Xanthomonas hortorum pv. gardneri TAL effector AvrHah1 is necessary and sufficient for increased persistence of Salmonella enterica on tomato leaves

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Salmonella enterica is ubiquitous in the plant environment, persisting in the face of UV stress, plant defense responses, desiccation, and nutrient limitation. These fluctuating conditions of the leaf surface result in S. enterica population decline. Biomultipliers, such as the phytopathogenic bacterium Xanthomonas hortorum pv. gardneri (Xhg), alter the phyllosphere to the benefit of S. enterica. Specific Xhg-dependent changes to this niche that promote S. enterica persistence remain unclear, and this work focuses on identifying factors that lead to increased S. enterica survival on leaves. Here, we show that the Xhg transcription activator-like effector AvrHah1 is both necessary and sufficient for increased survival of S. enterica on tomato leaves. An Xhg avrHah1 mutant fails to influence S. enterica survival while addition of avrHah1 to X. vesicatoria provides a gain of function. Our results indicate that although Xhg stimulates a robust immune response from the plant, AvrHah1 is not required for these effects. In addition, we demonstrate that cellular leakage that occurs during disease is independent of AvrHah1. Investigation of the interaction between S. enterica, Xhg, and the plant host provides information regarding how an inhospitable environment changes during infection and can be transformed into a habitable niche.

Salmonella enterica is a human enteric pathogen that causes disease in approximately 1.2 million Americans annually (CDC). Numerous multi-state outbreaks of salmonellosis have occurred from the consumption of fresh produce every year since 2000 (National Outbreak Reporting System (NORS;1–4)). S. enterica survives on multiple agricultural crops after root or leaf immigration5–7, and the bacteria persist in soil for months6,8,9. Despite the continued isolation of S. enterica from the agricultural environments associated with outbreaks, S. enterica has relatively poor fitness in the plant environment. Bacterial populations steadily decline in the phyllosphere, or above-ground parts of plants8,9.

The rapidly fluctuating conditions on the leaf surface provide a harsh environment for epiphytic bacteria, and survival depends on the ability to adapt to abrupt changes in UV irradiation and the availability of water and nutrients. Upon arrival to the leaf surface, S. enterica migrates to protected sites like trichomes, stomates, hydathodes, and epidermal cell wall junctions7,10–12. These locations are often associated with a local increase in nutrient abundance due to cracks or leakage through the cuticular layer and may provide protection from desiccation or UV stress10,13. The accessibility of these sites likely explains both how S. enterica persists on leaves over long periods of time and why eventually populations decline. This failure to replicate faster than cellular death is most likely because S. enterica cannot access the nutrient-rich interior of plant tissues on its own.

Recent studies involving S. enterica colonization of plants have centered on the identification and characterization of biomultipliers, factors that lead to increased S. enterica survival. S. enterica exploits changes to the plant environment imparted by other organisms, including infestation with phytophagous insects and infection.

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with bacterial phytopathogens. Plants infected with Xanthomonas spp. lead to increased persistence of S. enterica on tomato (Solanum lycopersicum) leaves. Four lineages of Xanthomonas cause bacterial spot of tomato: X. hortorum pv. gardneri (hereafter referred to as Xhg), X. euvesicatoria pv. euvesicatoria, X. euvesicatoria pv. perforans, and X. vesicatoria. These xanthomonads colonize tomato leaf surfaces and enter plant tissue through natural openings to circumvent the epidermis, the first layer of plant defense. Once inside, Xanthomonas colonizes the apoplast where it multiplies and eventually spreads to neighboring leaves, and ultimately other plants. Bacterial spot disease manifests as lesions on leaves, fruit, and stems of tomato plants and ultimately leads to loss of leaves and damaged fruit, resulting in substantial agricultural impact.

Although four Xanthomonas lineages cause the same disease on tomato, only a subset of these lineages has a beneficial effect on S. enterica populations. In plants colonized by Xhg, X. euvesicatoria pv. euvesicatoria, or, to a lesser extent, X. euvesicatoria pv. perforans, S. enterica populations on tomato leaves remain steady or even increase over time. Contrarily, S. enterica populations decline in the presence of X. vesicatoria or on healthy plants. Core Type III secretion system (T3SS) effectors are shared among the four Xanthomonas species, yet each species also has unique effectors. Of particular interest for this study, Xhg has a transcription activator-like effector (TALE) AvrHah1 that is not found in X. vesicatoria. TALEs induce host transcription by directly binding to the promoter region of target genes using a modular, central repeat domain that recognizes specific host effector binding elements. Xhg AvrHah1 has over 4,000 potential, predicted binding sites in tomato.

AvrHah1 induces a water soaking phenotype during disease, which is characterized by dark green lesions on leaves that reflect an influx of fluid into the apoplast. This water soaking is not observed in X. vesicatoria-infected tissues. In tomato, AvrHah1 is required for the direct induction of two beta helix loop helix (bHLH) transcription factors, bHLH3 and bHLH6. The spike in transcription for these two regulators leads to indirect induction of at least two additional downstream genes, including a pectate lyase (PL) and pectinesterase (PE). The current model suggests that PL alters hygroscopicity in the plant cell wall by releasing oligosaccharides, resulting in an influx of fluid through breaks in the epidermis and the observed water-soaked phenotype. Because the Xhg avrHah1 mutant reaches the same population level as wildtype Xhg in planta, it is thought that AvrHah1 is involved in pathogen dispersal or entry into leaf tissue as opposed to acquisition of nutrients during infection.

