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Research Letter

RESEARCH LETTER – Food Microbiology

Salmonella enterica serovar Typhimurium ATCC 14028S is tolerant to plant defenses triggered by the flagellin receptor FLS2

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One sentence summary: This study demonstrates that FLS2-mediated plant defenses are ineffective in preventing growth of *Salmonella enterica* strain 14028S.

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ABSTRACT

Salmonellosis outbreaks associated with sprouted legumes have been a food safety concern for over two decades. Despite evidence that *Salmonella enterica* triggers biotic plant defense pathways, it has remained unclear how plant defenses impact *Salmonella* growth on sprouted legumes. We used *Medicago truncatula* mutants in which the gene for the flagellin receptor FLS2 was disrupted to demonstrate that plant defenses triggered by FLS2 elicitation do not impact the growth of *Salmonella enterica* serovar Typhimurium ATCC 14028S. As a control, we tested the growth of *Salmonella enterica* serovar Typhimurium LT2, which has a defect in *rpoS* that increases its sensitivity to reactive oxygen species. LT2 displayed enhanced growth on *M. truncatula* FLS2 mutants in comparison to wild-type *M. truncatula*. We hypothesize that these growth differences are primarily due to differences in 14028S and LT2 reactive oxygen species sensitivity. Results from this study show that FLS2-mediated plant defenses are ineffective in inhibiting growth of *Salmonella entrica* 14028S.

Keywords: *Salmonella*; flagellin; FLS2; plant–microbe interactions

INTRODUCTION

Salmonella enterica contamination of sprouted legume seeds, such as alfalfa, has been a food safety concern for decades (Mahon *et al.* 1997; Dechet *et al.* 2014). Seed stocks are often implicated as the source of contamination (Mahon *et al.* 1997) and the favorable growth conditions provided by the sprout production environment lead to a rapid increase in *Salmonella* numbers (Howard and Hutcheson 2003). *Salmonella* serovars display multiple logs of growth on sprouting legume seeds and in the surrounding sprouted seed exudate, which contains a rich mixture of nutrients, including sugars and amino acids (Howard and Hutcheson 2003; Jayaraman *et al.* 2014; Kwan *et al.* 2015). Inhibition of attachment factor production, such as O-antigens, aggregative fimbria or cellulose, reduces *Salmonella*

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attachment and subsequent growth on sprouting legumes indicating that *Salmonella* closely associates with sprouting legumes (Barak *et al.* 2005, 2007). Furthermore, *Salmonella* can establish persistent endophytic populations in sprouted legume seedlings with inocula containing as few as two colony forming units (Jayaraman *et al.* 2014).

The presence of *Salmonella* triggers transcriptional changes in the plant, including the activation of plant defense genes. In *Arabidopsis thaliana*, inoculation with *Salmonella* activates defense pathways (Schikora *et al.* 2008). *Salmonella* inoculation of the model legume *Medicago truncatula* also induces significant transcriptional changes, including the up-regulation of multiple defense genes (Jayaraman *et al.* 2014). The interaction between *Salmonella* and plants has been further characterized on a mechanistic level through investigation of the role that *Salmonella* flagellin plays in triggering plant responses to *Salmonella*. An epitope of bacterial flagellin (referred to as flg22) is a Microbe Associated Molecular Pattern (MAMP) that is recognized by a variety of plant species through the LRR receptor-like kinase FLS2. (Felix *et al.* 1999; Gómez-Gómez and Boller 2000). Recognition of this eliciting epitope stimulates the Pathogen Triggered Immunity response in plants (Jones and Dangl 2006). Stimulation of Pathogen Triggered Immunity through the FLS2 receptor in *Arabidopsis* triggers many plant defense responses, including the production of reactive oxygen species (ROS) (Apel and Hirt 2004), deposition of callose (Luna *et al.* 2011) and closure of stomata (Zeng and He 2010). Work by Meng and colleagues demonstrated that synthetic flg22 peptide representing the FLS2 eliciting portion of *Salmonella* flagellin elicits an immune response, including the production of ROS, in tomato and *Nicotiana benthamiana* (Meng, Altier and Martin 2013). Immune response stimulation by *Salmonella* flg22 has also been demonstrated in *Arabidopsis* (Garcia *et al.* 2014); however, it has not been shown if recognition of flagellin through the FLS2 receptor impacts *Salmonella* growth in sprouted legume seeds.

