Identification of pathways required for *Salmonella* to colonise alfalfa using TraDIS-*Xpress*.

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Abstract

Enteropathogenic bacteria, such as *Salmonella*, have been linked to numerous fresh produce outbreaks, posing a significant public health threat. *Salmonella*'s ability to persist on fresh produce for extended periods is partly attributed to its capacity to form biofilms, which pose a challenge to food decontamination and can increase pathogenic bacterial load in the food chain. Preventing *Salmonella* colonisation of food products and food processing environments is crucial for reducing the incidence of foodborne outbreaks. Understanding the mechanisms of establishment on fresh produce will inform the development of decontamination approaches. We used Transposon-Directed Insertion site Sequencing (TraDIS-*Xpress*) to investigate the mechanisms employed by *Salmonella* enterica serovar Typhimurium to colonise and establish on fresh produce over time. We established an alfalfa colonisation model and compared the findings to those obtained from glass surfaces. Our research identified distinct mechanisms required for *Salmonella* establishment on alfalfa compared to glass surfaces over time. These include the type III secretion system (*sirC*), Fe-S cluster assembly (*iscA*), curcumin degradation (*curA*) and copper tolerance (*cueR*). Shared pathways across surfaces included NADH hydrogenase synthesis (*nuoA, nuoB)* , fimbrial regulation *(fimA, fimZ)*, stress response *(rpoS),* LPS O-antigen synthesis *(rfbJ),* iron acquisition *(ybaN*) and ethanolamine utilisation (*eutT, eutQ*). Notably, flagella biosynthesis differentially impacted colonisation of biotic and abiotic environments over time. Understanding the genetic underpinnings of *Salmonella* establishment on both biotic and abiotic surfaces over time offers valuable insights that can inform the development of targeted antibacterial therapeutics, ultimately enhancing food safety throughout the food processing chain.

Importance

Salmonella ranks as the second most costly bacterial foodborne illness in the UK, accounting for £0.2 billion annually, with numerous outbreaks linked to fresh produce such as leafy greens, cucumbers, tomatoes, and alfalfa sprouts. *Salmonella*'s ability to colonise and establish itself in fresh produce poses a significant challenge, hindering decontamination efforts and increasing the risk of illness. Understanding the key mechanisms *Salmonella* uses to colonise plants over time is key to finding new ways to prevent and control contamination of fresh produce. This study identified genes and pathways important for *Salmonella* colonisation of alfalfa and compared those to colonisation of glass using a genome-wide screen. Genes with roles in flagella biosynthesis, lipopolysaccharide production, and stringent response regulation varied in their significance between plants and glass. This work deepens our understanding of the requirements for plant colonisation by *Salmonella*, revealing how gene essentiality changes over time and in different environments. This knowledge is key to developing effective strategies to reduce the risk of foodborne disease.

Funding

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Data availability

Nucleotide sequence data supporting the analysis in this study has been deposited in ArrayExpress under the accession number E-MTAB-13495 (colonisation of alfalfa plants) and E-MTAB-11765 (colonisation of glass beads). The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files.

Introduction

Enteropathogenic bacteria present an evolving threat to public health. Historically, these pathogens were predominantly linked to meat products. However in recent years, fresh produce is emerging as a major cause of these outbreaks, being implicated in over a third of reported outbreaks in certain countries (1). The majority of cases are associated with ready-to-eat crops, although some cases have been attributed to the mishandling of vegetables that are typically subjected to cooking processes (2). Certain human pathogens, such as *Salmonella*, exhibit increased adaptability to colonising various ecological niches and surviving outside their primary host (3). *S. enterica* has been implicated in numerous recent multistate outbreaks associated with contaminated fruits and vegetables, including lettuce, tomatoes, alfalfa, cucumbers, and melons (4, 5, 6, 7). Recent studies have demonstrated *Salmonella*'s ability to actively colonise plant tissues employing specific mechanisms (8). *Salmonella* has been found to persist in produce for extended periods, with viability lasting over six months after initial colonisation to the pathogen (9).

Salmonella's adaptive strategy to persist in the challenging plant environment includes the formation of biofilms. Biofilms are structured, aggregated communities of microorganisms encased in an extracellular matrix and attached to surfaces (10). These communities play a critical role in enabling pathogenic bacteria to adhere to fresh produce increasing the risk of enteric disease transmission (11). Bacteria within biofilms exhibit intrinsic tolerance to high concentrations of antimicrobials, biocides, and disinfectants, which complicates decontamination efforts and poses challenges for ensuring food safety (12). Previous studies have contributed valuable insights into the mechanisms underlying *Salmonella's* biofilm formation and its ability to persist on plants, highlighting the significance of these processes in the context of food safety and public health (13, 14, 15, 16).

Transposon sequencing approaches have previously been used to determine the mechanisms through which bacteria survive in different environments. Tn-seq was used to identify the genes involved in *Pseudomonas simiae* colonisation of plant roots, which highlighted the importance of genes involved in flagella production, cell envelope biosynthesis, carbohydrate metabolism and amino acid transport and metabolism (17). A similar Tn-Seq approach was used to determine which genes are required for *Salmonella* colonisation of tomatoes, identifying a high abundance of mutants associated with amino acid biosynthesis (18). We have previously used another transposon sequencing approach, TraDIS-*Xpress*, to find the genes involved in biofilm formation in *Escherichia coli* (19) and *Salmonella enterica* serovar Typhimurium (20) on glass over time. TraDIS-*Xpress* builds on conventional transposon sequencing approaches by using larger denser transposon mutant libraries and by incorporating an outwards-transcribing promoter into the transposon element (21). Induction of this promoter enables increased expression of genes downstream of transposon insertions thereby facilitating investigation into how expression, as well

as gene disruption, affects survival of the mutant in a given condition. This approach also allows for the analysis of essential genes which do not tolerate insertional inactivation by transposons and can therefore not be assayed with conventional tools.