In this study, we tested the hypothesis that AvrHah1 defines the differential ability between Xhg and X. vesicatoria to enable S. enterica persistence in the phyllosphere. We showed that lack of this effector reduced the ability of Xhg to promote S. enterica persistence on tomato leaves. Correspondingly, the addition of avrHah1 to X. vesicatoria allowed this phytopathogen to promote S. enterica survival. The data presented here provide evidence that AvrHah1-dependent water soaking may be the mechanism by which the phytopathogenic bacterium benefits the human enteric pathogen.

**Results**

AvrHah1 is both necessary and sufficient for Xhg-dependent effects on S. enterica persistence. Previously, we had shown that infection with Xhg promotes survival of S. enterica on the leaves of tomato plants. To determine the importance of AvrHah1 for Xhg-dependent effects on S. enterica, we examined the effects of co-inoculation with the Xhg AvrHah1 DNA-binding domain mutant avrHah1ΔDBD and S. enterica. Bacterial populations were monitored on tomato plants dip-inoculated with S. enterica in the presence or absence of wildtype Xhg or the Xhg avrHah1ΔDBD mutant. The presence of wildtype Xhg led to approximately ten-fold higher S. enterica populations than the Xhg avrHah1 alone treatment by 6 dpi (Fig. 1a; P = 0.0058). Conversely, co-inoculation of S. enterica with the Xhg avrHah1ΔDBD mutant had no significant impact on S. enterica (Fig. 1a; P = 0.889). Xanthomonas populations were monitored over time, and there were no significant differences between treatments (Fig. 1b; P > 0.556 for all comparisons).

To determine if avrHah1 is sufficient for Xanthomonas-mediated effects on S. enterica persistence, X. vesicatoria, was transformed with a plasmid carrying the avrHah1 gene (pUFR034 (avrHah1)) and tested for its effects on S. enterica. Bacterial populations were determined in tomato plants dip-inoculated with S. enterica in the presence or absence of Xhg, X. vesicatoria + pUFR034 (vector alone control), or X. vesicatoria + pUFR034 (avrHah1). Co-inoculation of Xhg or X. vesicatoria + pUFR034 (avrHah1) with S. enterica resulted in approximately ten-fold higher S. enterica populations at 6 dpi than in plants co-inoculated with X. vesicatoria + pUFR034 and S. enterica or inoculated with S. enterica alone (Fig. 2a; Xhg P = 6.40 × 10^{-7} and 1.74 × 10^{-6}, respectively; X. vesicatoria + pUFR034 (avrHah1) P = 8.17 × 10^{-5} and 0.00019, respectively). There was no significant difference between the X. vesicatoria + pUFR034 and S. enterica alone treatments (P = 0.8203). Xanthomonas populations were monitored over time, and there were no significant differences between treatments (Fig. 2b; P > 0.97). These data demonstrate that the presence of AvrHah1 in Xanthomonas spp. can increase the persistence of S. enterica in co-inoculated leaves.

The AvrHah1 targets bHLH3, bHLH6, PL, and PE are transcribed to similar levels in plants infected with wildtype Xhg or the Xhg avrHah1ΔDBD mutant. Previous work has shown that AvrHah1 activates expression of multiple tomato genes, including two direct targets bHLH3 and bHLH6 and two indirect targets PL and PE. These targets provide the foundation for the current model of AvrHah1 water soaking. To examine whether these targets play a role in the mechanism by which Xhg enhances S. enterica persistence, we monitored transcription of the genes in leaf samples after inoculation with S. enterica in the presence or absence of wildtype Xhg or the Xhg avrHah1ΔDBD mutant. Under our experimental conditions, plants infected with the Xhg avrHah1ΔDBD mutant had the same or higher levels of the four targets compared to plants infected with wildtype Xhg at 1, 3, and 6 dpi (Table 1). In contrast, published results show that wildtype Xhg Xg153 significantly induces transcription of these genes (~90–1,300-fold) compared to a corresponding avrHah1ΔDBD mutant.
mutant when infiltrated into tomato Heinz 1706 leaves for 48 hours. To determine the effect of inoculation method (dip-inoculation vs infiltration) on our differing results, we infiltrated MoneyMaker tomato leaves with \textit{X. hortorum} wildtype and \textit{avrHah1}ΔDBD mutant following published protocols. Leaf samples were collected 48 h post-infiltration, and plant gene expression was measured using quantitative PCR (qRT-PCR). As with the dip-inoculation experiments, there were no significant reductions in plant gene expression in plants inoculated with the \textit{X. hortorum} \textit{avrHah1}ΔDBD mutant compared to plants inoculated with wildtype \textit{X. hortorum} for \textit{bHLH3, PL,} or \textit{PE} (Table 1). The only observed reduction in expression in plants inoculated with the \textit{X. hortorum} \textit{avrHah1}ΔDBD mutant was a ~25-fold reduction in \textit{bHLH6} transcription compared to plants inoculated with wildtype \textit{X. hortorum} (Table 1). These data indicate that \textit{AvrHah1} may target other host genes in the mechanism that leads to increased \textit{S. enterica} persistence in this system.