While multiple studies have demonstrated that *Salmonella* activates plant defense pathways, the high levels of *Salmonella* growth in the sprouted legume seed environment suggest that this immune response may not significantly impact *Salmonella* proliferation. Here, we used *M. truncatula* mutants, in which the gene for the FLS2 receptor has been disrupted by transposon insertion, to elucidate the impact that the exposure of *M. truncatula* to *Salmonella* flagellin has on *Salmonella* growth on sprouted legume seedlings. By manipulating the presence of the plant FLS2 receptor, we were able to investigate this interaction without adding the confounding variable of bacterial motility. Defects in bacterial motility reduce plant colonization by growth-promoting bacteria (de Weger *et al.* 1987; Broek, Lambrecht and Vanderleyden 1998; Gao *et al.* 2016; Rossi *et al.* 2016). Motility enhances the invasiveness of the plant pathogen *Pseudomonas syringae* (Haefele and Lindow 1987). Conflicting results of the role of flagellar motility in *Salmonella* plant colonization have been reported. In *A. thaliana*, *Medicago sativa* and lettuce, *Salmonella* colonization or internalization is reduced in motility mutants (Cooley, Miller and Mandrell 2003; Kroupitski *et al.* 2009; Cowles *et al.* 2016); however, a study done in *M. truncatula* found enhanced colonization by motility mutants (Iniguez *et al.* 2005). Due to the demonstrated importance of bacterial motility in plant colonization and the conflicting findings regarding the role of motility in *Salmonella* plant colonization, the role of FLS2 triggered plant defenses using *M. truncatula fls2* mutants instead of *Salmonella* flagella mutants were investigated.

MATERIALS AND METHODS

Bacterial serovars and inoculum preparation

Strains used in this work were *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028S and *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2. Strains were stored in nutrient broth (BD Biosciences, Sparks, MD) with 15% (vol/vol) glycerol at -70°C. Working stocks were maintained on Luria-Bertani (LB) agar (BD Biosciences) at 4°C.

Single colonies were stab inoculated into motility agar and cultured at 37°C for use in plant colonization assays. Plant inoculum was prepared by dissolving four 5 mm punches of agar from a motility agar plate into 50 mL sterile water.

Plant material

M. truncatula seeds were acid scarified using sulfuric acid, surface sterilized for 2 minutes using commercial bleach, and germinated on 1% agar plates supplemented with 1 μg/ml gibberellic acid. Seeds were vernalized at 4°C for 1–3 days before germination. Colonization assays and formazan precipitation assays were performed with 1-day-old seedlings. *M. truncatula* R108 seeds were used as wild type.

M. truncatula FLS2 was identified based on homology to *A. thaliana* FLS2. *M. truncatula fls2* mutants were identified by PCR-based reverse genetics screening (Cheng *et al.* 2014) a population of *M. truncatula* transposon (*Tnt1*) insertion lines (Tadege *et al.* 2008). Mutants were self-pollinated, and the F2 generation was screened by PCR for homozygous mutants. *Tnt1* insertion site was mapped by PCR and confirmed by Sanger sequencing using a primer embedded in the *Tnt1* and a primer in the flanking chromosome region (Fig S1, Supporting Information).

Sprouted seedling *Salmonella* growth assay

One-day-old *M. truncatula* seedlings were placed in 1.5 ml of inoculum prepared from *Salmonella* grown in motility agar. The *Salmonella* suspension was used to inoculate four to six replicates containing four seedlings each of either R108, *fls2–1* or *fls2–2*. The *Salmonella* inoculum was prepared fresh for each trial and contained an average of 10^6 colony forming units (CFU). Replicate colonization was normalized to the average colonization of R108 for each respective inoculum. Inoculated seedlings were kept at 25°C without light for 18 hours. These conditions were chosen to emulate the conditions in which commercial legume sprouts are produced. Following incubation, the seedlings were collected, weighed and homogenized in $1 \times$ PBS, pH 7.2, with 20% glycerol. Serial dilution in $1 \times$ PBS, pH 7.2 with 20% glycerol and enumeration of CFU were performed to determine colonization levels. Colonization levels were normalized to seedling weight. In colonization assays using flg22, the peptide was used at a concentration of 10 μM.