In this study, we established an alfalfa plant colonisation model that was used in conjunction with TraDIS-*Xpress* to investigate gene essentiality in *Salmonella* establishment on alfalfa over time. A library of *S.* Typhimurium transposon mutants was cultivated on sprouted alfalfa plants and cells were isolated at different stages to identify the genes involved in establishment on plants development *in planta* over time. Comparisons were made with findings from our previous study focusing on biofilm formation on glass surfaces (20). This allowed for the identification of plant-specific and glass-specific mechanisms used by *S.* Typhimurium to establish in biotic and abiotic surfaces, as well as conserved genes that play crucial roles on both surfaces.

We showed variations in the importance of factors including flagella biosynthesis, LPS production, and stringent response regulation in establishment on plants versus glass surfaces. Understanding the genes involved in colonisation of both biotic and abiotic surfaces over time provides valuable insights for the development of targeted antibacterial therapeutics to enhance food safety throughout the food processing chain.

Results

Establishment of an alfalfa plant colonisation model

To assess the ability of *S.* Typhimurium to establish and proliferate on plant hosts, an alfalfa seedling model was established (Figure 1). Initially, seeds underwent sterilisation and were allowed to germinate in Murashige-Skoog (MS) medium for three days (Figure 1 A,B). Following this germination period, the seedlings were inoculated at the root-shoot intersection with a *S.* Typhimurium strain marked with the *lacZ* reporter gene (14028*S::lacIZ*) for blue colony selection and counting (Figure 1 C,D).

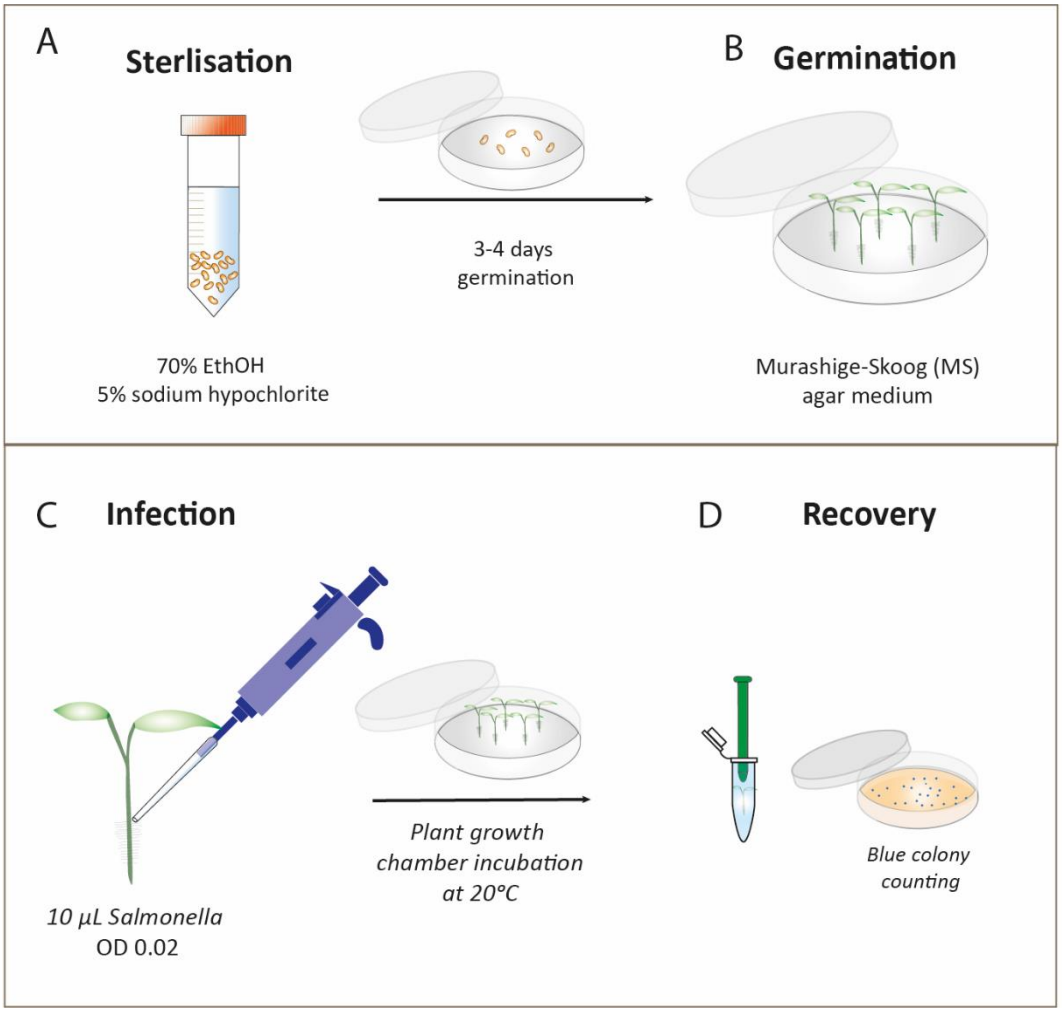

Figure 1: Alfalfa Plant Colonisation Model. A. Alfalfa seeds were sterilised by immersion in 70% ethanol for 30 seconds, followed by a 3-minute wash in 5% sodium hypochlorite. B. Subsequently, the sterilised seeds were left to germinate in darkness at 20°C in Murashige-Skoog (MS) agar medium for 3-4 days. C. Inoculation of the seedlings was performed at the root-shoot intersection using 10 µL of Salmonella inoculum, normalised to an optical density (OD) of 0.02. Inoculated seedlings were then transferred to fresh MS plates and incubated in a benchtop plant growth chamber at 20°C. D. To facilitate selection via blue colony screening, Salmonella recovery and quantification were performed over time using the 14028S::lacIZ strain. Inoculated seedlings were homogenised by mechanical disruption using a pestle to release the bacterial cells. Cell suspensions were subjected to serial dilution and plated onto X-gal/IPTG LB plates for further analysis.

Salmonella effectively colonises alfalfa sprouts and increases in numbers over time.