\textit{X. hortorum} and \textit{X. vesicatoria} elicit different immune responses in tomato leaves, which are further affected by co-inoculation with \textit{S. enterica}. To test the hypothesis that infection with \textit{X. hortorum} alters the plant immune response to the benefit of \textit{S. enterica}, plant defense gene expression was monitored over time in tomato leaves inoculated with \textit{S. enterica}, \textit{X. hortorum}, \textit{X. vesicatoria}, or a combination of each xanthomonad with \textit{S. enterica}. The SA-inducible pathogenesis related protein gene \textit{pr1a1} and the JA-inducible proteinase inhibitor

**Figure 1.** TALE \textit{AvrHah1} is required for \textit{X. hortorum}-dependent effects on \textit{S. enterica} persistence. Bacterial populations (\textit{S. enterica}, (a); \textit{Xanthomonas}, (b)) on tomato leaves were monitored following treatment with \textit{S. enterica} (Se; black circles), \textit{X. hortorum} (Xhg; open green triangles), \textit{X. hortorum} \textit{avrHah1}ΔDBD mutant (Xhg ΔAvr; open orange diamonds), \textit{S. enterica} + \textit{X. hortorum} (SeXhg; closed green triangles), or \textit{S. enterica} + \textit{X. hortorum} \textit{avrHah1}ΔDBD mutant (SeXhg ΔAvr; closed orange diamonds). All data points from three independent experiments are presented as log CFU/cm². Lines (black, \textit{S. enterica}, Se; dashed green, \textit{X. hortorum}, Xhg; dashed orange, \textit{X. hortorum} \textit{avrHah1}ΔDBD mutant, Xhg ΔAvr; solid green, \textit{S. enterica} + \textit{X. hortorum}, SeXhg; solid orange, \textit{S. enterica} + \textit{X. hortorum} \textit{avrHah1}ΔDBD mutant, SeXhg ΔAvr) correspond to a linear regression model with 95% confidence intervals represented by the shaded areas. Letters denote significant differences between treatments for the regression lines according to the linear regression model \((P < 0.01)\). Combining three independent experiments, \(n = 9\) plants per treatment per time point.
Figure 2. AvrHah1 is sufficient for Xanthomonas-dependent increases in S. enterica populations. Bacterial populations (S. enterica, (a); Xanthomonas (b)) on tomato leaves were monitored following treatment with S. enterica (Se; black circles), X. vesicatoria pUFR034 (Xv; open blue squares), X. vesicatoria pUFR034 (avrHah1) (Xv Avr; open cyan squares), S. enterica + Xhg (SeXhg; closed green triangles), S. enterica + X. vesicatoria pUFR034 (SeXv; closed blue squares), or S. enterica + X. vesicatoria pUFR034 (avrHah1) (SeXv Avr; closed cyan squares). All data points from three independent experiments are presented as log CFU/cm². Lines (black, S. enterica, Se; dashed green, Xhg, Xh; dashed blue, X. vesicatoria pUFR034, Xv; dashed cyan, X. vesicatoria pUFR034 (avrHah1), Xv Avr; solid green, S. enterica + Xhg, SeXhg; solid blue, S. enterica + X. vesicatoria, SeXv; solid cyan, S. enterica + X. vesicatoria pUFR034 (avrHah1), SeXv Avr) correspond to a linear regression model with 95% confidence intervals represented by the shaded areas. Letters denote significant differences between treatments for the regression lines according to the linear regression model (P < 0.01). Combining three independent experiments, n = 9 plants per treatment per time point.

Table 1. Transcription of AvrHah1 targets in tomato leaves after infection with the Xhg avrHah1ΔDBD mutant.

| Gene | Dip-inoculation | Infiltration |
|------|-----------------|--------------|
|      | 1 dpi*          | 3 dpi        | 6 dpi         | 48 h       |
| bHLH3| 3.28 ± 2.60     | 1.10 ± 0.21  | 1.21 ± 0.82   | 1.71 ± 0.14** |
| bHLH6| 1.88 ± 0.70*    | 0.76 ± 0.17  | 1.52 ± 1.26   | 0.04 ± 0.01* |
| PL   | 2.00 ± 1.42     | 1.20 ± 0.39  | 4.38 ± 4.75   | 9.99 ± 2.17* |
| PE   | 1.63 ± 1.02     | 0.85 ± 0.26  | 2.49 ± 2.71   | 0.90 ± 0.19  |

*Gene expression is displayed as the relative expression ratio of transcription levels after infection with the Xhg avrHah1ΔDBD mutant using the plant response to wildtype Xhg as the calibrator (i.e., the response to wildtype Xhg is set to 1.0). *Asterisks indicate statistical differences in plant gene expression after treatment with the Xhg avrHah1ΔDBD mutant compared to plant gene expression after treatment with wildtype Xhg using t-test (P < 0.05).
gene pin1 were used as established markers\(^{31,32}\) to indirectly monitor these two plant defense pathways with qRTPCR.

At 1 dpi, there were few differences in pr1a1 expression between treatments (Fig. 3a). The Xhg with S. enterica co-inoculation treatment was significantly different from the negative control (plants treated with water) ($P = 0.0018$), but all other treatments were statistically the same as the negative control at this time point (Fig. 3a). Compared to the negative control, tomatoes that were inoculated with Xhg, Xhg and S. enterica, or X. vesicatoria showed significant increases in pr1a1 expression by 3 dpi (Fig. 3a; $P = 0.0205$, 0.0005, and 0.0032, respectively). Contrastingly, tomato plants treated with X. vesicatoria and S. enterica or S. enterica alone had no change in pr1a1 expression compared to the water control at 3 dpi (Fig. 3a; $P = 0.901$ and 0.889, respectively). By 6 dpi, pr1a1 levels had increased in plants inoculated with Xhg, Xhg and S. enterica, or X. vesicatoria with changes reaching approximately 100–10,000-fold compared to water controls (Fig. 3a; $P = 5 \times 10^{-7}$, < $1 \times 10^{-7}$, and $7.46 \times 10^{-5}$, respectively). Plants treated with X. vesicatoria and S. enterica or S. enterica alone had no change in pr1a1 expression compared to the water control at 6 dpi (Fig. 3a; $P = 0.244$ and 1.00, respectively). Plants inoculated with X. vesicatoria and S. enterica had pr1a1 levels that were statistically the same as both the water control and the X. vesicatoria alone treatment (Fig. 3a; $P = 0.244$ and 0.084, respectively).