Hydrogen peroxide survival assay

14028S and LT2 were grown in sprouting seed exudates for 18 hours at 25°C. Cultures were diluted in sterile water to a concentration of approximately 10^7 CFU/ml and challenged with 30 mM hydrogen peroxide at 25°C without shaking. CFU enumeration was performed before the addition of hydrogen peroxide and at 30 and 60 minutes after the addition of hydrogen peroxide. Dilution and plating were conducted as described above.

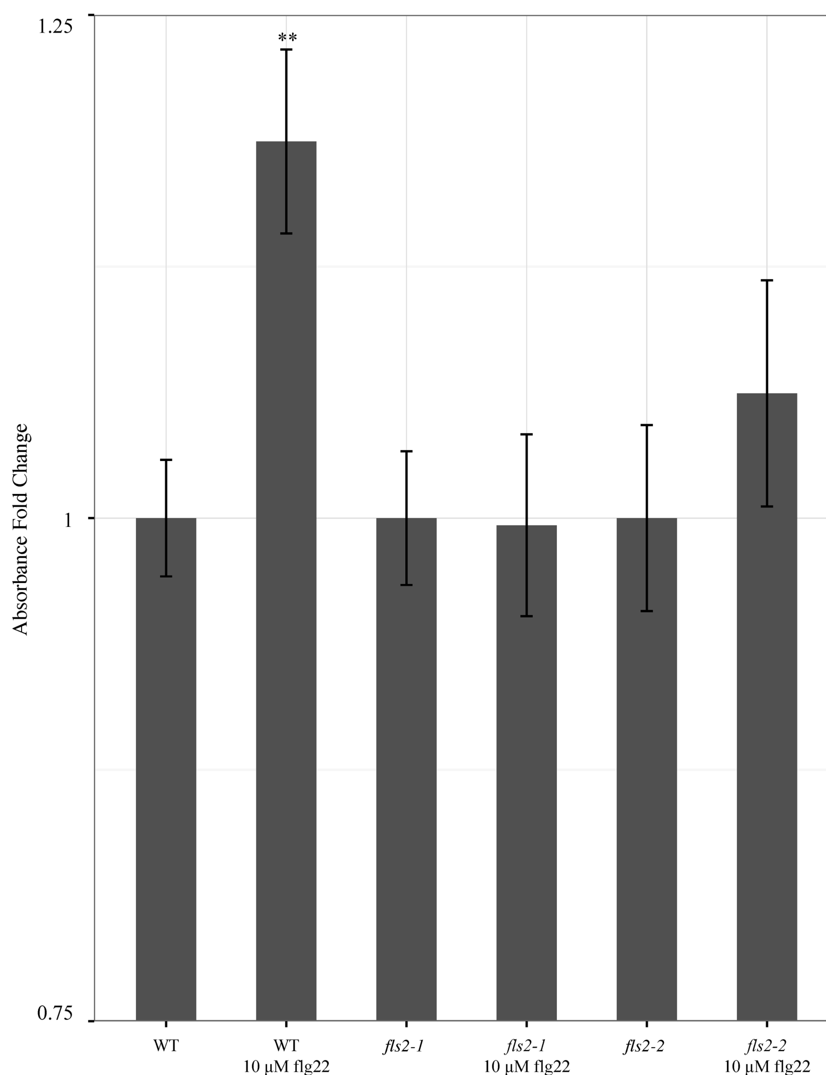

Figure 1. The absorbance of precipitated formazan following elicitation with 10 μM flg22 for 24 hours was used to determine production of ROS in wild-type *M. truncatula* R108, *fls2–1* mutant and *fls2–2* mutant. For each genotype, fold change in absorbance was calculated between plants in control solution (2 mM NBT) and plants in eliciting solution (2 mM NBT with 10 μM flg22). **Indicates *P* value $<$ 0.01 between control and flg22 treatment. N $\geq$ 15 for all genotypes and treatments.