To investigate the effectiveness of *Salmonella* colonisation in alfalfa seedlings, a strain tagged with *lacZ* (14028*S::lacIZ*) (22) was used to inoculate seedlings three days after germination. Following inoculation, the seedlings were homogenised and CFU/mL per seedling was quantified. Cells were recovered after 8, 24, 48 and 72-hours growth, demonstrating a significant increase in *S*. Typhimurium colonisation of alfalfa over time (see Figure 2).

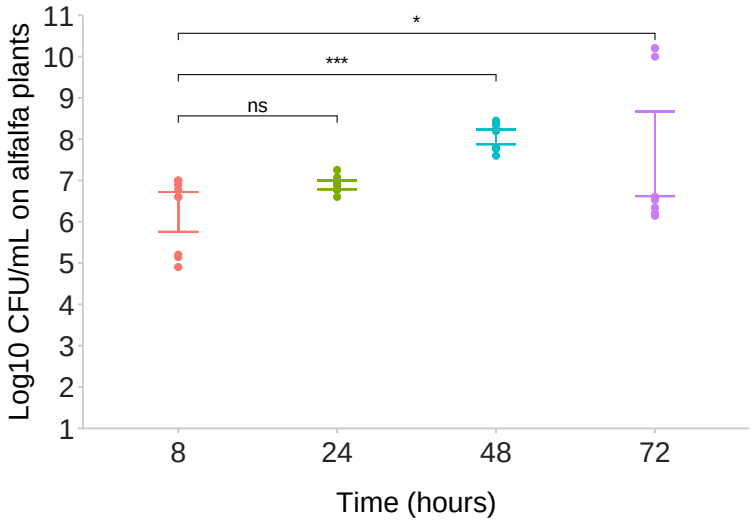

Figure 2: Salmonella *effectively colonises the alfalfa model. S. Typhimurium was isolated from alfalfa seedlings following 8-, 24-, 48- and 72-hours post-inoculation, and CFU/mL was determined at each time point. Points represent three biological and three technical replicates, and error bars show 95% confidence intervals. Asterisks show significant difference (Student's* t-test) *in CFU/mL from the 8 hour time point: ns not significant,* * p < *0.05,* ** p < *0.01,* *** p < *0.001,* **** p < *0.0001.*

Genes involved in *Salmonella* establishment on alfalfa over time.

TraDIS-*Xpress* was used to identify genes involved in alfalfa colonisation by *S.* Typhimurium over 3 days (24-, 48- and 72-hours post-seeding). These timepoints were carefully considered to capture the potentially diverse mechanisms required by *Salmonella* at different stages of alfalfa colonisation. This includes the early stages involving initial attachment and microcolony formation (at 24 hours) and the subsequent phases of *Salmonella* establishment on alfalfa (spanning 48 to 72 hours). We identified 69 genes in total involved in *S.* Typhimurium colonisation and establishment on alfalfa sprouts over time (supplementary table 1). These included genes involved in LPS biosynthesis, DNA housekeeping, respiration and responding to stress (Figure 3). Variation in insertion frequency per gene between replicates was low, indicating low experimental error (supplementary figure 1).

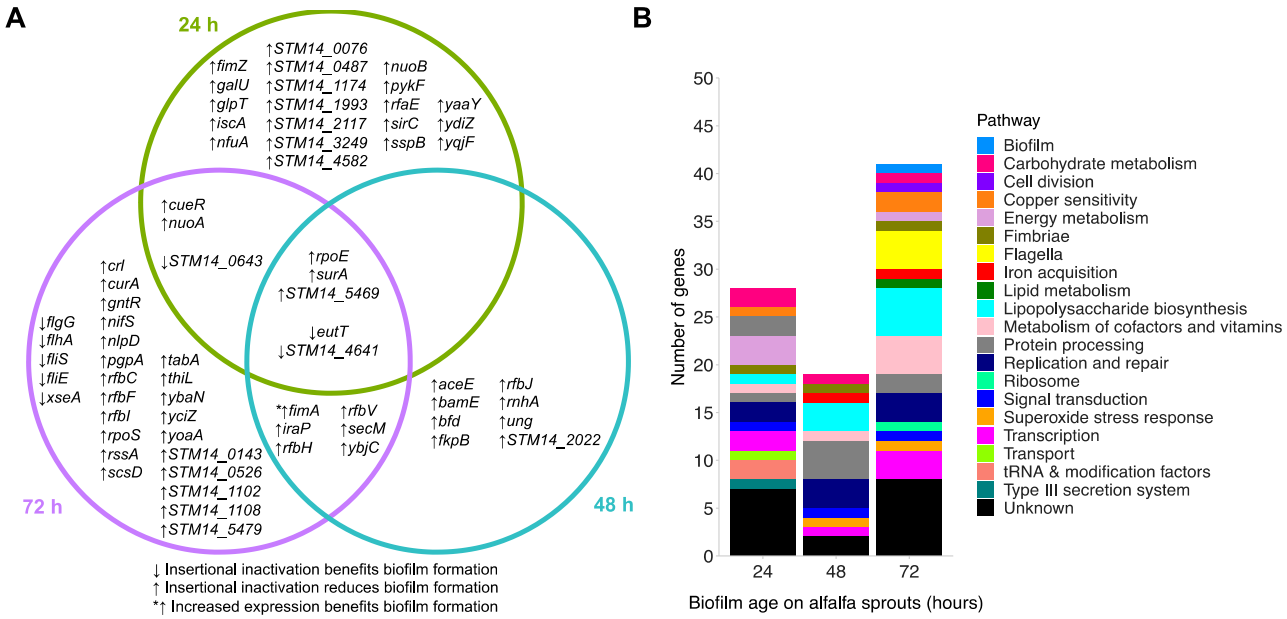

*Figure 3: A) Genes and B) pathways identified by TraDIS-*Xpress *to be involved in alfalfa colonisation 24-, 48- and 72-hours post-inoculation.*

Genes involved in adhesion were identified as beneficial after 24 hours growth, including previously reported genes, such as a negative fimbrial regulator *fimZ* (23) and type III secretion system component *sirC* (24). After 48 hours, genes involved in DNA housekeeping (*rnhA* and *ung*) (25, 26), iron storage (*bfd*) (27) and outer membrane protein assembly (*bamE*) (28) benefit the further establishment of *Salmonella* on alfalfa. After 72 hours of growth, genes associated with roles in LPS O-antigen production (*rfbF, rfbI, rfbC, rfbV* and *rfbH*) (29), flagella biosynthesis (*flgG, flhA, fliS* and *fliE*) (30) and responding to stress (*rpoS, iraP* and *crl*) (31) were identified.

