Salmonella Biofilms Tolerate Hydrogen Peroxide by a Combination of Extracellular Polymeric Substance Barrier Function and Catalase Enzymes

Mark M. Hahn1,2, Juan F. González1,2 and John S. Gunn1,2,3*

1 Center for Microbial Pathogenesis, Abigail Wexner Research Institute at Nationwide Children’s Hospital, Columbus, OH, United States, 2 Infectious Diseases Institute, The Ohio State University, Columbus, OH, United States, 3 Department of Pediatrics, The Ohio State University College of Medicine, Columbus, OH, United States

The ability of Salmonella enterica subspecies enterica serovar Typhi (S. Typhi) to cause chronic gallbladder infections is dependent on biofilm growth on cholesterol gallstones. Non-typhoidal Salmonella (e.g. S. Typhimurium) also utilize the biofilm state to persist in the host and the environment. How the pathogen maintains recalcitrance to the host response, and oxidative stress in particular, during chronic infection is poorly understood. Previous experiments demonstrated that S. Typhi and S. Typhimurium biofilms are tolerant to hydrogen peroxide (H2O2), but that mutations in the biofilm extracellular polymeric substances (EPSs) O antigen capsule, colanic acid, or Vi antigen reduce tolerance. Here, biofilm-mediated tolerance to oxidative stress was investigated using a combination of EPS and catalase mutants, as catalases are important detoxifiers of H2O2. Using co-cultured biofilms of wild-type (WT) bacteria with EPS mutants, it was demonstrated that colanic acid in S. Typhimurium and Vi antigen in S. Typhi have a community function and protect all biofilm-resident bacteria rather than to only protect the individual cells producing the EPSs. However, the H2O2 tolerance deficiency of a O antigen capsule mutant was unable to be compensated for by co-culture with WT bacteria. For curli fimbriae, both WT and mutant strains are tolerant to H2O2 though unexpectedly, co-cultured WT/mutant biofilms challenged with H2O2 resulted in sensitization of both strains, suggesting a more nuanced oxidative resistance alteration in these co-cultures. Three catalase mutant (katE, katG and a putative catalase) biofilms were also examined, demonstrating significant reductions in biofilm H2O2 tolerance for the katE and katG mutants. Biofilm co-culture experiments demonstrated that catalases exhibit a community function. We further hypothesized that biofilms are tolerant to H2O2 because the physical barrier formed by EPSs slows penetration of H2O2 into the biofilm to a rate that can be mitigated by intra-biofilm catalases. Compared to WT, EPS-deficient biofilms have a heightened response even to low-dose (2.5 mM) H2O2 challenge, confirming that resident bacteria of EPS-deficient biofilms are under greater stress and have limited...
protection from H₂O₂. Thus, these data provide an explanation for how *Salmonella* achieves tolerance to H₂O₂ by a combination of an EPS-mediated barrier and enzymatic detoxification.

**Keywords:** *Salmonella*, extracellular polymeric substances (EPSs), biofilms, innate immunity, hydrogen peroxide, chronic infection

## INTRODUCTION

The pathoadaptive properties of *Salmonella enterica* subspecies *enterica* serovar Typhi (S. Typhi) allow the etiologic agent of Typhoid fever to exist in its human host in both the planktonic and biofilm growth states (Parry et al., 2002; Crawford et al., 2010b; Gonzalez-Escobedo et al., 2010; Gonzalez-Escobedo and Gunn, 2013). With at least 14.3 million cases of Typhoid fever claiming the lives of 136,000 individuals each year (Stanaway et al., 2019), there is significant need to eradicate this disease. However, this illness remains endemic in part because individuals with latent infections can unknowingly transmit S. Typhi to others by fecal-oral contamination (Parry et al., 2002; Crump et al., 2004; Gonzalez-Escobedo et al., 2010; Ruby et al., 2012; Kirk et al., 2015). In fact, chronic carriers represent the only known biological reservoir of S. Typhi (Ruby et al., 2012; Stanaway et al., 2019) and there is a clear link between biofilm growth on gallstone surfaces in the gallbladder and chronic carriage (Crawford et al., 2010a; Crawford et al., 2010b; Gonzalez-Escobedo et al., 2010; Gunn et al., 2014; Marshall et al., 2014; Adcox et al., 2016). *Salmonella enterica* subspecies *enterica* serovar Typhimurium (S. Typhimurium) also forms environmental biofilms and biofilms on cholesterol gallstones/cholesterol surfaces and causes chronic disease in mice similar to S. Typhi chronic infections in humans (Coburn et al., 2007; Crawford et al., 2010b; Moraes et al., 2018; Sakarikou et al., 2020). S. Typhimurium and other non-typhoidal serovars also form biofilms at intestinal sites and, in immune-compromised individuals, have the ability to invade and cause systemic disease or establish chronic biofilm infections in distal body sites similar to S. Typhi (Barthel et al., 2003; Gordon, 2008; Gordon et al., 2010; Joseph et al., 2016). Thus, this non-typhoidal serovar provides a useful model for studying biofilm phenotypes *in vitro* and *in vivo*. Despite an appreciation for how it is transmitted, the mechanism for S. Typhi biofilm recalcitrance to the immune response during chronic carriage is poorly understood (Hay and Zhu, 2016).

Throughout its infectious cycle, S. Typhi encounters oxidative stress in the host environment. Planktonic S. Typhi first encounter products of the oxidative burst upon invasion of M cells at Peyer’s patches and entry into macrophages and neutrophils (Ibarra and Steele-Mortimer, 2009; Ruby et al., 2012; Behnsen et al., 2015). In a well-documented mechanism, S. Typhi is disseminated from the intestine by persistence in the *Salmonella* containing vacuole (SCV) inside macrophages (Hurley et al., 2014; van der Heijden et al., 2015; Kurtz et al., 2017). In response to intracellular *Salmonella* infection, phagocytes generate an oxidative burst through the NADPH oxidase (Imlay, 2003; Hébrard et al., 2009; Rhen, 2019). Superoxide (O₂⁻) produced by the oxidative burst is weakly reactive and unable to pass through bacterial cell walls; toxicity arises when superoxide dismutates (either enzymatically by superoxide dismutase or spontaneously) to hydrogen peroxide (H₂O₂) and molecular oxygen (O₂) (Farr and Kogoma, 1991; Tsolis et al., 1995; Janssen et al., 2003; Halliwell, 2006; Hébrard et al., 2009; Horst et al., 2010). H₂O₂ readily crosses bacterial membranes and enters the cytoplasm where it can undergo Fenton chemistry to form hydroxyl radicals (·OH) that damage macromolecules, such as DNA, proteins, and lipid membranes (Janssen et al., 2003; Hébrard et al., 2009; Horst et al., 2010).

Notably, *Salmonella* has multiple redundancies for mitigating oxidative stress. First and foremost, planktonic *Salmonella* within the SCV use SPI-2-encoded T3SS effector proteins to prevent Phox assembly on the SCV membrane, thereby reducing its exposure to oxidative species (Hensel et al., 1998; Vazquez-Torres et al., 2000; Gallois et al., 2001; Holden, 2002). Despite this activity, some oxidative species are still generated leading to a steady state H₂O₂ concentration of approximately 1-4 µM and no more than 2 mM in the SCV (Winterbourn et al., 2006; Slauch, 2011; Ortega et al., 2016), which the bacterium mitigates using two classes of enzymes. Peroxiredoxin-type peroxidases (peroxiredoxins) reduce organic hydroperoxides to alcohols and H₂O₂ to water at the expense of cellular reducing agents such as NADH and NADPH (Horst et al., 2010). Although peroxiredoxins are limited by the availability of reductants and thus limited in ability to mitigate the oxidative burst, their function is essential to virulence as mutations in genes encoding these enzymes severely limit the ability of *Salmonella* to degrade H₂O₂ to degrade H₂O₂, survive in macrophages, and infect mice (Hébrard et al., 2009; Horst et al., 2010; Ortega et al., 2016). *Salmonella* also has three unique catalase enzymes (KatG, KatE, and KatN), which remain highly catalytic in non-reducing environments and are thought to have a predominant role when H₂O₂ concentrations are in the millimolar range (Seaver and Imlay, 2001). However, the role of catalases in SCV-survival is not straightforward as only one of these enzymes is H₂O₂-inducible (katG, which is transcriptionally-regulated by OxyR) (Pardo-Esté et al., 2018). Contrarily, expression of katE and katN are growth phase-dependent and occurs at stationary phase as part of the RpoS regulon (Buchmeier et al., 1995; Ibanez-Ruiz et al., 2000; Robbe-Saule et al., 2001; Robbe-Saule et al., 2003; Hébrard et al., 2009).

Furthermore, the role of catalases in planktonic defense against the oxidative burst is dispensable as mutant *Salmonella* lacking all three catalases do not have a reduced growth rate or survival in macrophages and remain virulent in mice (Buchmeier et al., 1995; Hébrard et al., 2009).