Nitroblue tetrazolium (NBT) detection of reactive oxygen species

M. truncatula production of ROS was detected using a modified NBT staining protocol (Grellet Bournonville and Díaz-Ricci 2011). Seedlings were suspended in 50 mM potassium phosphate buffer pH 7.4 with 2 mM NBT +/− 10 μM flg22 peptide. Seedlings were incubated in the dark at 25°C for 18 hours. Following incubation, the supernatant containing precipitated formazan was collected, and absorbance was read at 600 nm.

Flagella stain

Flagella were visualized using Flagella stain (Hardy Diagnostics, Santa Maria, CA). 14028S and LT2 were grown overnight in motility agar. Plugs of agar were removed from these plates and suspended in LB for 15 minutes. Wet mounts were made by gently transferring cells to glass slides and applying coverslips. Flagella stain was applied to the cells through capillary action by placing the stain on the edge of the coverslip. Cells were visualized using an Olympus BHS microscope (Center Valley, PA) at 1000× magnification.

Statistical methods

Colonization numbers in each trial were normalized to the wild-type control and are reported as fold change relative to the wild-type control. Outliers were identified using Grubb's test. Student's *T*-test for independent groups was used to evaluate statistical significance ($P < 0.05$). The standard error of the mean is shown in all figures.

DISCUSSION AND RESULTS

Validation of *M. truncatula fls2* lines

Stimulation of the FLS2 receptor with bacterial flagellin leads to production of ROS (Apel and Hirt 2004). Following standard genotyping (Fig S1, Supporting Information), we validated our *M. truncatula fls2* lines using a NBT precipitation assay to assess