At 1 dpi, pin1 expression levels showed similar trends as pr1a1. There were few differences from the water control except for the X. vesicatoria with S. enterica treatment which showed tenfold higher levels of pin1 expression (Fig. 3b; $P < 1 \times 10^{-7}$). By 3 dpi, treatment with Xhg led to reduced levels of pin1 while treatment with X. vesicatoria and S. enterica resulted in increased pin1 expression compared to the water control (Fig. 3b; $P = 0.0011$ and 0.00097, respectively). Although inoculation with X. vesicatoria or S. enterica alone had no change in pin1 expression compared to the water-inoculated plants at 6 dpi (Fig. 3b; $P = 0.071$ and 0.939, respectively), inoculation with Xhg either with or without S. enterica led to a 1–3 log decrease in pin1 levels (Fig. 3b; $P < 1 \times 10^{-7}$ and $3 \times 10^{-7}$, respectively). Co-inoculation with X. vesicatoria and S. enterica led to a tenfold increase in pin1 expression at 6 dpi (Fig. 3b; $P = 0.00048$). Taken together, these data show that the two Xanthomonas spp. induce different plant immune responses.

To determine if there was a connection between AvrHah1 and the observed induction of pr1a1 transcription, we monitored pr1a1 levels at 6 dpi in the Xhg avrHah1\(^{ΔDBD}\) mutant. Wildtype Xhg infection led to increased pr1a1 expression (Fig. 3c; $P = 1 \times 10^{-7}$ and $5 \times 10^{-7}$). Inoculation with the Xhg avrHah1\(^{ΔDBD}\) mutant also induced pr1a1 expression compared to the water control (Fig. 3c; $P < 1 \times 10^{-7}$). Further, we measured both free and conjugated levels of SA in leaf tissue at 1 and 3 dpi after dip-inoculation with water, S. enterica, Xhg, Xhg avrHah1\(^{ΔDBD}\), X. vesicatoria, or S. enterica with each xanthomonad. SA levels between the treatments were not significantly different ($P > 0.05$; Fig. S1). These data demonstrate that avrHah1 is not required for the induction of pr1a1 during Xhg infection.

To examine the impact of AvrHah1 on plant gene expression in response to X. vesicatoria, leaf samples were taken at 6 dpi and examined for pr1a1 expression. Compared to the negative control, tomatoes that were inoculated with Xhg, Xhg and S. enterica, X. vesicatoria + pURF034, X. vesicatoria + pUFR034 (avrHah1), X. vesicatoria + pUFR034 (avrHah1) and S. enterica showed significant increases in pr1a1 expression by 6 dpi (Fig. 3d; $P = 1 \times 10^{-7}$, $8 \times 10^{-7}$, $0.00048$, $1.1 \times 10^{-5}$, and $7 \times 10^{-5}$, respectively). Contrastingly, tomato plants treated with X. vesicatoria + pUFR034 and S. enterica or S. enterica alone had no change in pr1a1 expression compared to the water control at 6 dpi (Fig. 3d; $P = 0.165$ and 0.174, respectively). Thus, addition of avrHah1 to X. vesicatoria alters the immune response to resemble the host response to Xhg.

**AvrHah1 is not required for electrolyte leakage in Xhg-infected tomato leaves.** Previous work has demonstrated that Xhg infection leads to more cellular damage in tomato leaves, as measured through electrolyte leakage, than X. vesicatoria infection\(^2\). From those data, it was hypothesized that this increase in cellular damage led to a resulting increase in S. enterica persistence in the phyllosphere\(^1\). Separately, it was shown that AvrHah1 is required for induction of electrolyte leakage in pepper leaves that have been infiltrated with Xhg\(^3\). To determine if AvrHah1 is linked to cellular damage and the resulting increases in S. enterica populations in tomato, plants were dip-inoculated with water, S. enterica, Xhg, Xhg avrHah1\(^{ΔDBD}\), X. vesicatoria, or S. enterica with each xanthomonad. Leaf samples were collected at 6 dpi. Plants treated with wildtype Xhg and S. enterica or X. vesicatoria with or without S. enterica had higher levels of electrolyte leakage than the water or S. enterica controls ($P < 0.01$, Fig. S2). The Xhg avrHah1\(^{ΔDBD}\) treatment resulted in intermediate electrolyte leakage levels that were statistically similar to the water and S. enterica controls and both the wildtype Xhg and X. vesicatoria treatments ($P > 0.01$; Fig. S2). The Xhg avrHah1\(^{ΔDBD}\) mutant and S. enterica treatment gave similar results as the avrHah1\(^{ΔDBD}\) mutant treatment except that it had statistically lower levels of electrolyte leakage than the two X. vesicatoria treatments ($P < 0.01$; Fig. S2). These results indicate that specific Xanthomonads cause different levels of electrolyte leakage, but these differences do not correlate with increased S. enterica persistence.