These findings raise the question of why S. Typhi, an organism characterized by its host specialization through genomic decay and extensive pseudogene formation (Wain...
et al., 2002; Dagan et al., 2006; Bäumer and Fang, 2013; Langridge et al., 2015; Ortega et al., 2016), would retain multiple redundancies in anti-oxidant function and, in particular, redundancies in catalase enzymes that are not required for planktonic resistance to the oxidative burst. The aforementioned maximum H$_2$O$_2$ concentration in the SCV of 2 mM is in agreement with our previously-reported minimum inhibitory concentration (MIC) of H$_2$O$_2$ against planktonic Salmonella spp. of 2.5 mM (Hahn and Gunn, 2020). While intracellular planktonic S. Typhi are capable of regulating their environment through modifications to the SCV membrane, S. Typhi biofilms develop in extracellular environments, such as the gallbladder lumen, and must have additional mechanisms to tolerate environmental oxidative stress. In particular, bile has been shown to be a potent source for oxidative stress and has pleiotropic effects on Salmonella gene regulation, membrane protein synthesis, and efflux systems (Gunn, 2000; Prouty et al., 2004; Begley et al., 2005; Merritt and Donaldson, 2009; Walawalkar et al., 2016). Many of these functions are dependent on upregulation of RpoS-dependent general stress pathways (Hernández et al., 2012). In a recent study (Walawalkar et al., 2016), biofilm SOD and catalase pathways were found to be specifically induced in response to ROS stress from bile and induction was dependent on a the autoinducer-2 quorum sensing pathway. Considering the abundance of stationary-phase cells in biofilms, growth- and stress-dependent regulation of catalase genes, and the ability of OxyR to directly sense H$_2$O$_2$ and induce numerous stress-response proteins, it is logical to expect an important role of S. Typhi catalases in biofilm recalcitrance to the host immune environment.

S. Typhi biofilms in the gallbladder consist of self-produced extracellular polymeric substances (EPSs) which anchor the biofilm to cholesterol gallstones (Crawford et al., 2010a; Crawford et al., 2010b; Gonzalez-Escobedo et al., 2010; Gunn et al., 2014) and protect resident bacteria from a variety of assaults including antibiotics and host immunity (Scher et al., 2005; Leid, 2009; Kostakioti et al., 2013; Gunn et al., 2016; González et al., 2018; González et al., 2019; Hahn and Gunn, 2020). Our previous study (Hahn and Gunn, 2020) on the innate immune response to Salmonella biofilms demonstrated the O antigen capsule, colanic acid, and the Vi antigen are necessary EPSs for Salmonella biofilm tolerance to H$_2$O$_2$. However, further investigation was need to determine the mechanism by which these EPSs resist H$_2$O$_2$ and protect biofilm resident bacteria from oxidative killing. By using wild-type (WT), EPS-deficient, and catalase mutant biofilms, we have defined the biofilm response to H$_2$O$_2$ and demonstrated a specific role of EPSs and catalase enzymes in H$_2$O$_2$ protection.

**MATERIALS AND METHODS**

**Bacterial Strains, Growth Conditions, and Biofilm Sample Preparation**

This study was conducted using the *Salmonella* parental WT strains or derivatives of S. Typhimurium ATCC 14028 (JSG210) and S. Typhi Ty2 (JSG4383) (Tables 1, 2). Tryptic soy broth (TSB) was used for all planktonic and biofilm cultures. When needed, antibiotics were used at the following concentrations: kanamycin (Kan), 45 µg/mL; ampicillin (Amp), 100 µg/mL. Planktonic bacteria were collected from 16-hour overnight broth cultures. Biofilms were initiated and cultured as previously described (Hahn and Gunn, 2020). Briefly, 96-well polypropylene microtiter plates were coated with 500 µg of cholesterol before inoculation to mimic gallstones. When inoculating mixed-strain biofilms (containing WT and mutant strain mixed together), planktonic bacteria were normalized to OD$_{490}$ = 0.65 then diluted 1:12 into mixed culture so that total

| Strain | Genotype | EPS Deficiency | Antibiotic Resistance | Reference Source |
|--------|----------|----------------|-----------------------|------------------|
| JSG210 | WT S. Typhimurium | – | – | ATCC14028 |
| JSG4681 | WT S. Typhimurium | – | Amp | This study |
| JSG3736 | ΔcsgA | Curli fimbriae | – | (Adcox et al., 2016) |
| JSG4608 | ΔcsgA | Curli fimbriae | Kan | This study |
| JSG4663 | ΔiucM | Colanic acid | – | (Adcox et al., 2016) |
| JSG4683 | ΔiucA | Colanic acid | Kan | This study |
| JSG4667 | ΔyhiO | O antigen capsule | – | (Adcox et al., 2016) |
| JSG4682 | ΔyhiO | O antigen capsule | Kan | This study |
| JSG3838 | ΔbcsE | Cellulose | – | (Adcox et al., 2016) |
| JSG4608 | ΔbcsE | Cellulose | K | This study |
| JSG3790 | ΔcsgΔiucM | Curli fimbriae, Colanic acid | – | (Adcox et al., 2016) |
| JSG4684 | ΔcsgΔiucM | Curli fimbriae, Colanic acid | Kan | This study |
| JSG4682 | ΔcsgΔiucMΔyhiO | Curli fimbriae, Colanic acid, O antigen capsule | – | (Adcox et al., 2016) |
| JSG4685 | ΔcsgΔiucMΔyhiO | Curli fimbriae, Colanic acid, O antigen capsule | Kan | This study |
| JSG3841 | ΔcsgΔiucMΔyhiOΔbcsE | Curli fimbriae, Colanic acid, O antigen capsule, Cellulose | – | (Adcox et al., 2016) |
| JSG4686 | ΔcsgΔiucMΔyhiOΔbcsE | Curli fimbriae, Colanic acid, O antigen capsule, Cellulose | Kan | This study |
| JSG4383 | WT S. Typhi rpoS$^+$ | – | – | (Santander et al., 2007) |
| JSG4687 | WT S. Typhi rpoS$^+$ | – | Amp | This study |
| JSG4695 | S. Typhi ΔviB rpoS$^+$ | Vi antigen | – | This study |
| JSG4696 | S. Typhi ΔviB rpoS$^+$ | Vi antigen | Kan | This study |

Kan, Kanamycin; Amp, Ampicillin.
bacteria starting in the biofilm was equivalent to single-strain biofilms (diluted 1:6). Biofilms were begun by inoculation of 200 µL/well and cultures were maintained at 30°C on a nutator for 96 hours. Supernatants were replaced with fresh media once every 24 hours. Prior to each experiment, biofilm samples were washed 2× with phosphate-buffered saline (PBS) to remove unattached and planktonic bacteria.

Biofilm aggregates were used in single- and co-culture phenotypic experiments examining the tolerance of EPS and catalase mutant bacteria. To create aggregates, mature biofilms were mechanically collected by scraping microtiter plate biofilms with pipette tips and normalized by total protein quantification (Bradford method) (Hahn and Gunn, 2020). Biofilms used for reverse-transcription quantitative PCR (RT-qPCR) and Western blot experiments were washed 2× with PBS then exposed to H₂O₂ without prior disruption. After 1 or 2 hours of H₂O₂ exposure, biofilm samples were washed 2× with PBS and cultures were maintained at 30°C on a nutator for 96 hours. Supernatants were replaced with fresh media once every 24 hours. Prior to each experiment, biofilm samples were washed 2× with phosphate-buffered saline (PBS) to remove unattached and planktonic bacteria.

Mutant Generation
Mutation to Vi antigen (tviB) in was constructed in rpoS Ty2 S. Typhi (JSG4383) using λ-Red mutagenesis (Datsenko and Wanner, 2000) with the use of primers JG2934-JG2935 (Table 3). Briefly, S. Typhi carrying the λ-Red recombinase (JSG4393) was transformed with a Kan resistance cassette with tviB homology sequence tags. Subsequently, Kan resistance was removed by transformation with pCP20 carrying the FLP recombinase (Cherepanov and Wackernagel, 1995). The deletion was confirmed by PCR using primers JG2936 and JG2937 (Table 3) and analysis by gel electrophoresis before temperature-mediated removal of pCP20. Catalase activity in JSG4695 was confirmed to be phenotypically equivalent to the WT (JSG4383) by placing one colony of each strain on a glass slide and exposing to ~20 µL of 3% H₂O₂ then observing for reactive bubbling.