Five genes were shared among the time points tested; these were *eutT, surA, rpoE*, *STM14_4641* and *STM14_5469*. Preventing the function of the *eut* operon through disruption of *eutT* (32) was beneficial to *S.* Typhimurium establishment at all time points tested. Transcription of *STM14_4641* encoding an RNA-directed DNA polymerase was detrimental to colonisation throughout its growth

on alfalfa sprouts. There were fewer transposon mutants across all time points in *surA* (outer membrane protein chaperone (33)), *rpoE* (sigma factor involved in responding to misfolded protein stress (34)) and *STM14_5469* (unknown function) relative to planktonic controls, which suggests these genes are beneficial throughout all stages of alfalfa colonisation.

Conserved pathways crucial for *Salmonella* establishment on alfalfa sprouts and glass

We have previously identified genes essential for biofilm formation on glass over time using the same *S.* Typhimurium transposon mutant library as used in this study (20). The library used has 500,000 unique insertion sites, corresponding to approximately one insertion every eight base pairs. Insertion frequencies in mutant libraries colonising glass or plant surfaces were both compared to planktonic cultures grown for the same amount of time. This acted as a standard to demonstrate where transposon insertions affected surface colonisation relative to planktonic growth, and the subsequent gene lists for bacterial communities at the same developmental stages on each surface were then compared. This found pathways involved in *S.* Typhimurium establishment on both surfaces included flagella biosynthesis, LPS production, respiration, iron storage and responding to stress. Seven genes were found to be conserved between growth on alfalfa sprouts and on glass (figure 4). These were *nuoA* and *nuoB*, involved in synthesis of the first NADH hydrogenase in the electron transport chain (35),(35), fimbrial subunit *fimA* and its regulator *fimZ* (23), *rfbJ* involved in LPS O-antigen synthesis (29), *ybaN* predicted to have a role in iron acquisition (36), and stress response sigma factor *rpoS* (37). The ethanolamine utilisation pathway played an important role in *S.* Typhimurium establishment on both alfalfa sprouts (*eutT*) and on glass (*eutQ*) at all time points tested, with disruption of each gene seen to aid colonisation. Together, this reveals a core set of pathways involved in colonisation of both biotic and abiotic surfaces (Figure 4).

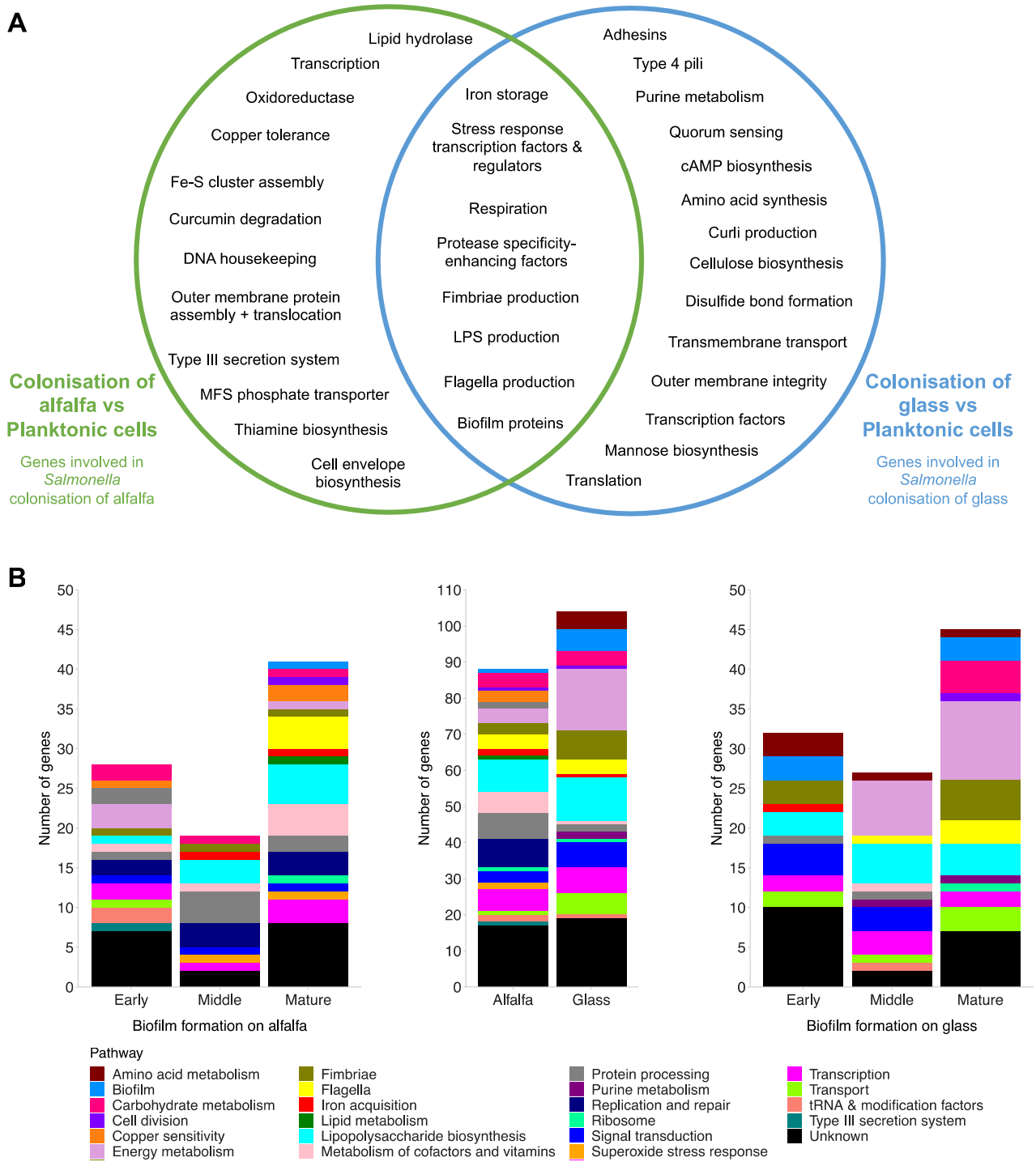

Figure 4: A) Conserved and surface-specific pathways involved in S. Typhimurium colonisation of alfalfa sprouts and glass. B) Abundance of genes in each pathway for Salmonellagrown on alfalfa sprouts or glass over time.