**Discussion**

As foliar pathogens, *Xanthomonas* species survive on both the leaf surface and in the leaf interior. To succeed on the surface, epiphytic bacteria tolerate varying degrees of stress, including UV exposure, desiccation, and nutrient limitation. By actively moving to the leaf interior, xanthomonads can avoid these stresses and thrive. Two goals of this work were to identify which aspect(s) of the Xhg disease cycle benefit *S. enterica* and to characterize the mechanism that explains the observed phenotypic differences between *S. enterica* populations on Xhg- and *X. vesicatoria*-infected plants.

For this study, we hypothesized that one or more modifications to the leaf caused by Xhg infection benefit other leaf community members and lead to increased *S. enterica* survival. The infection process includes aggregation on the leaf surface, entry into the leaf interior, promotion of a water-soaked apoplast, and suppression of...
Figure 3. Xanthomonas species differentially impact the plant immune responses. Plant gene expression was quantified at days 1, 3, and 6 dpi with water (purple circles), S. enterica (Se; black circles), Xhg (Xh; green, open triangles), Xhg + S. enterica (SeXhg; green, closed triangles), X. vesicatoria (Xv; blue, open squares), X. vesicatoria + S. enterica (SeXv; blue, closed squares), Xhg avrHah1ΔDBD mutant (XhΔAvr; open, orange diamonds), X. vesicatoria pUFR034 (avrHah1) (Xv pAvr; open, cyan squares), or S. enterica + X. vesicatoria pUFR034 (avrHah1) (SeXv pAvr; closed, cyan squares). Data for the SA-inducible prl1a (a,c,d) and JA-inducible pin1 (b) are displayed as log-transformed relative expression ratios (RER) using water-treated plants as the calibrator. Each symbol represents transcription levels in one tomato plant, and the dashed lines indicate a two-fold change relative to the water control. Means for each treatment at each time point are depicted with horizontal black lines. Transcription was measured in plants from three biological replicates, sampling from three plants per treatment per timepoint for each replicate. Letters denote significant differences between treatments within a single time point using Tukey’s HSD test (P < 0.05). Combining three independent experiments, n = 9 plants per treatment per timepoint.

In that region (data not shown). While these results do not completely preclude a role for tomato variety, they suggest that the appearance or increased frequency of the water-soaked lesions resulted in increased S. enterica survival (Fig. 4). Alternatively, or concurrently, the SA-inducible prl1a gene begins to show signs of induction as early as 1 dpi with Xhg (Fig. 3a). The rapid impact of Xhg on the plant immune response could manifest as increased S. enterica survival six days later. Regardless of the mechanism, it takes less than a week in the Xhg infection process to significantly affect the persistence of S. enterica in this environment.

As Xhg impacts S. enterica in the early stages of disease (Fig. 1), the TSSS effectors that have been linked to the acquisition of nutrients and the suppression or evasion of the plant immune response (for review, see33) could be factors that affect this process. We chose to focus on AvrHah1 because this effector has been linked to the water soaking phenotype in Xhg and is absent from the X. vesicatoria genome6,30. Thus, its specificity in Xhg made it a potential factor that could explain the different effects of these two species on S. enterica survival. As shown in this work, AvrHah1 is both necessary and sufficient for Xanthomonas-dependent increases in S. enterica persistence. The Xhg avrHah1ΔDBD mutant is no longer beneficial towards S. enterica survival (Fig. 1), and X. vesicatoria carrying a plasmid-borne copy of avrHah1 demonstrates a gain of function for this phenotype (Fig. 2).

To characterize the mechanism by which AvrHah1 leads to increased S. enterica persistence, we examined downstream host targets of AvrHah1. Following dip-inoculation, we saw no significant changes in gene expression for bHLH3, bHLH6, PL, or PE (Table 1). This result contrasted with previous work demonstrating that infiltration of wildtype Xhg induced significantly higher transcription of these four genes compared to tomato plants that were infiltrated with the Xhg avrHah1ΔDBD mutant39. The two studies differ in several aspects of experimental design, including Xhg strain, tomato genotype, and inoculation method (dip-inoculation vs infiltration). We found that infiltration of wildtype Xhg mildly induced expression of only one of the four genes, bHLH6, compared to the Xhg avrHah1ΔDBD mutant (Table 1). An alignment of the predicted AvrHah1-binding sites upstream of bHLH3 and bHLH6 showed that the two tomato varieties (Heinz 1706 and MoneyMaker) are 100% identical in that region (data not shown). While these results do not completely preclude a role for tomato variety, they suggest the idea that bacterial strain likely impacts the observed differences in expression between experiments (Table 1; 28). Regardless, these data demonstrate that AvrHah1 plays a key role in the ability of Xhg to increase S. enterica persistence. In this system, AvrHah1 may have different targets that may influence the leaf environment. Schwartz et al. identified 4,106 potential AvrHah1 binding sites in tomato29. Future characterization of potential targets could provide a more detailed mechanism for AvrHah1-mediated increases in S. enterica persistence.