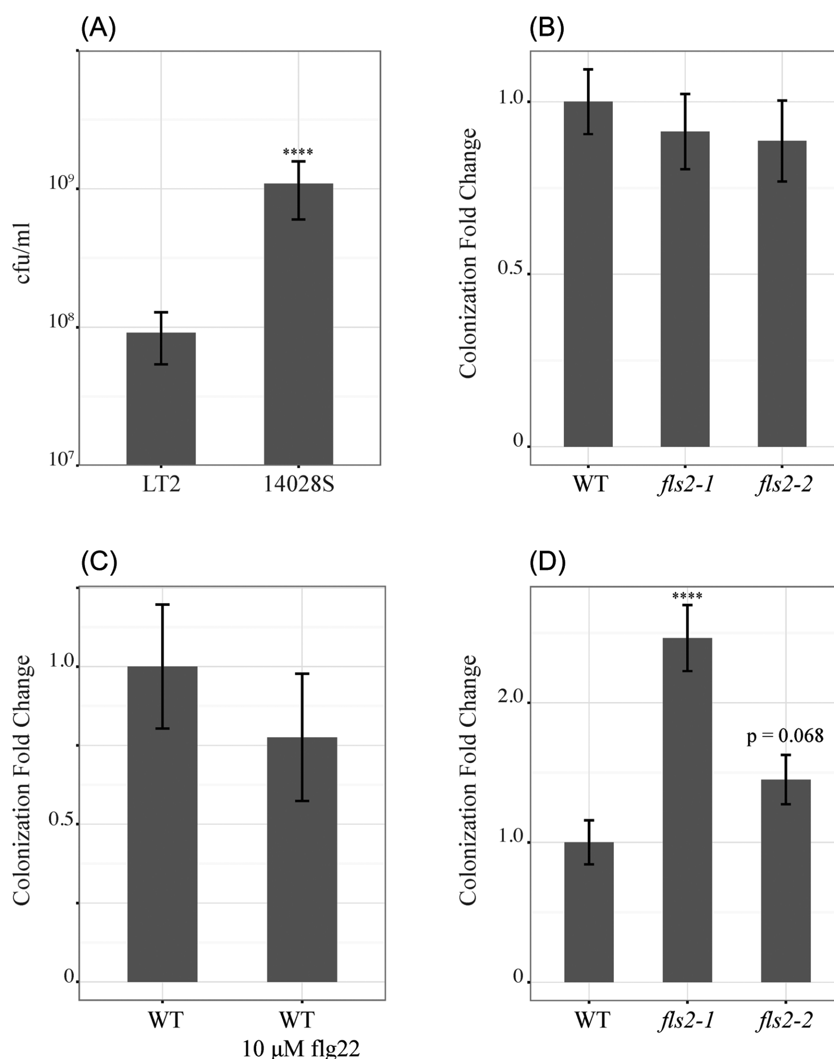

Figure 2. Growth of *Salmonella enterica* 14028S or LT2 on *M. truncatula* R108, *fls2–1* mutant and *fls2–2* mutant was determined by colony enumeration. **(A)** *Salmonella enterica* 14028S growth compared to LT2 growth on wild-type *M. truncatula* R108. Growth shown in colony forming units per gram of fresh plant weight; $N \geq 10$. **(B)** *Salmonella enterica* 14028S growth on *M. truncatula fls2–1* or *M. truncatula fls2–2* as compared to growth on wild-type *M. truncatula*; $N = 15$. **(C)** Growth of *Salmonella enterica* 14028S on *M. truncatula* R108 treated with 10 μM flg22 as compared to growth on untreated plants; $N = 10$. **(D)** Growth of *Salmonella enterica* LT2 on *M. truncatula fls2–1* and *fls2–2* as compared to growth on wild-type *M. truncatula*; $N \geq 15$. ****Indicates P value < 0.0001.

ROS production (Grellet Bournonville and Díaz-Ricci 2011). Wild-type *M. truncatula* plants showed an increase in precipitated formazan when elicited with flg22, indicating an increase in the production of ROS. The two independent *Tnt1* insertion lines, *M. truncatula fls2–1* and *fls2–2*, did not exhibit an increase in precipitated formazan when exposed to flg22, indicating little to no ROS production (Fig 1). This confirms that FLS2-dependent ROS production is inactivated in both *M. truncatula fls2* mutant lines.

Salmonella triggers a FLS2 dependent immune response, but *Salmonella* 14028S is not susceptible to this response

Inoculation with *Salmonella* stimulates immune responses in both *A. thaliana* and *M. truncatula* (Schikora *et al.* 2008; Jayaraman *et al.* 2014), and treatment with *Salmonella* flg22 stimulates immune responses in tomato, *N. benthamiana* and *A. thaliana* (Meng, Altier and Martin 2013; Garcia *et al.* 2014). It is unclear what impact this activation of plant defense pathways has on *Salmonella* on sprouted legumes. Our data suggest that growth of *Salmonella* 14028S on sprouted legumes is not influenced by defenses triggered by bacterial flagellin through the plant FLS2 receptor.

Salmonella 14028S numbers on both *M. truncatula fls2* lines were equivalent to those on wild-type *M. truncatula* (Fig 2B). To ensure that this was not a result of 14028S failing to stimulate the FLS2 receptor, we treated wild-type *M. truncatula* with 10 μM flg22 peptide in conjunction with 14028S inoculation. This concomitant elicitation and inoculation did not result in a change in 14028S final numbers (Fig 2C). However, treatment of *A. thaliana* with flg22 results in significantly reduced growth of *P.s syringae pv. tomato DC3000* (Zipfel *et al.* 2004), demonstrating that exogenous elicitation of the FLS2 receptor can impact bacteria that are sensitive to FLS2 triggered defense reactions. In contrast to the sprouted legume seedlings used in this work, growth of syringe inoculated *Salmonella* was reduced on *N. benthamiana* leaves that were pretreated by syringe infiltration of flg22 (Meng, Altier and Martin 2013).