Transduction of Catalase Mutations
The following reagents were obtained through BEI Resources, NIAID, NIH: Salmonella enterica subspecies enterica, strain 14028s (Serovar Typhimurium) Single-Gene Deletion Mutant Library, Plate SGD_011/012_Kan, NR-29404; Plate SGD_164/165_Kan, NR-42853; Plate SGD_156/157_Kan, NR-42849 (Porwollik et al., 2014). The three catalase mutations (a putative catalase protein, ΔkatE, and ΔkatG, respectively) were transduced into S. Typhimurium (JSG210) by P22 HT-int phage

| Primer | Sequence | Purpose |
|--------|----------|---------|
| JG2934 | 5′—ATTTAGTAAAGGATTTGCTG—3′ | Forward tviB sequence tag |
| JG2935 | 5′—GTCCTGCTAGTTATGTCG—3′ | Forward tviB sequence tag |
| JG2936 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Forward verification tviB |
| JG2937 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Reverse verification tviB |
| JG3144 | 5′—AGCCTGCTAGTTATGTCG—3′ | Forward putative catalase protein flanking |
| JG3145 | 5′—GTCCTGCTAGTTATGTCG—3′ | Reverse putative catalase protein flanking |
| JG3147 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Forward katE flanking |
| JG3148 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Reverse katE flanking |
| JG3149 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Forward katG flanking |
| JG3150 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Reverse katG flanking |
| JG2081 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Forward rpoB (qPCR) |
| JG2082 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Reverse rpoB (qPCR) |
| JG3165 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Forward putative catalase protein (qPCR) |
| JG3166 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Reverse putative catalase protein (qPCR) |
| JG3167 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Forward katE (qPCR) |
| JG3168 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Reverse katE (qPCR) |
| JG3169 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Forward katG (qPCR) |
| JG3170 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Reverse katG (qPCR) |

TABLE 2 | Catalase mutant strains used in this study.

| Strain | Background | Mutation source strain | Catalase Deficiency | Antibiotic Resistance | Reference Source |
|--------|------------|------------------------|---------------------|----------------------|------------------|
| JSG4588 | JSG210 | SGD_011/012, well A09 | Putative catalase protein (ACY88561.1) | Kan | This study |
| JSG4590 | JSG210 | SGD_164/165, well E03 | ΔkatE (ACY88079.1) | Kan | This study |
| JSG4592 | JSG210 | SGD_156/157, well B08 | ΔkatG (ACY91293.1) | Kan | This study |

These catalase mutants have no known deficiencies in extracellular polymeric substances. Kan, Kanamycin.

TABLE 3 | Oligonucleotide primers used in this study.

| Primer | Sequence | Purpose |
|--------|----------|---------|
| JG2934 | 5′—ATTTAGTAAAGGATTTGCTG—3′ | Forward tviB sequence tag |
| JG2935 | 5′—GTCCTGCTAGTTATGTCG—3′ | Forward tviB sequence tag |
| JG2936 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Forward verification tviB |
| JG2937 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Reverse verification tviB |
| JG3144 | 5′—AGCCTGCTAGTTATGTCG—3′ | Forward putative catalase protein flanking |
| JG3145 | 5′—GTCCTGCTAGTTATGTCG—3′ | Reverse putative catalase protein flanking |
| JG3147 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Forward katE flanking |
| JG3148 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Reverse katE flanking |
| JG3149 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Forward katG flanking |
| JG3150 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Reverse katG flanking |
| JG2081 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Forward rpoB (qPCR) |
| JG2082 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Reverse rpoB (qPCR) |
| JG3165 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Forward putative catalase protein (qPCR) |
| JG3166 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Reverse putative catalase protein (qPCR) |
| JG3167 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Forward katE (qPCR) |
| JG3168 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Reverse katE (qPCR) |
| JG3169 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Forward katG (qPCR) |
| JG3170 | 5′—GAGGAGGGAATGCTGATAGATTAG—3′ | Reverse katG (qPCR) |
transduction. In short, 3 mL of overnight broth cultures of the catalase mutant donors grown in the presence of P22 phage were harvested by the addition of 500 μL chloroform and pelleted at 5000xg (5 minutes). Dilutions of the aqueous layer (containing phage lysate) were then used to infect overnight broth cultures of S. Typhimurium. Infections were incubated at 37°C for 25 minutes before the addition of LB + 10 mM EGTA and additional incubation at 37°C for 60 minutes. Cultures were spread on LB agar containing 10 mM EGTA and Kan and incubated at 37°C overnight to select for transductants. After two rounds of isolation streaking on selection plates (LB, 10 mM EGTA, Kan) transductants were screened for phage loss on Evans Blue-Uranine plates. Appropriate colonies were selected for genomic DNA isolation (GenElute Bacterial Genomic DNA; Sigma-Aldrich; St. Louis, MO) and confirmed to carry the transduced catalase mutation by PCR amplification using the gene-specific primers JG3144-JG3150 (Table 3).

**Antibiotic Markers to Test Mixed-Community Biofilms**

WT and EPS mutant strains were differentially antibiotic resistance-marked for use in co-culture experiments. The empty vector plasmids pWSK29 and pWSK129 (carrying Amp<sup>R</sup> or Kan<sup>R</sup> cassettes, respectively) were isolated from overnight broth cultures of E. coli DH5α (SG047 and JSG133, respectively) using the QIAprep Spin Miniprep kit (Qiagen; Germantown, MD). The WT of both serovars was transformed with pWSK29 and all mutants were transformed with pWSK129 and selected on LB supplemented with appropriate antibiotics and incubated at 37°C. One resistant colony from each transformation was selected for further use in co-culture experiments (Table 1).

**Growth Rate, MIC, and Planktonic Sensitivity to H<sub>2</sub>O<sub>2</sub>**

All mutants generated by conjugal transfer, transduction, or transformation were evaluated for growth rate and MIC of H<sub>2</sub>O<sub>2</sub>. Growth rate was determined by 16-hour growth curve conducted in microtiter plates at 37°C from a starting culture of approximately 2.0 × 10<sup>6</sup> colony forming units per milliliter (CFUs/mL). Growth was monitored by OD<sub>600</sub> readings every 30 minutes using a SpectraMax M3 plate reader. The MIC of H<sub>2</sub>O<sub>2</sub> was tested as previously described (Hahn and Gunn, 2020) with starting cultures of 2.0 × 10<sup>6</sup> CFUs/mL and H<sub>2</sub>O<sub>2</sub> concentration ranging from 10 mM to 0.156 mM.

Overnight EPS or catalase mutant planktonic cultures were normalized to 2.0 × 10<sup>6</sup> CFUs/mL, mixed 1:1 with fresh 2× TSB, and incubated at 37°C and 200 rpm for 3 hours using a shaker (200 rpm) at 37°C. H<sub>2</sub>O<sub>2</sub> was supplied at 0 mM, 1.25 mM, 12.5 mM, 31.25 mM, or 62.5 mM for single-strain catalase mutant experiments or at 0 mM, 2.5 mM, 2.5 mM, or 125 mM for WT-catalase mutant co-culture experiments. As for single-strain EPS mutant experiments, WT-EPS mutant co-culture experiments were conducted using 0 mM, 2.5 mM, or 125 mM H<sub>2</sub>O<sub>2</sub> challenges for S. Typhimurium strains and 0 mM, 2.5 mM, or 25 mM H<sub>2</sub>O<sub>2</sub> challenges for S. Typhi strains. The values were selected to represent a 0×, 1×, 10×, 25×, or 50× increase from the previously published WT MIC (2.5 mM) (Hahn and Gunn, 2020). Challenge concentrations for catalase mutant biofilms were adjusted to represent proportional fold-differences as these mutants had a reduced MIC. Because co-culture experiments involved strains with differing MICs, all H<sub>2</sub>O<sub>2</sub> concentrations are expressed henceforth as millimolar. Single-culture experiments were enumerated by serial dilution plating on LB agar and co-culture samples were plated twice on LB + Amp or LB + Kan to independently enumerate WT and mutant bacteria in the sample (respectively).

**Supernatant Transfer**

Single-culture biofilms of WT or EPS mutants were started as described. In experiments involving S. Typhimurium ΔωcaM, S. Typhimurium ΔyihO, or S. Typhi ΔrtiB, WT biofilms were designated supernatant-source biofilms and mutant biofilms were designated as receiving biofilms. In trials involving S. Typhimurium ΔcsgA and S. Typhimurium ΔbcgE, the opposite designations were made. Each time biofilm supernatant was replaced (once every 24 hours), supernatant from the receiving biofilms was removed and discarded. Spent TSB from supernatant-source biofilms was removed by pipetting, filter sterilized (by PES membranes), mixed in a 1:1 ratio with fresh 2× TSB, and used to replenish media on the receiving biofilms. Supernatant-source biofilms received fresh TSB. The PES membranes used are rated to have low protein-binding activity. Mixing spent supernatant with 2× TSB ensured all biofilms received equal nutrient concentrations each day while allowing receiving biofilms to be exposed to soluble factors and waste produced by supernatant-source biofilms. Receiving biofilms were washed and challenged with H<sub>2</sub>O<sub>2</sub> as described for single-culture biofilm aggregates.

**RNA Isolation**

Collected biofilm samples were pooled in 3.2 mL PBS then pelleted at 4,000 rpm (10 minutes, 4°C) and the supernatant
was removed prior to freezing pellets at -80°C. RNA was isolated from the frozen pellets using the hot phenol method. Pellets were resuspended in 475 µL AE buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.2) then added to 475 µL phenol and 40 µL 20% SDS. Tubes were incubated at 65°C for 10 minutes, shaking every minute. Samples were then placed on ice (5 minutes) and centrifuged at 10,000 rpm (15 minutes, 4°C) to pellet debris. Aqueous phases were then transferred to new tubes containing 475 µL chloroform, mixed, and centrifuged at 2,000 rpm (10 minutes, 4°C). Second aqueous phases were transferred to new tubes and RNA was precipitated with 500 µL isopropanol and 50 µL 2M sodium acetate. RNA was pelleted at 12,000 rpm (20 minutes, 4°C), washed with 250 µL 70% cold ethanol then repelleted at 12,000 rpm (5 minutes, 4°C) before discarding the ethanol supernatant and air-drying on ice for 15 minutes. Finally, pellets were resuspended in 20 µL nuclease-free water (NFW), analyzed for yield, and treated with DNase I (New England Biolabs; Ipswich, MA) for 10 minutes at 37°C according to manufacturer guidelines (protocol M0303). One µL SUPERase-In RNase Inhibitor (Invitrogen; Carlsbad, CA) was added to each 100 µL reaction to stabilize the samples. After the 10 minute incubation, RNA was re-isolated with isopropanol precipitation/ethanol wash as described above then resuspended in 50 µL NFW.