Differential Flagella and Lipopolysaccharide Biosynthesis in Alfalfa vs. Glass

Deletion mutants were constructed in targets identified by TraDIS-*Xpress* to investigate their effects on colonisation and establishment on the two surfaces (biotic and abiotic). These mutants were subjected to competitive colonisation experiments with wild type *S.* Typhimurium strains on both glass and alfalfa surfaces. Equal numbers of mutant and wild type CFU/mL were inoculated onto glass beads and alfalfa plant sprouts. Subsequently, the percentage change in mutant CFU within the recovered populations from each surface was determined over time.

TraDIS-*Xpress* indicated that inactivation of genes involved in flagella biosynthesis was beneficial for plant colonisaton after 72 hours growth (Figure 5A). We predicted that because flagella are detected by the plant's immune system, aflagellated cells will have a competitive advantage in these communities during colonisation. Our previous work suggested aflagellated cells were disadvantaged at colonising glass surfaces (20). To characterise the role of flagella in *S.* Typhimurium establishment on both environments, a deletion mutant of the main flagella biosynthetic regulator (*flhDC*) and a component of the flagella export machinery (*flhA*) were grown on glass and alfalfa sprouts in competition with wild type *S.* Typhimurium. At the initial stages of colonisation (24 hours post-inocculation), Δ*flhDC* and Δ*flhA* exhibited a significantly enhanced competitive advantage at colonising glass but were competitively disadvantaged at colonising alfalfa plants (Figure 5B), contrary to the TraDIS-*Xpress* findings.

LPS core and O-antigen biosynthesis genes were beneficial for growth on alfalfa sprouts, however the impact of different LPS biosynthesis genes on *S.* Typhimurium colonisation varied. Some exhibited beneficial effects when inactivated during glass colonisation, while others had detrimental impacts. Based on the TraDIS-*Xpress* data, *rfbJ* was beneficial for growth and establishment on alfalfa sprouts, whereas inactivation of the gene was beneficial for establishment on glass. We created a deletion mutant of *rfbJ* in *S.* Typhimurium to investigate its effect on glass and plant colonisation. Deletion of *rfbJ* resulted in reduced colonisation of both glass and plant over time (Figure 5B). This indicates the importance of this gene for adhesion and colonisation of both surfaces.