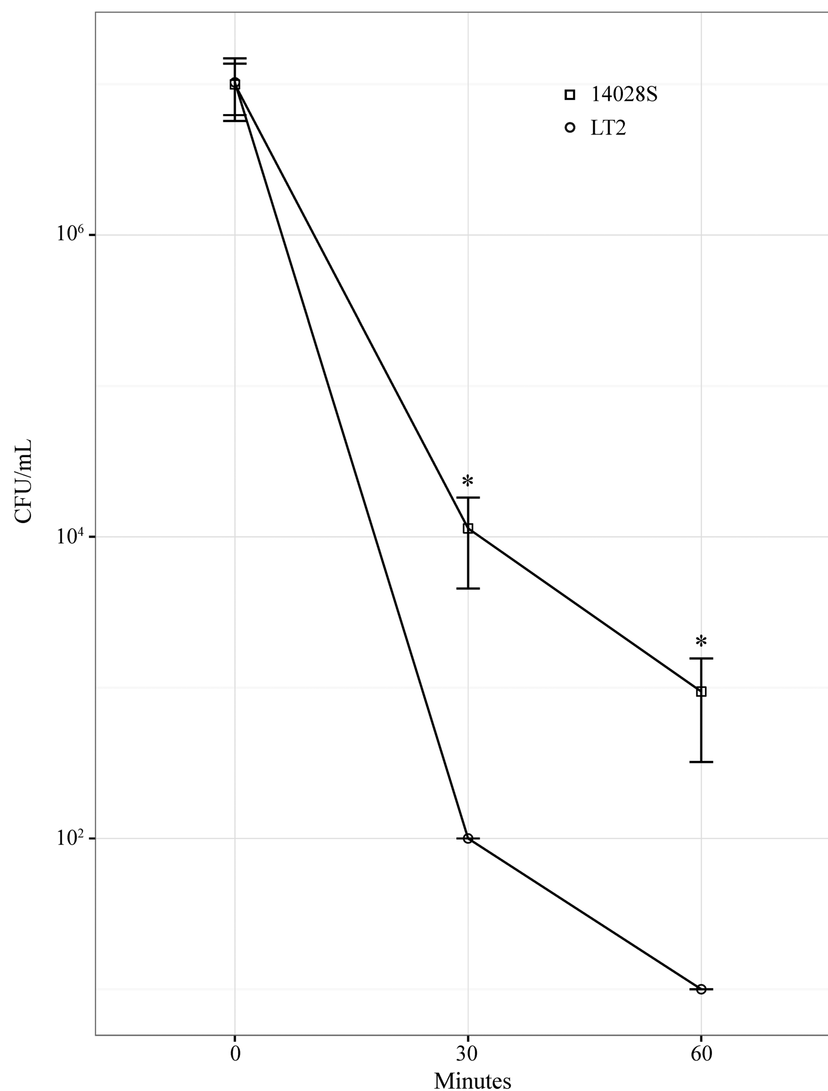

Figure 3. *Salmonella enterica* 14028S and LT2 grown in sprouted seedling exudates for 18 hours were challenged with 30 mM hydrogen peroxide. Survival was determined by culture sampling and colony enumeration. LT2 measured below the limit of detection at 30 and 60 minutes, and was scored at the limit of detection. *Indicates P value < 0.05. LT2 N = 3, 14 028 N = 6.

As an additional control, both to validate our *fls2* lines and to ensure that *Salmonella* is recognized by *M. truncatula* through the FLS2 receptor, we conducted *in planta* growth experiments with *Salmonella enterica* LT2. LT2 is more than 98% identical in sequence to 14028S, differing mainly in prophage regions, but is avirulent due to a disruption in *rpoS*, which encodes for the general stress response transcriptional regulator σ^s (Swords, Cannon and Benjamin 1997; Jarvik *et al.* 2010). Since σ^s promotes transcription of oxidative stress response genes, in addition to other stress response genes, LT2 was used as a control strain for FLS2-triggered plant defenses. The LT2 strain had greater growth on *M. truncatula fls2* lines ($P < 0.0001$ on *fls2–1* and $p = 0.068$ on *fls2–2*) than on the wild-type plant, demonstrating that it is sensitive to FLS2-triggered defenses (Fig 2D). This result further validated disruption of FLS2 in the *M. truncatula fls2* lines and shows that *Salmonella* triggers FLS2-dependent defense responses.