**cDNA Synthesis and Quantitative PCR**

RNA was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen; Carlsbad, CA). Initial priming was conducted with 19.25 ng/µL random primers and 769 µM dNTPs in a 13 µL reaction heated to 65°C for 5 minutes then placed on ice for 1 minute. An additional 7 µL cDNA synthesis master mix (prepared for each reaction as: 4 µL 5X Buffer, 1 µL 0.1M DTT, 1 µL Suprase-IN, and 1 µL SuperScript-RT III or NFW) was added to each sample which was then incubated sequentially at 25°C (5 minutes), 50°C (60 minutes) then 70°C (15 minutes). The additional step of adding 1 µL (2 units) of the kit-provided E. coli RNase H to samples and incubating at 37°C for 20 minutes was conducted to remove RNA remaining complementary to the cDNA.

Catalase gene quantitative PCR (qPCR) was conducted with PowerUp SYBR Green Master Mix (Applied Biosystems; Foster City, CA) and gene specific primers (Table S3; 500 nM each). The reference gene was rpoB. All samples were run in triplicate using an Applied Biosystems 7500 Real Time PCR System. Copy numbers were calculated by the Livak method (Livak and Schmittgen, 2001).

**Western Blot**

Biofilm samples were pooled in 3.2 mL PBS and centrifuged at 4,000 rpm (10 minutes, 4°C). The pellet was resuspended in 60 µL PBS and boiled at 95°C for 10 minutes. Protein concentration was measured by the Bradford method then samples were normalized to 30 µg/µL in Laemmli sample buffer and boiled for an additional 15 minutes. Following a brief vortex and centrifugation to collect tube contents, a total of 750 µg protein from each sample was loaded into the wells of a Criterion TGX stain-free 4-15% gel (Bio-Rad; Hercules, CA). The Precision Plus Protein WesternC molecular weight ladder (Bio-Rad; Hercules, CA) was also included in each gel and proteins were electrophoresed at 200 V for 45-60 minutes until the dye front migrated to the bottom of the gel. Proteins were transferred to a methanol (MeOH)-activated polyvinylidene difluoride (PVDF) membrane (0.45 µm) using Trans-Blot Turbo Transfer System (Bio-Rad; Hercules, CA) set to 2.5A and 25V for 7 minutes. Membranes were blocked immediately after transfer in 5% bovine serum albumin (BSA) prepared in Tris-buffered saline + Tween 20 (TBST) for 1 hour at room temperature. Blocked membranes were probed with polyclonal rabbit anti-catalase peroxidase antibody (Agrisera AS08 374; Vännäs, Sweden) diluted 1:3000 in 5% BSA/TBST for 16 hours at 4°C. The following day, secondary goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Bio-Rad STAR124P) diluted 1:2000 in 5% BSA/TBST was applied along with Precision Protein StrepTactin-HRP conjugate (Bio-Rad; Hercules, CA) diluted 1:5000 for 60 minutes at room temperature. Membranes were washed in TBST 3× (5 minutes each wash) after each antibody incubation. Protein-antibody complexes were visualized with Clarity Western Blotting Substrate (Bio-Rad; Hercules, CA) and chemiluminescent signals were captured using a C400 gel imager (Azure Biosystems; Dublin, CA). Protein and background signals were quantified using ImageJ software (Schneider et al., 2012). After background values were subtracted, protein signal values were normalized to baseline conditions (WT protein at t=0, 0 mM H₂O₂).

**RESULTS**

**EPS-Associated Tolerance to H₂O₂ Is Partially a Community Behavior**

**Presence of WT EPSs Protects Some, but Not All, EPS Mutants**

We previously assayed *Salmonella* biofilm tolerance to H₂O₂ by testing each WT and EPS mutant against 0 mM, 2.5 mM, 25 mM, 62.5 mM, and 125 mM challenges. In developing this assay, we reported that *S. Typhimurium* EPSs enable biofilm tolerance to H₂O₂ at least 50-fold (125 mM) the planktonic MIC (2.5 mM) and that *S. Typhi* EPSs enable biofilm tolerance 10-fold (25 mM). Additional experimentation demonstrated the primary EPSs responsible for this phenotype are the O antigen capsule, colanic acid, and (for *S. Typhi*) the Vi antigen as mutation to these EPSs resulted in loss of tolerance specifically at 125 mM (for EPSs mutated in *S. Typhimurium*) or 25 mM (for EPSs mutated in *S. Typhi*) (Hahn and Gunn, 2020). In order to further investigate these findings, each of the WT and EPS mutant strains were marked with different antibiotic resistances. The introduction of antibiotic resistance had no discernable effect on any of the planktonic phenotypes tested (Supplementary Figure 1). The O antigen capsule and colanic acid were again shown to be responsible for biofilm tolerance to H₂O₂, which was independent of antibiotic function (Figures 1A–C). Furthermore, the elimination of curli fimbriae alone or cellulose alone does not affect tolerance to H₂O₂ (Figures 1D, E) while multiple EPS mutation eliminated tolerance (Figures 1F–H).
While the experiments described above significantly advanced the understanding of the role each EPS has in biofilm tolerance to H$_2$O$_2$, they did not address the mechanism by which these EPSs protect biofilm-resident bacteria or if the presence of biofilm EPSs may be a community behavior. To this end, antibiotic-marked WT and EPS mutant bacteria were co-cultured in a biofilm. They were then challenged with H$_2$O$_2$ to determine if the presence of WT EPSs in the biofilm could protect mutant bacteria from otherwise lethal doses of H$_2$O$_2$ or if the tolerant phenotype is attributed to an individual cell only protecting itself by EPS production. The equal survival of WT and mutant bacteria at 125 mM H$_2$O$_2$ (normally lethal to the EPS mutant) and suggestive of a community behavior mediated by the production of EPSs, whereas the latter result would be evident by survival of the WT only and indicate the protective EPS must be cell-associated to provide tolerance.

In order to demonstrate the protective function by WT is specific to the biofilm growth state, planktonic S. Typhimurium and S. Typhimurium ΔcsgAΔwcaMΔyihOΔbcsE were co-cultured in 5 mM H$_2$O$_2$, which confirmed both strains are rapidly eliminated by a concentration of H$_2$O$_2$ that is sub-lethal to S. Typhimurium biofilms (Supplementary Figure 1). To address biofilms, 1:1 mixtures of WT S. Typhimurium and EPS mutants were grown and collected as biofilm aggregates for challenge with H$_2$O$_2$. The equal survival of WT and colanic acid-deficient bacteria (ΔwcaM) upon 125 mM H$_2$O$_2$ challenge indicates production of colanic acid by WT bacteria enhances tolerance to H$_2$O$_2$ for all biofilm-resident bacteria and that colanic acid does not need to be cell-associated to enact a tolerance phenotype (Figure 2A). However, for biofilm aggregates of WT and ΔyihO (O antigen capsule), the opposite result was observed in that the WT-produced O antigen capsule was not able to protect the ΔyihO mutant from killing upon 125 mM H$_2$O$_2$ challenge (Figure 2B). In fact, even the WT in any aggregate mix with the ΔyihO mutation lost its ability to tolerate 125 mM H$_2$O$_2$ challenge (Figures 2B, F, G). Also surprisingly, aggregates of WT co-cultured with curli fimbiae mutants (WT/ΔcsgA) or a curli, colanic acid double mutants (WT/ΔcsgAΔwcaM) eliminated biofilm tolerance at 125 mM H$_2$O$_2$ for both WT and mutant bacteria (Figures 2C, E). These results were unexpected for two reasons. First, the ΔcsgA mutation alone in single-culture biofilm (Figure 1D) did not have a discernable effect on tolerance to H$_2$O$_2$, so it was expected to survive equally with WT when co-cultured. Secondly, co-culture biofilms of WT and ΔwcaM (Figure 2A) indicate the WT is able to complement the mutant strain with respect to H$_2$O$_2$ tolerance, so the additional loss of curli fimbiae (ΔwcaMΔcsgA) was not expected to compound any tolerance defect from ΔwcaM. As expected because of its lack of involvement in H$_2$O$_2$ tolerance (Figure 1E) (Hahn and Gunn, 2020), biofilms co-cultured with WT and cellulose mutant bacteria (WT/ΔbcsE) survived challenge with 125 mM H$_2$O$_2$ equally (Figure 2D).