Hosts, such as Xhg, have restricted access to the leaf interior through natural openings such as stomates, hydathodes, and wounds. Entry through stomates brings the bacteria to the apoplast, an air-filled, intercellular space4,39. During infection with wildtype Xhg, the apoplast is inundated with host cellular constituents, creating the water-soaked lesions seen in infected tomato plants (Fig. 4). The water soaking phenotype is absent in plants infected with the Xhg avrHah1ΔDBD mutant. A water-soaked apoplast leads to higher Xanthomonas populations, spread of pathogens into host tissues, suppression of host defense responses, and potentially promotes availability of nutrients40. As part of the defense response, plants cause local desiccation to limit pathogen replication (reviewed in34), and the AvrHah1-dependent water soaking may benefit S. enterica by reducing desiccation stress in this niche. In Arabidopsis thaliana, virulent P. syringae pv tomato DC3000 causes water potentials that promote pathogen growth while avirulent DC3000 are associated with higher levels of desiccation that inhibit bacterial growth36,37. In that study, desiccation was measured using a reporter fusion to the proU promoter, which was induced under low water potentials. Utilizing a reporter to monitor water potentials in future experiments could prove to be informative regarding the role of AvrHah1-mediated water soaking in resulting water stress levels.

In addition to desiccation, epiphytic bacteria also experience nutrient limitation on the leaf surface due to the cuticle that restricts diffusion of nutrients from inside the leaf39. Migration to the leaf interior may provide...
access to the many nutrients (sugars, amino acids, etc.) that are transported through the apoplast and into phloem tissue. Although carbon sources such as glucose, fructose, GABA, succinate, and others accumulate in the apoplast, they are typically sequestered in forms that are bound in the cell wall or within cellular vacuoles. Thus, we hypothesized that \(Xhg\) water soaking could release cellular constituents, nutrients, and electrolytes in an accessible form due to changes in host cell membrane permeability (Fig. 4). This result would not be the first example where \(S.\) enterica utilizes host-derived molecules made available by other bacteria. Plant cell wall breakdown by the soft-rotting pathogen \(Pectobacterium\) carotovorum produces oligosaccharides that are thought to be scavenged by \(S.\) enterica. There are also several examples of phytopathogens altering host cell membrane permeability for growth. \(Pantoea\) stewartii subsp. \(stewartii\) causes water-soaked lesions on corn leaves by damaging cell membranes to release water and nutrients. Water soaking caused by the endophytic pathogen \(P.\) syringae pv. \(phaseolicola\) releases \(Ca^{2+}, Fe^{2+/3+},\) and \(Mg^{2+}\) into the apoplast of bean leaves. Our conductance data suggests that electrolyte leakage does not correlate with increased \(S.\) enterica persistence. Plants co-inoculated with either \(Xhg\) or \(X.\) vesicatoria and \(S.\) enterica have increased electrolyte leakage compared to the water and \(S.\) enterica controls (Fig. 2). However, unlike \(Xhg\), \(X.\) vesicatoria infection has no impact on \(S.\) enterica populations. Although overall differences in electrolyte leakage cannot explain the \(X.-gardneri\) specific effects on \(S.\) enterica survival, these experiments could have missed changes in the levels of specific electrolytes. Fluctuations in ions of low abundance, like iron, wouldn’t have been detected using this approach. Iron has repeatedly been identified as a host-limited nutrient, and pathogens, including \(S.\) enterica, have multiple mechanisms to scavenge iron from their environment. An increase in iron availability could indicate that host manipulations by \(Xhg\) release this critical factor for \(S.\) enterica growth. Future experiments examining the release of specific ions could elucidate more details of this process.

Invading microorganisms face a number of plant defense responses (antimicrobials, reactive oxygen species (ROS), ‘pathogenesis related’ (PR) proteins, etc.) that must be overcome to survive in intercellular spaces. The differential ability of \(S.\) enterica to colonize a range of tomato cultivars suggests an active plant response to the enteric pathogen. Leaf discs treated with the \(S.\) enterica flagellar epitope flg22 showed increased levels of ROS, and dip-inoculation of tomatoes with \(S.\) enterica resulted in a transient induction of the SA-inducible \(prl1\) defense gene. Other work has demonstrated that the presence of a virulent pathogen can protect another non-pathogenic epiphyte from host immunity. A nonpathogenic \(X.\) euvesicatoria T3SS mutant regains the ability to...
grow in planta if co-inoculated with pathogenic X. euvesicatoria. The authors showed that the pathogenic X. euvesicatoria suppressed host immunity, prevented recognition of LPS from both bacterial strains, and inhibited host defense responses. Further, manipulation of the immune system has been linked to water soaking and T3SS in other phytopathogens. P. syringae pv. tomato induces water soaking and suppresses SA-dependent responses in A. thaliana using two T3SS effectors HopM1 and AvrE. Recent work also demonstrated that HopM1 and AvrE induce abscessic acid (ABA) production to close stomata and produce water soaked lesions. We hypothesized that Xhg may similarly manipulate the immune system, providing protection for S. enterica on tomato leaves. Co-inoculation of S. enterica with Xhg resulted in sustained induction of prl1a and repression of the JA-inducible pin1. As induction of prl1a contrasts with suppression or no change in expression of prl genes by other pathogens in wheat or A. thaliana, respectively, our results suggest that Xhg host immune manipulation may occur through a different mechanism. Contrastingly to Xhg, co-inoculation of S. enterica with X. vesicatoria showed no changes in prl1a transcription and induced expression of pin1. Despite these differences in prl1a gene expression between treatments, there was no effect on levels of free or conjugated SA at one and three dpi for any treatment (Fig. S1). Induction of the SA pathway typically results in a spike in hormone levels followed by a rapid return to basal levels. Thus, it is possible that we missed a transient increase in SA that could have led to prl1a induction in the Xhg-treated plants. Even a short burst of SA production could have beneficial effects on S. enterica. Although SA is normally considered to be a mediator of defense responses against bacteria, increased SA levels can result in increased S. enterica antibiotic resistance which could enhance S. enterica resistance to stresses in this niche. Alternatively, SA levels may remain constant, and prl1a may be induced by a different signaling pathway, such as ethylene. However, because the Xhg avrHah1 mutant induced prl1a expression to the same extent as wildtype Xhg, we concluded that prl1a induction does not contribute to the role of AvrHah1 in increasing S. enterica persistence. Although AvrHah1 is not involved, the importance of SA and other plant hormones in S. enterica survival requires further study as there were still significant differences between Xhg and X. vesicatoria in these initial experiments.