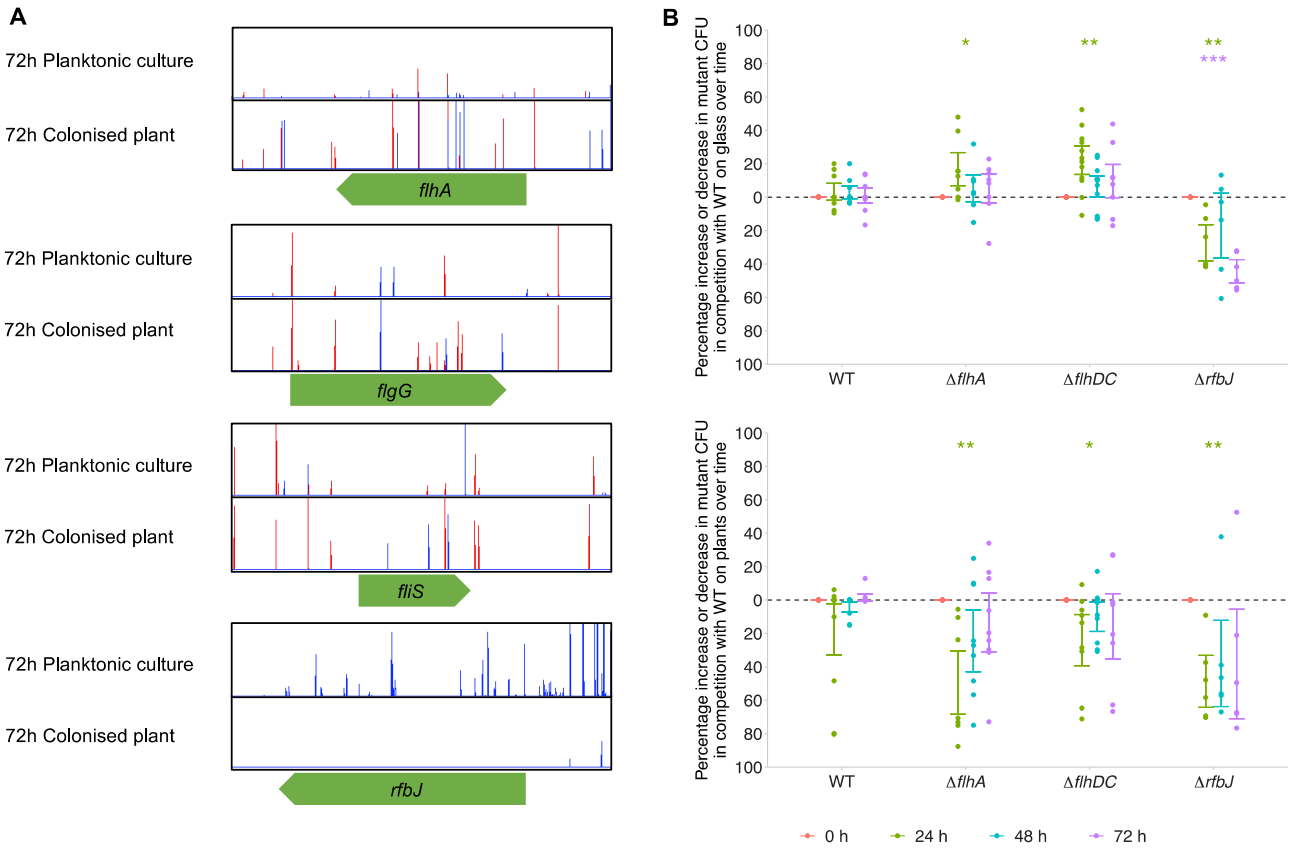

*Figure 5: A) Insertion loci and frequency in and around genes involved in flagella biosynthesis (flhA, flgG and fliS) and LPS O-antigen biosynthesis (rfbJ) following growth on alfalfa sprouts relative to planktonic growth. Red lines indicate the transposon-located promoter is facing left-to-right and blue lines indicate it is oriented right-to-left. Images are representative of two independent replicates. B) Percentage increase or decrease in flhA, flhDC and rfbJ deletion mutants in biofilms formed on glass (top panel) and alfalfa plant sprouts (bottom panel) in competition with wild type (WT) S. Typhimurium. Points show changes in the percentage of mutant CFU relative to time point 0, and show 3 technical and 4 biological replicates. Error bars denote 95% confidence intervals and asterisks show significant differences (One-sample t-test, change from 0) of each mutant from time point 0, where time points are distinguished by colour: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.*

<u>Genes involved in copper tolerance, type III secretion regulation and curcumin degradation conferred a competitive advantage to *Salmonella* establishment on alfalfa.</u>

Analysis of the TraDIS-*Xpress* data found pathways involved in *S.* Typhimurium establishment on alfalfa plants that were not involved during biofilm formation on glass. These included type III secretion regulation (*sirC*) (24) and Fe-S cluster assembly (*iscA*) (38), which were beneficial at the early stages of colonisation of alfalfa. Curcumin degradation (*curA*) (39) was beneficial following 72 hours growth on alfalfa and copper tolerance (*cueR*) (40) was beneficial following 24 and 72 hours growth on alfalfa.

Gene deletion mutants were made in these genes and grown in the presence of wild type *S.* Typhimurium on glass and alfalfa plants to investigate their effects on colonisation. Deletion of *iscA* resulted in a competitive disadvantage for colonisation of both glass and alfalfa plants, supporting

the TraDIS-*Xpress* findings (figure 6B). Deletion of *cueR* caused a competitive disadvantage in colonisation of alfalfa plants, but there was no significant change in glass colonisation, demonstrating that expression of *cueR* is only beneficial for colonisation of plant surfaces and not glass surfaces. There was no significant change in the percentage of Δ*sirC* or Δ*curA* mutants over time on either glass or plants, suggesting the effects of these genes on colonisation observed in the TraDIS-*Xpress* data cannot be quantified by this assay.