Similar to other EPSs, the Vi antigen has an important role in S. Typhi biofilm tolerance which is independent of antibiotic resistance (Figures 3A, B). Previous investigation of S. Typhi biofilms deficient in Vi antigen were conducted using a tvIB mutant in the Ty2 background that also has a point mutation in rpoS (JSG1213), causing RpoS to be non-functional. While many studies have been conducted with S. Typhi Ty2, both Salmonella catalases and biofilm growth can be affected by RpoS (Fang et al., 1992; Santander et al., 2007; Burda et al., 2018). Thus, we took the additional step to re-construct the S. Typhi ΔtvIB mutation in an rpoS+ S. Typhi WT (Table 1). This change did not alter the tolerance phenotype of S. Typhi ΔtvIB biofilms, which were inhibited by 25 mM H$_2$O$_2$ (Figure 3B). Additionally, introduction of antibiotic resistance genes into each of the S. Typhi strains did not cause changes in growth rate (Supplementary Figure 2). Equal survival of both the WT and mutant in co-cultured biofilms (WT/ΔtvIB) challenged with 25
mM H$_2$O$_2$ indicate WT-produced Vi antigen is sufficient in protecting all biofilm-resident bacteria (Figure 3C). As observed previously for S. Typhimurium, this result is specific to the biofilm growth state (Supplementary Figure 2). Therefore, similar to colanic acid, the presence of Vi antigen in the biofilm serves a community function and protects all biofilm-resident bacteria.

To rule out the possibility that WT biofilm tolerance (and associated compensation in co-culture experiments) is due to non-EPS soluble signaling factors produced by the WT, supernatant transfer experiments were conducted with aggregates non-EPS soluble signaling factors produced by the WT, associated compensation in co-culture experiments) is due to resident bacteria.

Despite this intrinsic difference, catalase mutant biofilm protected all biofilm residents (S. Typhimurium, this result is specific to co-culture experiments) is due to resident bacteria. To determine if WT catalase activity could compensate for catalase mutant bacteria in a biofilm, mixed-strain biofilms (WT/ catalase mutant) were challenged with 62.5 mM and 125 mM H$_2$O$_2$. These two challenge concentrations are equal to 50-fold the catalase mutant or WT planktonic MIC, respectively and are concentrations where ΔkatE and ΔkatG mutants demonstrated reduced tolerance with no CFUs detectable at 31.25 mM or 62.5 mM H$_2$O$_2$, respectively (Figures 4B, C). Since the catalase mutants have no known EPS differences compared to WT, these data demonstrate that the high degree of tolerance associated with WT biofilms is due to combined action of EPSs and biofilm catalases.

To determine if WT catalase activity could compensate for catalase mutant bacteria in a biofilm, mixed-strain biofilms (WT/ catalase mutant) were challenged with 62.5 mM and 125 mM H$_2$O$_2$. These two challenge concentrations are equal to 50-fold the catalase mutant or WT planktonic MIC, respectively and are concentrations where ΔkatE and ΔkatG mutants demonstrated reduced tolerance with no CFUs detectable at 31.25 mM or 62.5 mM H$_2$O$_2$, respectively (Figures 4B, C). Since the catalase mutants have no known EPS differences compared to WT, these data demonstrate that the high degree of tolerance associated with WT biofilms is due to combined action of EPSs and biofilm catalases.

Bacterial Catalases Have a Role in Biofilm Tolerance

While there is a clear role for EPSs in tolerance to H$_2$O$_2$, the contribution for bacterial catalases in detoxifying the biofilm environment was also investigated using three catalase mutant S. Typhimurium strains cultured in a biofilm [putative catalase (ACY88561.1), katE, and katG]. As expected, the H$_2$O$_2$ MIC was reduced for each of the planktonic catalase mutants while growth rates of each mutant were not altered (Supplementary Figure 4). Despite this intrinsic difference, catalase mutant biofilms retained tolerance when challenged with H$_2$O$_2$, although not necessarily to the same degree as WT biofilm (survival >125 mM H$_2$O$_2$; Figure 1A, Figure 4). The putative catalase mutant retained the most tolerance as CFUs were recovered up to 62.5 mM H$_2$O$_2$ challenge (Figure 4A) whereas the ΔkatE and ΔkatG mutants demonstrated reduced tolerance with no CFUs detectable at 31.25 mM or 62.5 mM H$_2$O$_2$, respectively (Figures 4B, C). Since the catalase mutants have no known EPS differences compared to WT, these data demonstrate that the high degree of tolerance associated with WT biofilms is due to combined action of EPSs and biofilm catalases.

To determine if WT catalase activity could compensate for catalase mutant bacteria in a biofilm, mixed-strain biofilms (WT/ catalase mutant) were challenged with 62.5 mM and 125 mM H$_2$O$_2$. These two challenge concentrations are equal to 50-fold the catalase mutant or WT planktonic MIC, respectively and are concentrations where ΔkatE and ΔkatG mutants demonstrated reduced tolerance with no CFUs detectable at 31.25 mM or 62.5 mM H$_2$O$_2$, respectively (Figures 4B, C). Since the catalase mutants have no known EPS differences compared to WT, these data demonstrate that the high degree of tolerance associated with WT biofilms is due to combined action of EPSs and biofilm catalases.

Bacterial Catalases Have a Role in Biofilm Tolerance

While there is a clear role for EPSs in tolerance to H$_2$O$_2$, the contribution for bacterial catalases in detoxifying the biofilm environment was also investigated using three catalase mutant S. Typhimurium strains cultured in a biofilm [putative catalase (ACY88561.1), katE, and katG]. As expected, the H$_2$O$_2$ MIC was reduced for each of the planktonic catalase mutants while growth rates of each mutant were not altered (Supplementary Figure 4). Despite this intrinsic difference, catalase mutant biofilms retained tolerance when challenged with H$_2$O$_2$, although not necessarily to the same degree as WT biofilm (survival >125 mM H$_2$O$_2$; Figure 1A, Figure 4). The putative catalase mutant retained the most tolerance as CFUs were recovered up to 62.5 mM H$_2$O$_2$ challenge (Figure 4A) whereas the ΔkatE and ΔkatG mutants demonstrated reduced tolerance with no CFUs detectable at 31.25 mM or 62.5 mM H$_2$O$_2$, respectively (Figures 4B, C). Since the catalase mutants have no known EPS differences compared to WT, these data demonstrate that the high degree of tolerance associated with WT biofilms is due to combined action of EPSs and biofilm catalases.
resident bacteria and that tolerance is dependent on a certain threshold of catalase activity that WT cells cannot fully restore when they make up only 50% of the biofilm.

**Mechanism of EPS-Mediated Recalcitrance to H₂O₂ Assault**

The inability for WT cells to fully complement mutant EPS functions and the role of bacterial catalases in biofilm tolerance led to the hypothesis that *Salmonella* biofilms are tolerant to H₂O₂ because EPSs slow penetration of H₂O₂ into the intra-biofilm space to a rate that can be mitigated by catalases in that location to keep H₂O₂ levels tolerable to resident bacteria. However, without sufficient abundance of certain EPSs, H₂O₂ diffuses rapidly and accumulates in the intra-biofilm space thereby having more potent effects against all resident bacteria regardless of EPS-producing abilities. To investigate this...
hypothesis, the biofilm response to H$_2$O$_2$ challenge was evaluated in real time by gene and protein expression with the prediction that EPS-deficient biofilms would respond to H$_2$O$_2$ challenge in less time and/or to a greater degree because the H$_2$O$_2$ was accessing the intra-biofilm space more rapidly.

**H$_2$O$_2$ Challenge Increases Catalase Expression in EPS Mutant Biofilms**

To test the hypothesis that EPS mutant biofilms allowed H$_2$O$_2$ diffusion into the intra-biofilm space faster or to a greater extent, WT*S. Typhimurium* and S. Typhimurium ΔcsgAΔwcaMAΔyihOΔbcsE biologs were challenged separately with H$_2$O$_2$ and assayed for the catalase response. Because CFU experiments demonstrated that EPS mutant biologs (S. Typhimurium ΔcsgAΔwcaMAΔyihOΔbcsE) are eradicated by a 2-hour exposure to 125 mM H$_2$O$_2$, RNA and protein samples were collected at 1 hour (as well as 2 hours) so that potential differences in the biofilm response to either the tolerable concentration (2.5 mM) or the challenge concentration (125 mM) could still be observed.

qPCR was used to assess the transcriptional response of each of the selected catalase genes in the EPS mutant compared to the WT (presented as fold-change of mutant vs. WT; Figure 6). For each catalase target, the mutant and WT biofilms had similar levels of expression under control conditions (t=0 and 0 mM) (Figure 6). Challenge with 2.5 mM H$_2$O$_2$ had a minimal effect on the expression of the putative catalase gene (Figure 6A). However, this condition led to moderate up-regulation of katE in the mutant at 2 hours post challenge and dramatic fold-changes in katG transcription at 1 and 2 hours post challenge (Figures 6B, C). Challenge with 125 mM H$_2$O$_2$ led to increased expression of each gene in mutant biofilms though for katE and katG the overall response was less robust than in 2.5 mM conditions (Figure 6). This limited detection was likely due to lethality associated with the 125 mM challenge. Given that KatG is the primary inducible catalase for *Salmonella*, the increased expression observed by 2.5 mM H$_2$O$_2$ challenge indicates mutant biofilms are experiencing a response consistent with our hypothesis of H$_2$O$_2$ entering the intra-biofilm space to a much greater extent. Even though the EPS mutant biofilms survive 2.5 mM H$_2$O$_2$ challenge (Figures 1–3), the induction of katG as early as 1 hour and sustained up-regulation at 2 hours indicates a more-stressed population of intra-biofilm *Salmonella* due to limited protection afforded by the EPS-deficient biofilm.