In summary, this work identifies one Xhg factor, AvrHah1, which is both necessary and sufficient for providing conditions that lead to increased S. enterica persistence. Although the mechanism by which AvrHah1 promotes S. enterica survival remains unclear, several potential explanations have been described and will require further exploration. Xhg could provide increased nutrient availability through water soaking and/or defense against the plant immune response. Interestingly, X. perforans strains carrying the avrHah1 gene have recently been identified in the southeastern United States, and these strains have gained the ability to cause water soaking in pepper leaves. While X. perforans is typically found in the southeastern United States, Xhg is commonly isolated in the midwestern United States. The recent emergence of another species, with different geographic distribution, carrying avrHah1 suggests selective pressure for horizontal transfer of the avrHah1-containing extrachromosomal plasmid between species. Acquisition of avrHah1 may promote Xanthomonas disease and raises the concern that this genetic transfer could lead to an increased risk of S. enterica contamination of crops around the country. Continued research in this area will provide fundamental knowledge of how an inhospitable environment changes during infection, resulting in survival of human pathogens and an increased risk of human disease.

Materials and methods
Bacterial strains, media, and culture conditions. A kanamycin-resistant strain of S. enterica serovar Typhimurium 14028s and nalidixic acid strains of X. vesicatoria 1111 (ATCC 35937), and Xhg 444 (spontaneous Nal-resistant strain; this study) were used as wildtype strains in this study. S. enterica was made kanamycin resistant by inserting the adaA gene at the attTn7 site. The Xhg avrHah1 mutant was obtained from J. Jones and the X. vesicatoria pUFR034 and pUFR034 (avrHah1) strains were made by transforming X. vesicatoria 1111 with the Kanamycin-resistant plasmids pUFR034 and pUFR034 (avrHah1). Bacterial cultures were grown in lysogeny broth (LB) for S. enterica at 37 °C or nutrient broth (NB) for Xanthomonas spp. at 28 °C with shaking at 200 rpm. The antibiotics nalidixic acid (Nal) and Kanamycin (Kan) were used at concentrations of 20 and 50 μg ml⁻¹, respectively. Strains used in this study are shown in Table 2.

Plant inoculation. Tomato cultivar MoneyMaker seeds were purchased commercially (Eden Brothers). No plant material was collected. This study complies with the relevant institutional, national, and international guidelines and legislation for experimental research on plants. Seedlings were cultivated in Professional Growing Mix (Sunshine Redi-earth) with a 16 h photoperiod at 24 °C for five weeks. For colonization assays, Xanthomonas bacterial cultures were grown for two days in NB (NB with Kan for pUFR strains) at 28 °C, and S. enterica cultures were grown overnight in LB at 37 °C. Bacterial strains were normalized to an optical density at 600 nm (OD₆₀₀) of 0.2 (for S. enterica and X. vesicatoria strains), 0.25 (for the Xhg avrHah1 mutant), and 0.3 (for wildtype Xhg) in sterile water. These OD₆₀₀ values correspond to a bacterial population level of ~10⁸ CFU/ml for the respective strains. Normalized cultures were diluted 1:100 in sterile water for an inoculum level of ~10⁶ CFU/ml. Treatments consisted of individual bacterial strains mixed with equal parts water (S. enterica, Xhg, X. vesicatoria, Xhg avrHah1 mutant, X. vesicatoria pUFR034, or X. vesicatoria pUFR034 (avrHah1)), S. enterica mixed 1:1 with each individual Xanthomonas strain, or water alone. Prior to inoculation, 0.025% Silwett was added to water or the bacterial inoculum. Tomato plants were dip-inoculated by inverting plants in either sterile water or the bacterial inoculum for 30 s with agitation to prevent bacterial cell settlement. Plants were incubated at high humidity for 48 h in lidded, plastic bins under grow lights with a 16 h photoperiod at room temperature (~26 °C). After 48 h, plants were exposed to low humidity conditions (bin lids were removed) during the day and high humidity conditions (bin lids were replaced) during the night. At multiple time points post-inoculation, leaf samples were taken using destructive sampling to determine bacterial populations or collect samples for tomato RNA extraction. For infiltration experiments, MoneyMaker tomato leaves were infil-
treated with Xhg wildtype or the avrHah1ΔDBD mutant at OD_{600} = 0.25, following published protocols^{29}. At 48 h post-infiltration, two 79 cm^2 leaf discs were taken from each of two leaflets on middle leaves and combined for a total of four leaf discs per plant. Samples from four plants per treatment were collected and frozen at −80 °C for further processing, as described below.

