced alfalfa seedling colonization at 48 h compared to the wild-type. To our knowledge, STM0650 is the first *S. enterica* gene to be identified as important for initial attachment, but not continued colonization of seedlings. The other mechanisms identified as important for *S. enterica* initial attachment, namely thin aggregative fimbriae, O-antigen capsule, and cellulose (Barak *et al.*, 2005, 2007), are also important for continued colonization. The promoters for both STM0278 and STM0650 were expressed at 4 h following inoculation of 3-day-old seedlings and at 72 h after inoculation of alfalfa seeds. Since STM0650 is expressed at 72 h post-inoculation but its mutant did not show reduced colonization at 48 h, we hypothesize that STM0650 is less important or is duplicated by other mechanisms at this time point. In contrast, STM0278 is necessary for both

initial attachment and continued colonization. Our work to date, including this study, supports the conclusion that several mechanisms are utilized by *S. enterica* to persist on seedlings. What is novel in this study is the discovery that different mechanisms are employed over time.

Examining the role of STM0278 and STM0650 on seedlings led us to examine other phenotypes in our mutant analysis. Flagella and motility play a role in fitness and colonization of alfalfa seedlings by *L. monocytogenes* (Gorski *et al.*, 2009). Neither the STM0278 nor the STM0650 mutant was affected in the ability to swim, but neither could swarm comparably to wild-type. In *S. enterica*, swarm cells are elongated, hyper-flagellated cells that can migrate across semi-solid surfaces and more viscous media (Harshey & Matsuyama, 1994), which may be encountered in the sprouting system. Since both mutants could swim similarly to wild-type, we concluded that they did not have flagellar assembly defects. The length and number of flagella were similar between mutants and wild-type. Thus the failure to swarm was not due to an inability to hyper-flagellate or an obvious defect in the flagella.

The swarming dysfunction of each mutant was distinct. The STM0278 mutant failed to swarm, while swarming of the STM0650 mutant was delayed. Swarming bacteria produce a surfactant as a wetting agent (Toguchi *et al.*, 2000), or an osmotic agent that extracts water from the underlying agar and provides a thin layer of fluid to aid the

To determine whether *S. enterica* swarms on alfalfa seedlings, we used alfalfa exudates to induce swarming. Alfalfa exudates added to low-percentage agar failed to induce swarm cell differentiation. *S. enterica* cells that were induced to swarm maintained their swarming ability and actively migrated across alfalfa exudates (Fig. 3). Swarm-cell differentiation and maintenance in *S. enterica* require different essential nutrients (Kim & Surette, 2004). When propagated on M9 minimal medium supplemented with glucose (M9G), vegetative cells do not undergo swarmer differentiation; however, differentiated swarmers can maintain their ability to migrate on M9G, unlike LB without the addition of glucose. Our data support the conclusion that alfalfa exudates from 48 h and 72 h seedlings can maintain efficient utilization of the full tricarboxylic acid cycle and enhanced *de novo* biosynthetic pathways for cells to maintain the swarm state, similar to what was shown for M9G (Kim & Surette, 2004).

S. enterica swimmers also display an increased production of the universally recognized signalling molecule AI-2 and sensitivity to *N*-acylhomoserine lactones (AHLs), signalling molecules that *Salmonella* does not produce (Kim & Surette, 2006). Although AHLs have not been identified in the human gastrointestinal tract, where *S. enterica* causes disease, AHL mimics have been detected from rice, soybean, tomato, crown vetch and *Medicago truncatula* (Teplitski *et al.*, 2000). *sdia*, the *Salmonella* AHL receptor, is not active during transit through chicks, pigs, mice, guinea pigs, calves or rabbits (Smith *et al.*, 2008); however, it may be active outside an animal host. *S. enterica* swimmers also display an adaptive antibiotic resistance to a wide variety of structurally and functionally distinct classes of antibiotics (Kim *et al.*, 2003), which would be advantageous among the diverse plant microbiota and as protection from the plant itself. We hypothesize that swarming in the plant environment would offer *Salmonella* an ecological advantage: (1) motility to explore and locate preferential colonization sites; (2) heightened sensitivity for intra- and interspecies communication; and (3) protection from antimicrobials.

In addition to swarming, mutants of STM0278 and STM0650 are affected in biofilm formation. *S. enterica* forms biofilm on the roots and root hairs of alfalfa seedlings (Charkowski *et al.*, 2002), the roots of mung bean seedlings (Mohle-Boetani *et al.*, 2009), and lettuce roots (Klerks *et al.*, 2007). We have previously identified *S. enterica* genes, *agfA* and *bcsA*, that play a role in seedling colonization via biofilm formation (Barak *et al.*, 2007). We hypothesize that STM0278 and STM0650 may be involved with seedling colonization through swarming and biofilm formation, but at different times. Swarming and biofilm formation seem in contrast, motile versus sedentary. Bacteria on plant leaf surfaces can be either solitary or in aggregates – biofilms – and appear to play different roles in these modes. On leaves it is hypothesized that the aggregated population serves to protect bacteria under stressful conditions and the solitary cells may foster spread to newly colonizable sites (Boureau *et al.*, 2004). *S. enterica* has been reported to require flagella production and movement to travel from the roots of *Arabidopsis thaliana* along the surface of the plant to its leaves and flowers (Cooley *et al.*, 2003). Our data identify STM0278 and STM0650 as two of the *S. enterica* genes involved in both swarming and biofilm formation and suggest that these are the mechanisms used during plant colonization.

We identified nucleotide sequences similar to both STM0278 and STM0650 in plant-associated bacteria. We interpret this as further evidence of the importance of these genes in the life cycle of *S. enterica* outside a warm-blooded host. A phylogenetic analysis of the amino acid sequence of STM0650 revealed that plant-associated bacteria from diverse classes, i.e. Alpha- and Betaproteobacteria, have similar sequences (Fig. 4). We hypothesize that these putative hydrolase proteins in *Agrobacterium tumefaciens*, *Ralstonia solanacearum* and *Bradyrhizobium japonicum*

may also be involved in root colonization of these plant-associated bacteria, pathogens and symbiont.

As more information is learned about the molecular mechanisms utilized by *S. enterica* to colonize and survive on seedlings, multicellular behaviour appears to be important. Previous studies have shown the importance of AgfD-regulated behaviour that produces a matrix of Tafi and cellulose (Barak *et al.*, 2005). This study identified a second multicellular behaviour, swarming, that appears to be involved in seedling colonization. Plant roots may be a natural part of the life cycle of *S. enterica*. Biofilm formation is by its nature a multicellular behaviour. Mutations in *agfD*, or either of the matrix components, disrupt biofilm formation at 30 °C (Barak *et al.*, 2007) while mutations in STM0278 and STM0650 lead to increased biofilm formation at this temperature (this study). Thus, careful coordination of several multicellular behaviours allows *S. enterica* to successfully attach to and colonize seedlings.

Successful colonization of plants that may be eaten by a desirable host may be an additional benefit to *S. enterica*. Plants have become a significant vector for *S. enterica* to move from host to host, as seen by the numerous salmonellosis outbreaks caused by the consumption of contaminated produce, including seedlings. The *S. enterica* genes involved in plant colonization and survival outside a warm blooded host are most likely among the ‘function unknown’ genes, as the mechanisms used in these environments differ greatly from the well-studied environment of animals or laboratory media.

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