Western blot assays were conducted to evaluate protein-level differences in biofilm catalases during the same challenge period (Figure 7). The limited availability of commercial antibodies only

![FIGURE 6](image_url)
permitted detection of general catalase proteins. Since protein content was normalized prior to loading each gel, gel quantification of each lane was normalized to control conditions (t=0, 0 mM H2O2). The representative gel (Figure 7A) and gel quantification (Figures 7B, C) demonstrate that both WT and EPS mutant biofilms had similar catalase content present in biofilms at each H2O2 concentration from 0-2 hours. While each biofilm responded to H2O2 exposure by increasing catalase expression, the responses of WT and EPS-deficient biofilms varied greatly. In WT biofilms, a modest increase in biofilm catalase was evident as early as 1 hour after H2O2 exposure; this response was sustained at 2 hours but only slight increases in catalase were evident for both the 2.5 mM and 125 mM conditions. By contrast, EPS-deficient biofilms had less catalase content after 1-hour exposure to H2O2 compared to initial conditions. However, exposure to 2.5 mM or 125 mM H2O2 caused a 3.3-fold or 1.7-fold increase (respectively) in biofilm catalase content from 1 to 2 hours of H2O2 exposure.

**DISCUSSION**

*S. Typhi* produces EPSs to protect biofilm bacteria from many stressors, such as antibiotics and host defenses (Scher et al., 2005; Leid, 2009; Kostakioti et al., 2013; Gunn et al., 2016; Gonzalez et al., 2018; Gonzalez et al., 2019; Hahn and Gunn, 2020). We have demonstrated that at least one of these host defenses thwarted by *S. Typhi* biofilms is oxidative stress from H2O2 and our work furthers understanding of how each of these EPSs protect against this host antimicrobial. Our findings confirmed *S. Typhi* biofilms rely on Vi antigen (Figures 3A, B) and *S. Typhimurium* biofilms utilize the O antigen capsule and colanic acid to tolerate H2O2 doses well-above planktonic-lethal conditions (Figures 1A–C). Cellulose, which was demonstrated to be a dispensable EPS (Figures 1E, 2D), must not affect biofilm integrity in the same manner as the other EPSs tested. While EPSs other than Vi antigen may protect *S. Typhi* against H2O2, this possibility remains to be investigated. Since *S. Typhimurium* biofilms rely on the O antigen capsule for tolerance, we predict this EPS may also contribute to *S. Typhi* tolerance. However, colanic acid will not be a protective EPS in *S. Typhi*, as all *S. Typhi* strains have mutations in colanic acid biosynthetic genes (Nuccio and Bäumler, 2014; Pando, 2017).

To begin investigating the mechanism for EPS-mediated biofilm tolerance, co-culture experiments were used to determine if WT-derived EPSs serve a community function by providing protection for all biofilm resident bacteria regardless of EPS-producing ability. These experiments yielded mixed results in which Vi antigen and colanic acid produced by WT *Salmonella* protected both the WT and mutants deficient in either of their corresponding EPSs (Figures 2A, 3C). These results indicate that WT bacteria produce and secrete/slug off sufficient quantities of Vi antigen or colanic acid for community protection or by sufficient production of other compensatory EPSs by WT and mutants alike. On the other hand, co-culture experiments using WT and O antigen capsule mutants resulted in elimination of both WT and the mutant (Figure 2B) indicating the O antigen capsule was not directly protective for the WT cells producing it despite having a critical function in biofilm tolerance. This result suggests that there is insufficient production of the O antigen capsule to protect either the WT or O antigen mutant, or that there is no compensatory EPS production for the lack of O antigen capsule.

The initial finding that *S. Typhi* and *S. Typhimurium* biofilms lose tolerance without Vi antigen or the O antigen capsule (respectively) seemed to be congruent given that both are capsular polysaccharides and that the O antigen capsule has previously been found to have analogous functions in *S. Typhimurium* as the Vi antigen in *S. Typhi* (Sharma and Qadri, 2004; Raffatellu et al., 2005; Gibson et al., 2006; Winter et al., 2008; Marshall and Gunn, 2015; Hiyoshi et al., 2018). However, as discussed, co-culture experiments addressing H2O2 tolerance demonstrated WT *S. Typhi* was able to compensate for Vi antigen mutants but WT *S. Typhimurium* was not able to compensate for O antigen capsule mutants. This difference highlights a key fitness advantage conferred to *S. Typhi* by its ability to produce Vi antigen. Furthermore, our data suggest *S. Typhimurium* biofilms rely primarily on colanic acid to confer biofilm H2O2 tolerance as it was the only other EPS included in our analysis that could be fully complemented by the presence of WT in the biofilm. This finding is significant because colanic acid, which is not produced by typhoidal serovars (as mentioned above), is immunostimulatory (Hahn and Gunn, 2020) and its production in vivo would defeat the stealth-like behavior of *S. Typhi*. Reliance on colanic acid and not capsular polysaccharide represents a key difference between the two serovars studied here and may suggest why *S. Typhimurium* and other non-typhoidal serovars are less likely to form chronic biofilm infections in human hosts.
Results involving curli fimbriae mutants further complicated our understanding of EPS-mediated protection, as the presence of a ΔcsgA mutant in co-cultured WT/ΔcsgA biofilms resulted in sensitization of both the WT and mutant despite the fact that biofilms consisting of purely ΔcsgA bacteria (and of course the WT) tolerated H₂O₂ challenge conditions (Figures 1D, 2C). For curli fimbriae, our rationale that EPSs provide either a benefit only to the bacteria producing the EPS or a community benefit to the whole biofilm does not explain why WT/ΔcsgA biofilms have a tolerance defect and represents an active area of investigation. Because curli fimbriae is a major component of biofilm biomass (Adcox et al., 2016), we hypothesize that the co-culture leads to a global change in biofilm properties or H₂O₂ susceptibility that are yet to be determined. Similarly, in strains with combinations of EPS mutations (Figures 2E–G), all of which have ΔcsgAΔwcaM as part of the mutant repertoire, the co-cultured biofilms behave like WT/ΔcsgA and not WT/ΔwcaM regarding H₂O₂ susceptibility. Thus the WT/ΔcsgA phenotype, in which both WT and mutant strains become susceptible to H₂O₂ at concentrations where they were previously resistant, is dominant. Overall, given the variability between EPS mutants in single- and co-culture biofilm experiments, it is clear that EPS development and biofilm tolerance is governed by more than one pathway. This work is beginning to unveil what is likely a series of complex interactions which must be addressed in the future to enhance understanding of biofilm development and recalcitrance in vivo.

The fact that biofilms missing all major EPSs (S. Typhimurium ΔcsgAΔwcaMΔyihOΔbcsE) still survive up to 62.5 mM H₂O₂ challenge (Hahn and Gunn, 2020) indicated additional mechanisms were important for biofilm tolerance against H₂O₂. Given the unexplained redundancies of S. Typhi catalases, it was logical to focus on this enzyme class. Catalase biofilms have no known EPS deficiencies so the reduced tolerance to H₂O₂ in the catalase mutants indicates each of these enzymes are involved in mitigating oxidative stress. KatE is regulated by RpoS in stationary phase. Therefore, it is expected to be present at peak levels only after biofilms have fully developed and, consistently, biofilms with a ΔkatE mutation had the greatest reduction in tolerance indicating a pivotal role for KatE in biofilm survival in the presence of H₂O₂. Furthermore, the loss of WT tolerance and lack of compensatory activity in WT/ΔkatE co-culture biofilms shows that the total amount of the KatE enzyme in the biofilm is essential for the tolerance phenotype and that other catalases cannot make up for this deficiency. Mutation to KatG, which is inducible by the OxyR-mediated stress response, also reduced biofilm H₂O₂ tolerance indicating that resident bacteria in mature WT biofilms are able to rapidly sense and induce a protective catalase response via KatG when needed (e.g., if KatE present in the biofilms becomes saturated). In further support of this conclusion, WT bacteria in co-cultured biofilms were only able to compensate for ΔkatG mutants (Figure 5). However, there is still a limit to this inducible activity as 125 mM H₂O₂ challenge eliminated WT bacteria in WT/ΔkatG co-cultured biofilms and the inducible KatG response presumably occurs (albeit unsuccessfully) in WT/ΔkatE biofilms. Taken together, these data indicate a novel function of catalase enzymes that is essential for biofilm tolerance and provides a plausible explanation for why S. Typhi has retained multiple catalase enzymes though they are redundant and dispensable during acute infection and planktonic survival in the host (Buchmeier et al., 1995; Hébrard et al., 2009). Consistent with EPS co-culture experiments, we determined catalase-associated tolerance is also dependent on the enzymatic capacity of the biofilm unit and not necessarily the functionality of individual cells within the biofilm.