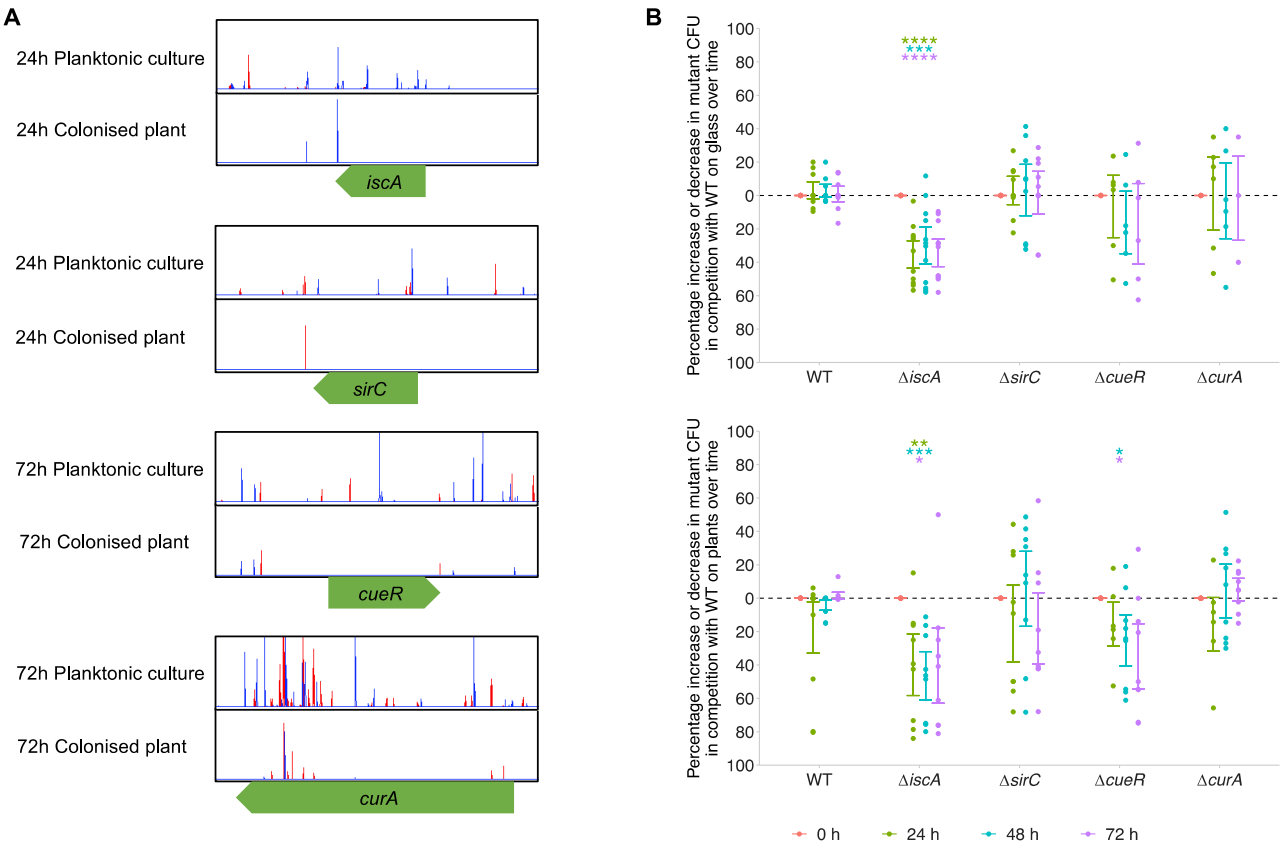

*Figure 6: a) Transposon insertions within and around iscA, sirC, cueR and curA in S. Typhimurium planktonic culture compared to Salmonella recovered from alfalfa after 24 or 72 hours. Lines show the insertion loci and the height of the lines shows the number of reads mapped to the loci. The colour of the line indicates the orientation of the promoter within the transposon: red lines denote the promoter is promoting transcription left-to-right, and blue lines denote right-to-left. Plot files shown are representative of two independent replicates. b) Percentage increase or decrease in iscA, sirC, cueR deletion mutants in biofilms formed on glass (top panel) and alfalfa plant sprouts (bottom panel) in competition with wild type (WT) S. Typhimurium. Points show changes in the percentage of mutant CFU relative to time point 0, and show 3 technical and 4 biological replicates. Error bars denote 95% confidence intervals and asterisks show significant differences (One-sample t-test, change from 0) of each mutant from time point 0, where time points are distinguished by colour: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.*

Discussion

The primary objective of this study was to identify the mechanisms employed by *S.* Typhimurium to colonise effectively and establish on fresh produce and compare these to the pathways required for colonisation and biofilm formation on glass, across various stages of colonisation. To achieve this, we established a fresh produce alfalfa colonisation model and used genome-wide transposon insertion sequencing (TraDIS-*Xpress*) to investigate *S*. Typhimurium establishment on alfalfa, comparing our findings to mechanisms previously identified for biofilm formation on glass surfaces (20). Our aim was to discern the extent to which these mechanisms are universally necessary for adhesion, colonisation, and establishment on biotic surfaces in contrast to abiotic surfaces. Our working hypothesis centred on the presence of both common and distinct mechanisms in the two tested environments. Several key findings emerge from this study.

We found differences in gene essentiality differed over time as *S*. Typhimurium colonised the alfalfa, in a similar way to which was seen on glass surfaces. Initially, we identified the importance of genes involved in adhesion and type III secretion systems, and over time genes involved in DNA housekeeping and envelope synthesis became more important for establishment. In the latest colonisation timepoint tested, genes involved in LPS synthesis, flagella synthesis and global stress response systems were key to *S.* Typhimurium establishment on alfalfa. We identified seven conserved genes important in *S.* Typhimurium establishment on both alfalfa sprouts and glass, highlighting the shared genetic elements critical for *S.* Typhimurium colonisation of diverse surfaces. These genes belong to various functional categories, including NADH hydrogenase synthesis (*nuoA* and *nuoB*), fimbrial regulation and production (*fimA* and *fimZ*), LPS O-antigen synthesis (*rfbJ*), iron acquisition (*ybaN*), and stress responses (*rpoS*). Ethanolamine utilisation genes, *eutT* and *eutQ*, were also identified to play an important role in *S.* Typhimurium establishment on both environments, with their disruption aiding colonisation of both surfaces. Notably, ethanolamine signalling has been reported to aid *S.* Typhimurium infection of mammalian cells (41). The identification of these conserved genes underscores their significance in surface colonisation, regardless of the surface material.

Flagella biosynthesis was found to affect colonisation of biotic and abiotic surfaces differently in our study. We showed that aflagellated mutants (*ΔflhDC* and *ΔflhA*) exhibit significantly enhanced glass colonisation at the early stages of colonisation (24 hours) but perform significantly worse on alfalfa. However, with time, these mutants regain their ability to grow on alfalfa. This demonstrates the potential role of the flagellum for initial stages of adhesion to alfalfa. We know that flagellar motility is essential for initial host colonisation in several bacterial species (42, 43). This contrasts with TraDIS-*Xpress* results, highlighting the complexity of the role of flagella at different stages of colonisation and the adaptive capabilities of *S.* Typhimurium over time (44).

We also found pathways involved in *S.* Typhimurium establishment on alfalfa seedlings that were not involved in biofilm formation on glass. Notably, genes related to type III secretion regulation (*sirC*), Fe-S cluster assembly (*iscA*), curcumin degradation (*curA*), and copper tolerance (*cueR*) confer a competitive advantage to *S.* Typhimurium during colonisation of alfalfa. Deletion of *cueR* reduced the ability of *S.* Typhimurium to colonise plants but had no effect on glass, demonstrating a conditional importance between surfaces. Metals play an important role in plant-pathogen interactions (45), and regulating the expression of copper export through *cueR* is therefore beneficial for colonisation and establishment on a plant. Deletion of *iscA* reduced colonisation on both glass and plant surfaces, and there was no difference in colonisation seen in Δ*sirC* or Δ*curA* deletion mutants. TraDIS-*Xpress* is able to determine very small changes in competitive fitness that may not always be seen in culture-based assays, therefore further characterisation is needed to determine how these genes affect plant colonisation.

The use of mixed pools of mutants in TraDIS-*Xpress* experiments offers several advantages, primarily by better simulating the complexity of environmental communities composed of multiple strains and species. This approach is more representative of real-world populations compared to isogenic populations typically studied *in vitro*. However, this comes with limitations, particularly for follow-up target characterisation. Differences between polygenic and isogenic populations can result in discrepancies when comparing data from whole gene deletion mutants and TraDIS-*Xpress* data. Microbes form complex communities and structures (such as biofilms) that can be influenced by various factors affecting their fitness over time. Consequently, differences between gene deletion mutants and the wild type may not always be readily detectable in simple culture-based assays.