**Bacterial population sampling.** Bacterial populations on leaves were determined as described^{14}. Briefly, at indicated times, one 79 cm^2 leaf disc was taken from a leaflet on middle leaves using a surface sterilized cork borer^{18}. Samples from three plants per treatment per time point were individually homogenized in 500 μl of sterile water in microfuge tubes using a 4.8 V rotary tool (Dremel, Mt. Prospect, IL) with microcentrifuge tube sample pestle attachment (Fisher Scientific). Homogenates were diluted as needed and spiral plated (Autoplate 4000, Spiral Biotech, Norwood, MA) on LB Kan (for *S. enterica* or NB NaI or NB Kan NaI (for *Xanthomonas* spp.) plates. Resulting colonies were counted after overnight incubation at 37 °C (for *S. enterica*) or after incubation for three days at 28 °C (for *Xanthomonas* spp.) to determine bacterial populations. Although not shown in the figures, bacterial samples were taken at Day 0 to confirm that all bacterial populations were equivalent at the beginning of each experiment. No statistical differences were noted between treatments for any experimental setup at Day 0 (P > 0.01). Experiments were performed with three biological replicates.

**RNA isolation.** Tomato gene expression levels were determined as described^{14}. At indicated time points, two 79 cm^2 leaf discs were taken from each of two leaflets on middle leaves and combined for a total of four leaf discs per plant. Samples from three plants per treatment per time point were collected and frozen at −80 °C for further processing. RNA was extracted using the Maxwell® RSC Plant RNA kit (Promega). Briefly, four leaf discs were homogenized with a mortar and pestle in the presence of liquid nitrogen. Ground tissue was transferred to a small weigh boat containing 450 μl homogenization solution, mixed with a pipette tip, and transferred to a microcentrifuge tube. Samples were then either stored at −80 °C until further processed or immediately processed according to manufacturer's instructions using a Maxwell® RSC (Promega). RNA samples were eluted in 50 μl volume of RNase-free water and were quantified by NanoDrop (Thermo Scientific). RNA was isolated from three technical replicates for each of three biological replicates.

**cDNA synthesis and real-time PCR.** cDNA synthesis and real-time PCR were performed as described^{14,30}. Briefly, cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR primers for *pin1*, *pr1a1*, *bHLH3*, *bHLH6*, *PL*, and *PE* were designed with Beacon Designer software (Premier Biosoft International) avoiding template secondary structure (Table 2). Primer efficiencies (Table 2) were calculated using serial dilutions of MoneyMaker genomic DNA and CFX Manager 3.0 software (Bio-Rad). Reference transcripts and primers were chosen based on published works: *act41* and *ubi3*^{40}. Stable expression between treatments was validated using the Best Keeper program and three independent RNA samples from each treatment per biological replicate. Real-time PCR experiments utilized the CFX96 Real-Time System, and data were analyzed with the CFX Manager 3.0 software (Bio-Rad). The mean Cq of each target transcript was normalized by the mean Cq of each reference gene using the formula: 

$$
2^{-(\text{Cq target} - \text{Cq reference})}
$$

As previously described^{40}, we determined the relative expression ratio (RER) of the target gene by dividing the normalized target RNA by a calibrator consisting of the average of the normalized values of the control samples (expression after water...
treatment in most experiments; expression after infection with wildtype Xhg in the AvrHah1 target experiments (Table 1)).

**Measurement of free and conjugated SA.** Plants were inoculated with *S. enterica, Xhg, X. vesicatoria, Xhg avrHah1ΔDBD* mutant, with *S. enterica* mixed 1:1 with each individual *Xanthomonas* strain, or with water alone, as described above. Leaf tissue samples were collected at multiple time points post-inoculation, weighed, flash frozen in liquid nitrogen, and extracted in 600 μl of H2O:1-propanol:HCL (1:2:0.005) with 200 ng of d4-SA as an internal standard. For total SA, 2 μl of glacial HCL was added, and the sample boiled for 30 min as in 53. Both free and total SA were phase partitioned with 1 ml MeCl2 and the MeCl2:1-propanol layer was collected and derivatized with trimethylsilyldiazomethane, collected by vapor phase extraction on SuperQ columns, eluted in MeCl2 and analyzed using GC–MS as in 81.

**Electrolyte leakage.** Plants were inoculated with *S. enterica, Xhg, X. vesicatoria, Xhg avrHah1ΔDBD* mutant, with *S. enterica* mixed 1:1 with each individual *Xanthomonas* strain, or with water alone, as described above. Leaf tissue samples were collected at 6 dpi for bacterial populations (as described above) and electrolyte leakage measurements. Three leaf discs taken from a leaflet from a middle leaf were pooled from each plant, and changes in electrolyte leakage (conductance) were quantified as previously described 13.

**Statistical analysis.** All statistical analyses were performed using R software (version 2.14.1; R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria [http://www.R-project.org]) as described 82. Briefly, three biological replicates were performed for each experiment, and samples taken from one replicate were considered as subsamples. Linear regression analysis was used to determine whether bacterial population results differed between treatments for *S. enterica* and *Xanthomonas* spp. For real-time PCR analysis, three samples were compared for each treatment at each time point using Tukey’s HSD test. Results were considered statistically significant at *P < 0.01.*

**Data availability** All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

Received: 4 January 2022; Accepted: 25 April 2022
Published online: 04 May 2022

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Acknowledgements
We would like to thank J. Jones and G. Minsavage for Xanthomonas strains and plasmids and J. Jones and A. Sharma for help with EBE analysis. We would also like to acknowledge JB’s lab members for helpful discussions.

Author contributions
K.C. and J.B. designed the experiments for this work, K.C. and A.B. performed the experiments, and all authors analyzed and interpreted the data. K.C. and J.B. wrote the main manuscript text, and all authors reviewed the manuscript.

Funding
Funding was provided by the Food Research Institute at the University of Wisconsin-Madison. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-11456-6.

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