Finally, to bring a model for biofilm H₂O₂ tolerance into focus, it was important to evaluate the role of EPSs and catalases in a unified experiment. This was conducted through qPCR and Western blot examination of the catalase response in WT and EPS mutant (S. Typhimurium ΔcsgAΔwcaMΔyihOΔbcsE) biofilms. The most prominent response measured by qPCR was from katG which was induced in EPS mutant biofilms by both the low and challenge concentrations of H₂O₂ (2.5 mM and 125 mM, respectively). This evidence of H₂O₂-mediated stress, even at 2.5 mM, shows that the absence of EPSs allows easier penetration of H₂O₂ into the biofilm, supporting our hypothesis. Furthermore, the sustained transcriptional response in the EPS mutant from 1 to 2 hours corresponded with a substantial increase in catalase protein 2 hours post challenge indicating the biofilms induce an enzymatic response to H₂O₂. Nonetheless, this response is inadequate at protecting biofilm bacteria as demonstrated by CFU experiments involving S. Typhimurium ΔcsgAΔwcaMΔyihOΔbcsE mutants (Figures 1, 2). While unexpected, the reduced catalase content in the EPS-deficient biofilms at 1-hour exposure to H₂O₂ could indicate that the weak biofilm-forming ability of the mutant prevents some biofilm-resident bacteria from progressing to stationary phase. This would prevent RpoS-mediated KatE production, leading to fewer total catalases present at experimental onset (further limiting the fitness of the EPS mutant) and predisposing the mutant to the stress-response observed by katG induction upon H₂O₂ influx. By contrast, the limited increase in catalase proteins from 1 to 2 hours observed for WT biofilms suggests that WT biofilms do not need to induce a large enzymatic response as they rely first on EPSs to maintain a steady state of H₂O₂ influx regardless of external H₂O₂ concentration (2.5 mM vs. 125 mM) that can be mitigated with existing catalase enzymes. From the CFU experiments (Figures 1, 3) it is apparent that this response, in combination with appropriate EPSs, is sufficient for robust biofilm tolerance.

CONCLUSION

We previously reported Salmonella biofilms cultured in vitro are tolerant to H₂O₂. Our work here moves the field forward as it provides an explanation for how Salmonella achieves this function using a combination of the physical barrier arising from certain EPSs and enzymatic mitigation. It has been known for quite some time that Salmonella EPSs can vary significantly depending on growth conditions and other environmental...
signals (Scher et al., 2005), however by attributing the tolerance phenotype to specific EPSs, we are able to predict which EPSs are likely to be essential for biofilm survival in vivo. We recognize that our challenge concentration of H$_2$O$_2$ (125 mM) is likely not encountered in vivo, although the true microenvironmental H$_2$O$_2$ concentration encountered by Salmonella in the gallbladder environment is not known. However, the ability of the WT biofilm to adequately sense and respond to H$_2$O$_2$ at extreme concentrations compared to the stress response observed from EPS mutant biofilms at planktonic-lethal levels indicates that Salmonella is well-suited for the host environment because of its biofilm lifestyle and that additional clearance mechanisms must be employed by the host in order to eliminate chronic infections.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

**AUTHOR CONTRIBUTIONS**

All authors contributed to the article and approved the submitted version. The following list describes the contributions of each author: Conceptualization, MH and JSG. Methodology, MH, JFG, and JSG. Investigation, MH. Formal analysis, MH and JSG. Writing (original draft preparation), MH. Writing (review and editing), JFG and JSG. Supervision, JSG. Funding acquisition, JSG.

**REFERENCES**

Adcox, H. E., Vasicek, E. M., Dwivedi, V., Hoang, K. V., Turner, J., and Gunn, J. S. (2016). Salmonella Extracellular Matrix Components Influence Biofilm Formation and Gallbladder Colonization. *Infect. Immun.* 84 (11), 3243. doi: 10.1128/IAI00352-16

Barthel, M., Hapfelmeier, S., Quintanilla-Martinez, L., Kremer, M., Rohde, M., Hogardt, M., et al. (2003). Pretreatment of Mice With Streptomycin Provides a Salmonella Enterica Serovar Typhimurium Colitis Model That Allows Analysis of Both Pathogen and Host. *Infect. Immun.* 71 (5), 2839–2858. doi: 10.1128/IAI.71.5.2839-2858.2003

Baumler, A., and Fang, F. C. (2013). Host Specificity of Bacterial Pathogens. *Cold Spring Harb. Perspect. Med.* 3 (12), a010041. doi: 10.1101/cshperspect.a010041

Begley, M., Gahan, C. G., and Hill, C. (2005). The Interaction Between Bacteria and Bile. *FEMS Microbiol. Rev.* 29 (4), 625–651. doi: 10.1016/j.femsre.2004.09.003

Behrens, J., Perez-Lopez, A., Nuccio, S.-P., and Raffatellu, M. (2015). Exploiting Host Immunity: The Salmonella Paradigm. *Trends Immunol.* 36 (2), 112–120. doi: 10.1016/j.it.2014.12.003

Buchmeier, N. A., Libby, S. I., Xu, Y., Loewen, P. C., Switala, J., Guiney, D. G., et al. (1995). DNA Repair is More Important Than Catalase for Salmonella Virulence in Mice. *J. Clin. Invest.* 95 (3), 1047–1053. doi: 10.1172/JCI117750

Burda, W. N., Brennan, K. E., Gonzales, A., and Curtiss, R. (2018). Conversion of RpoS- Attenuated Salmonella Enterica Serovar Typhi Vaccine Strains to RpoS+ Improves Their Resistance to Host Defense Barriers. *mSphere* 3 (1), e0006–e001018. doi: 10.1128/mSphere.0006-18

Cherepanov, P. P., and Wackernagel, W. (1995). Gene Disruption in Escherichia Coli: Tcr and KmR Cassettes With the Option of Flp-catalyzed Exision of the Antibiotic-Resistance Determinant. *Gene* 158 (1), 9–14. doi: 10.1016/0378-1119(95)00193-A

**FUNDING**

This research was supported by the grants R21AI156328, R21AI153752, and R01AI116917 from the National Institutes of Health to JSG and with additional funds provided to JSG by the Abigail Wexner Research Institute at Nationwide Children’s Hospital.

**ACKNOWLEDGMENTS**

We thank Roy Curtiss III at the University of Florida for constructing and sharing the RpoS+ S. Typhi Ty2 strain and Michael McClelland at the University of California, Irvine and Helene Andrews-Polymenis at Texas A&M University for generating and sharing the single-gene deletion mutant library used to transduce catalase mutants.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.683081/full?supplementary-material
Gibson, D., White, A., Snyder, S., Martin, S., Heiss, C., Azadi, P., et al. (2006). Salmonella Produces an O-antigen Capsule Regulated by AgfD and Important for Environmental Persistence. J. Bacteriol. 188 (22), 7722–7730. doi: 10.1128/JB.00809-06

González, J. F., Alberts, H., Lee, J., Doolittle, L., and Gunn, J. S. (2018). Biofilm Formation Protects Salmonella From the Antibiotic Ciprofloxacin In Vitro and In Vivo in the Mouse Model of Chronic Carriage. Sci. Rep. 8 (1), 222. doi: 10.1038/s41598-017-18516-2

Gonzalez-Escobedo, G., and Gunn, J. S. (2013). Gallbladder Epithelium as a Niche for Chronic Salmonella Carriage. Infect. Immun. 81 (8), 2920. doi: 10.1128/IAI.00258-13

Gonzalez-Escobedo, G., Marshall, J. M., and Gunn, J. S. (2010). Chronic and Acute Infection of the Gall Bladder by Salmonella Typhi: Understanding the Carrier State. Nat. Rev. Microbiol. 9 (1), nmrrc02490. doi: 10.1038/nmrrc02490

González, J. F., Kuzit, J., Bauer, D. L., Hitt, R., Fitch, J., Wetzel, A., et al. (2019). Establishment of Chronic Typhoid Infection in a Mouse Carriage Model Involves a Type 2 Immune Shift and T and B Cell Recruitment to the Gallbladder. mBio 10 (5), e02262–e02219. doi: 10.1128/mBio.02262-19

Gordon, M. A. (2008). Salmonella Infections in Immunocompromised Adults. J. Infect. 56 (6), 413–422. doi: 10.1016/j.jinf.2008.03.012

Gordon, M. A., Kankwati, A. M., Mwafailrwa, G., Walsh, A. L., Hopkins, M. J., Parry, C. M., et al. (2010). Invasive non-Typhoid Salmonellae Establish Systemic Intracellular Infection in HIV-infected Adults: An Emerging Disease Pathogenesis. Clin. Infect. Dis. 50 (7), 953–962. doi: 10.1086/651090

Gunn, J. S. (2000). Mechanisms of Bacterial Resistance and Response to Bile. Microbes Infect. 2 (8), 907–913. doi: 10.1016/S1286-4579(00)00392-0

Gunn, J. S., Bakaletz, L. O., and Wozniak, D. J. (2016). What

Infection of the Gall Bladder by Salmonella Typhi: Understanding the Carrier State. Nat. Rev. Microbiol. 9 (1), nmrrc02490. doi: 10.1038/nmrrc02490