In conclusion, this research provides a comprehensive understanding of the genetic determinants that influence *S.* Typhimurium colonisation and establishment on diverse surfaces. The findings emphasise the role of specific genes at different stages of *S.* Typhimurium colonisation of fresh produce, reflecting its adaptability and the conditional importance of certain pathways. Moreover, the identification of conserved genes highlights their significance in the pathogen's establishment on various substrates. This knowledge is invaluable in advancing our understanding of *Salmonella* pathogenesis and host-microbe interactions and may have implications for controlling *Salmonella* colonisation and infection.

Materials and Methods

Alfalfa seed sterilisation and germination

Alfalfa seeds were sterilised by immersion in 20 mL of 70% ethanol for 30 seconds, followed by three sequential rinses with 20 mL sterile water. Subsequently, the seeds were treated with 5% sodium hypochlorite (20 mL) for 3 minutes on a rolling platform. Three subsequent washes in water were carried out. For germination, sterilised seeds were transferred to square agar plates (20 mL) containing Murashige-Skoog (MS) agar medium. These seeds were positioned with sufficient spacing to allow for three days of germination, reaching an approximate size of 1 cm. Following germination, the seedlings were transferred to fresh MS plates and inoculated with *S.* Typhimurium. Adequate seedlings were included in the process to enable replication for experimental purposes.

Quantification of *Salmonella* on alfalfa seedlings

Three-day-old alfalfa seedlings were inoculated with 10 µL *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain 14028*S* tagged with the *lacZ* operon (14028*S::lacIZ*) (22), with the bacterial density normalised to an optical density (OD_{600nm}) of 0.02. The seedlings were incubated at 20 °C throughout the experiment's duration. After 8-, 24-, 48-, and 72-hours post-inoculation, three seedlings per timepoint were homogenised using a plastic pestle in PBS and then serially diluted in PBS. The dilutions were spotted on LB-agar plates supplemented with 40 µg/mL X-gal (-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) and 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside), which allows S. Typhimurium tagged with *lacZ* to appear blue. The prepared plates were incubated at 37 ºC overnight. Following overnight incubation, colony-forming units (CFU) were counted. Each time point included at least three technical replicates and three biological samples, ensuring robust and reliable quantification of *S.* Typhimurium populations.

Competition assays on alfalfa seedlings and glass

Single gene deletion mutants were made following the gene doctoring protocol (46) using plasmids constructed via Golden Gate assembly (47). Mutants were validated by whole genome sequencing on NextSeq2000 (Illumina), aiming for a 60x coverage to confirm loss of the gene of interest. Sequencing files were assembled into contigs using Shovill (version 1.1.0) (48) and mapped against a reference genome (CP001363) to validate loss of the target gene. Primers for mutant construction are listed in supplementary table 2. For competition in alfalfa seedlings, three-day-old seedlings were inoculated with 10 µL of *S*. Typhimurium tagged with *lacZ* (14028*S::lacZ*) in a 1:1 ratio with deletion mutants, all adjusted to a final OD of 0.02 in 10 mM $MgCl_2$. Inoculated seedlings were subsequently transferred to fresh MS plates and incubated at 20°C. After 24-, 48-, and 72-hours post-inoculation, three seedlings per timepoint were homogenised using a plastic pestle in PBS and then serially diluted in PBS. The dilutions were spotted on LB-agar plates supplemented with 40 µg/mL X-gal and 1mM IPTG. For competition on glass beads, beads suspended in 5 mL of

LB-NaCl were inoculated with 50 µL of selected strains mixed with 14028*S::lacZ* in a 1:1 ratio, normalised to a final OD of 0.02. After incubation, three beads were recovered at 24-, 48-, and 72-hours post-inoculation, washed in PBS to eliminate planktonic growth, and the biofilm cells were recovered by vortexing in PBS. The recovered cells were serially diluted and spotted on LB agar plates supplemented with 40 µg/mL X-gal and 1 mM IPTG.

TraDIS-*Xpress* library preparation, sequencing and data analysis

Three-day-old alfalfa seedlings, grown on MS agar, were inoculated at the shoot-root junction with a 10 µL droplet of a *S.* Typhimurium transposon mutant library (described by Holden, Yasir (20), normalised to an OD_{600nm} of 0.01 with 1 mM IPTG to induce transcription from the transposon-located promoter. Seedlings were then allowed to grow at 30 ºC (for the results to be directly comparable to growth on glass beads) before sampling following 24-, 48- and 72-hours growth. Ten seedlings were processed per timepoint and were homogenised in 1 mL of sterile PBS using a plastic pestle. Samples were filtered through 5 µm syringe filters to isolate bacterial cells and eliminate plant cell contamination. Genomic DNA was extracted from these cells following the protocol described by Trampari, Holden (49). A Mu sSeek DNA fragment library preparation kit (ThermoFisher) was used to tagment genomic DNA and was then purified with AMPure XP beads (Beckman Coulter). DNA fragments were amplified using customised primers that anneal to the tagmented ends and biotinylated primers that anneal to the transposon. These PCR products were purified and biotinylated DNA was incubated for 4 hours with streptavidin beads (Dynabeads® kilobaseBINDER™, Invitrogen) to capture only DNA fragments containing the transposon. These fragments were amplified using barcoded sequencing primers that anneal to the tagmented ends and to the transposon (21). DNA fragments were then purified and size-selected using AMPure beads. Fragment length was quantified using a Tapestation (Aligent) and sequenced on a NextSeq500 using the NextSeq 500/550 High Output Kit v2.5 with 75 cycles. Fastq files were aligned to the *S*. Typhimurium 14028*S* reference genome (CP001363, modified to include chromosomally integrated *lacIZ*) using BioTraDIS (version 1.4.3) (50). Significant differences ($p < 0.05$, after correction for false discovery) in insertion frequencies between planktonic and *Salmonella* recovered from glass and alfalfa at each time point were found using BioTraDIS and AlbaTraDIS (version 1.0.1) (51). Amino acid sequences for genes of unknown function were analysed using EggNOG (version 5.0.0) (52) to determine predicted function.

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