Flagella staining of both LT2 and 14028S cells confirmed the presence of flagellated cells (Fig S2, Supporting Information). However, the peptide sequence of certain bacterial flagellin, including the flagellin of the plant pathogen *Agrobacterium tumefaciens* and the plant symbiont *Sinorhizobium meliloti*, do not elicit a response from the FLS2 receptor (Felix *et al.* 1999). *Salmonella* produces two distinct antigenic forms of flagella by switching between the expression of flagella genes *fliC* and *fljB* (Aldridge *et al.* 2006). Analysis of both flagella variants (*fliC* and *fljB*) in 14028S and LT2 showed homology within the FLS2 eliciting domain (Fig S3, Supporting Information), confirming that 14028S is not avoiding elicitation of FLS2 through antigenic variation of flagella (McClelland *et al.* 2001; Jarvik *et al.* 2010).

We hypothesize that the susceptibility to FLS2-triggered defenses displayed by LT2 is due to reduced tolerance for ROS (Fig 3). Furthermore, we suggest that the highly efficient and redundant mechanisms that *Salmonella* possesses for neutralizing ROS are one factor that allows it to proliferate to high levels in sprouts, despite triggering the plant immune response (Hébrard *et al.* 2009). In addition to the production of ROS (Apel and Hirt 2004), FLS2 stimulation triggers the deposition of callose (Luna *et al.* 2011) and stomata closure (Zeng and He 2010), although these defenses are unlikely to have a significant impact on

Salmonella in sprouted seedlings. Callose deposition is a strategy for fortifying internal plant tissue through the production of high molecular weight polymers that can contain antimicrobial compounds (Luna *et al.* 2011). While *Salmonella* does grow endophytically, it displays higher growth epiphytically and does not produce the enzymes required to degrade plant cell walls (Cooley, Miller and Mandrell 2003; Teplitski, Barak and Schneider 2009; Jayaraman *et al.* 2014). Callose deposition would be largely ineffective against epiphytic *Salmonella* growth, and *Salmonella* does not display the degradative endophytic growth that callose deposition would protect against. The impact of stomata closure would also be minimal, as *Salmonella* displays preferential growth in the rhizosphere (Cooley, Miller and Mandrell 2003). For these reasons, it is probable that the production of ROS is the primary FLS2 triggered defense response that is relevant to *Salmonella* growth on plants.

As an intracellular human pathogen capable of replicating within macrophage where it will encounter oxidative stress, *Salmonella* is well equipped to neutralize the ROS produced through elicitation of the FLS2 receptor. The *Salmonella* genome encodes for three catalases (KatE, KatG and KatN), two alkyl hydroperoxide reductases (AhpC and TsaA) and two superoxide dismutases (SodC1 and SodC2) which allow *Salmonella* to proliferate even when challenged with FLS2-triggered ROS (Ammendola *et al.* 2005; Hébrard *et al.* 2009). Furthermore, it is possible that the oxidative stress encountered on sprouted seedlings could impact virulence of *Salmonella*. While *Salmonella* 14028S grown on *Arabidopsis* did not display an increase in mammalian cell invasiveness as compared to *Salmonella* 14028S grown in rich media (Schikora *et al.* 2011), FLS2-triggered ROS production may select for cells with greater ROS tolerance in heterogeneous *Salmonella* populations.

The human pathogen *Salmonella enterica* shows rapid growth in the sprouted legume environment and has been responsible for outbreaks of salmonellosis involving the consumption of contaminated sprouts. While many groups have demonstrated that plants perceive *Salmonella* and activate defense pathways in response to it, these defenses do not influence the *in planta* growth of *Salmonella* 14028S. Defenses such as callose deposition and stomata closure are probably ineffective because they target bacteria with growth strategies and lifestyles that differ from *Salmonella*, while the defense offered by the production of ROS is ineffective against *Salmonella* due to ROS-inactivating enzymes.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflict of interest. None declared.

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