Hensel, M., Shea, J. E., Waterman, S. R., Mundy, R., Nikolaus, T., Banks, G., et al. (1998). Genes Encoding Putative Effector Proteins of the Type III Secretion System of Salmonella Pathogenicity Island 2 are Required for Bacterial Virulence and Proliferation in Macrophages. Mol. Microbiol. 30 (1), 163–174. doi: 10.1111/j.1365-2958.1998.00104.x

Hernández, S. B., Cota, I., Ducret, A., Assel, L., and Casadesús, J. (2012). Adaptation and Preadaptation of Salmonella Enterica to Bile. PloS Genet. 8 (1), e1002459. doi: 10.1371/journal.pgen.1002459

Hibbard, M., Viala, J. P., Miresse, S., Barras, F., and Ausset, L. (2009). Redundant Hydrogen Peroxide Scavengers Contribute to Salmonella Virulence and Oxidative Stress Resistance. J. Bacteriol. 191 (14), 4605–4614. doi: 10.1128/JB.00144-09

Hensel, M., Shea, J. E., Waterman, S. R., Mundy, R., Nikolaus, T., Banks, G., et al. (1998). Genes Encoding Putative Effector Proteins of the Type III Secretion System of Salmonella Pathogenicity Island 2 are Required for Bacterial Virulence and Proliferation in Macrophages. Mol. Microbiol. 30 (1), 163–174. doi: 10.1111/j.1365-2958.1998.00104.x

Hart, D. W. (2002). Tracking of the Salmonella Vacuole in Macrophages. Traffic 3 (3), 161–169. doi: 10.1034/j.1600-0852.2002.003030.x

Horst, S. A., Jaeger, T., Denkel, L. A., Rouf, S. F., Rhen, M., and Bange, F.-C. (2010). Thiol Peroxidase Protects Salmonella Enterica From Hydrogen Peroxide Stress In Vitro and Facilitates Intracellular Growth. J. Bacteriol. 192 (11), 2929–2932. doi: 10.1128/JB.0162-09

Hurley, D., McCusker, M. P., Fanning, S., and Martins, M. (2014). Salmonella–Host Interactions–Modulation of the Host Innate Immune System. Front. Immunol. 5, 481. doi: 10.3389/fimmu.2014.00481
Parry, C. M., Hien, T. T., Dougan, G., White, N. J., and Farrar, J. J. (2002). Typhoid Fever. *N. Engl. J. Med.* 347 (22), 1770–1782. doi: 10.1056/NEJMra02001

Porwollik, S., Santiviago, C. A., Cheng, P., Long, F., Desai, P., Fredlund, J., et al. (2014). Defined Single-Gene and Multi-Gene Deletion Mutant Collections in *Salmonella Enterica* Sb Typhimurium. *Pho* One 9 (7), e99820. doi: 10.1371/journal.pone.0099820

Prouty, A., Brodsky, I., Falkow, S., and Gunn, J. (2004). Bile-Salt-Mediated Induction of Antimicrobial and Bile Resistance in *Salmonella Typhimurium*. *Microbiology* 150 (4), 775–783. doi: 10.1099/mic.0.26769-0

Raffatellu, M., Chessa, D., Wilson, R. P., Dusold, R., Rubino, S., and Bäumler, A. J. (2006). The Vi Capsular Antigen of *Salmonella Enterica* Serotype Typhi Reduces Toll-like Receptor-Dependent Interleukin-8 Expression in the Intestinal Mucosa. *Infect. Immun.* 73 (6), 3367–3374. doi: 10.1128/IAI.73.6.3367-3374.2005

Rhen, M. (2019). *Salmonella* and Reactive Oxygen Species: A Love-Hate Relationship. *J. Innate Immun.* 11 (3), 216–226. doi: 10.1159/000496370

Robbe-Saule, V., Algorta, G., Rouilhac, I., and Norel, F. (2003). Characterization of the RpoS Status of Clinical Isolates of *Salmonella Enterica*. *Appl. Environ. Microbiol.* 69 (8), 4352–4358. doi: 10.1128/AEM.69.8.4352-4358.2003

Robbe-Saule, V., Coyaudt, C., Ibanez-Ruiz, M., Hernant, D., and Norel, F. (2001). Identification of a non-Haem Catalase from *Salmonella* and its Regulation by RpoS (σ5). *Mol. Microbiol.* 39 (6), 1533–1545. doi: 10.1046/j.1365-2958.2001.02340.x

Ruby, T., McLaughlin, L., Gopinath, S., and Monack, D. (2012). Salmonella’s Long-Term Relationship With its Host. *FEBS Microbiol. Rev.* 36 (3), 600–615. doi: 10.1111/j.1574-6976.2012.00332.x

Sakarikou, C., Kostoglou, D., Simões, M., and Giaouris, E. (2020). Exploitation of Plant Extracts and Phytochemicals Against Resistant *Salmonella* spp. in Biofilms. *Food Res. Int.* 128, 108806. doi: 10.1016/j.foodres.2019.108806

Santander, J., Wanda, S.-Y., Nickerson, C. A., and Curtis, R. (2007). Role of RpoS in Fine-Tuning the Synthesis of Vi Capsular Polysaccharide in *Salmonella Enterica* Serotype Typhi. *Infect. Immun.* 75 (3), 1382–1392. doi: 10.1128/IAI.00888-06

Scher, K., Romling, U., and Yaron, S. (2005). Effect of Heat, Acidification, and Chlorination on *Salmonella Enterica* Serovar Typhimurium Cells in a Biofilm Formed at the Air-Liquid Interface. *Appl. Environ. Microbiol.* 71 (3), 1163–1168. doi: 10.1128/AEM.71.3.1163-1168.2005

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 Years of Image Analysis. *Nat. Methods* 9 (7), 671–675. doi: 10.1038/nmeth.2089

Seaver, L. C., and Imlay, J. A. (2001). Alkyl Hydroperoxide Reductase is the Primary Scavenger of Endogenous Hydrogen Peroxide in Escherichia Coli. *J. Bacteriol.* 183 (24), 7173–7181. doi: 10.1128/JB.183.24.7173-7181.2001

Sharma, A., and Qadri, A. (2004). Vi Polysaccharide of *Salmonella Typhi* Targets T-lymph Cells. *Virol. Journal* 1 (50), 17492–17497. doi: 10.1072/pxn0.407536101

Slauch, J. M. (2011). How Does the Oxidative Burst of Macrophages Kill Bacteria? Still an Open Question. *Mol. Microbiol.* 80 (3), 580–583. doi: 10.1111/j.1365-2958.2011.07819.x

Stanaway, J. D., Reiner, R. C., Blacker, B. F., Goldberg, E. M., Khalil, I. A., Troeger, C. E., et al. (2019). The Global Burden of Typhoid and Paratyphoid Fevers: A Systematic Analysis for the Global Burden of Disease Study 2017. *Lancet Infect. Dis.* 19 (4), 369–381. doi: 10.1016/S1473-3099(18)30685-6

Tsolis, R. M., Bäumler, A. J., and Heffron, F. (1995). Role of *Salmonella Typhimurium* Mn-superoxide Dismutase (SodA) in Protection Against Early Killing by J774 Macrophages. *Infect. Immun.* 63 (5), 1739–1744. doi: 10.1128/IAI.63.5.1739-1744.1995

van der Heijden, J., Bosman, E. S., Reynolds, L. A., and Finlay, B. B. (2015). Direct Measurement of Oxidative and Nitrosative Stress Dynamics in *Salmonella* Inside Macrophages. *Proc. Natl. Acad. Sci.* 112 (2), 560–565. doi: 10.1073/pnas.1414569112

Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Holden, D. W., Lucia, S. M., Dinauer, M. C., et al. (2000). *Salmonella* Pathogenicity Island 2-Dependent Evasion of the Phagocyte NADPH Oxidase. *Science* 287 (5458), 1655–1658. doi: 10.1126/science.287.5458.1655

Wain, J., House, D., Parkhill, J., Parry, C., and Dougan, G. (2002). Unlocking the Genome of the Human Typhoid Bacillus. *Lancet Infect. Dis.* 2 (3), 163–170. doi: 10.1016/S1473-3099(02)00225-6

Walawalkar, Y. D., Vaidya, Y., and Nayak, V. (2016). Response of *Salmonella Typhi* to Bile-Generated Oxidative Stress: Implication of Quorum Sensing and Persister Cell Populations. *Pathog. Dis.* 74 (8), ftw090. doi: 10.1093/femspd/ftw090

Winterbourn, C. C., Hampton, M. B., Livesey, J. H., and Kettle, A. J. (2006). Modeling the Reactions of Superoxide and Myeloperoxidase in the Neutrophil Phagosome Implications for Microbial Killing. *J. Biol. Chem.* 281 (52), 39860–39869. doi: 10.1074/jbc.M605898200

Winter, S. E., Raffatellu, M., Wilson, R. P., Rüssmann, H., and Bäumler, A. J. (2008). The *Salmonella Enterica* Serotype Typhi Regulator TviA Reduces Interleukin-8 Production in Intestinal Epithelial Cells by Repressing Flagellin Secretion. *Cell. Microbiol.* 10 (1), 247. doi: 10.1111/j.1365-2958.2007.01037.x

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Copyright © 2021 Hahn, Gonzalez and Gunn. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*