Characterizing 5-oxoproline sensing pathways of *Salmonella enterica* serovar typhimurium

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5-Oxoproline (5OP) is a poorly researched ubiquitous natural amino acid found in all life forms. We have previously shown that *Salmonella enterica* serovar Typhimurium (*Salmonella*) responds to 5OP exposure by reducing cyclic-di-GMP levels, and resultant cellulose dependent cellular aggregation in a YfeA and BcsA dependent manner. To understand if 5OP was specifically sensed by *Salmonella* we compared the interaction of *Salmonella* with 5OP to that of the chemically similar and biologically relevant molecule, L-proline. We show that L-proline but not 5OP can be utilized by *Salmonella* as a nutrient source. We also show that 5OP but not L-proline regulates cellulose dependent cellular aggregation. These results imply that 5OP is utilized by *Salmonella* as a specific signal. However, L-proline is a 5OP aggregation inhibitor implying that while it cannot activate the aggregation pathway by itself, it can inhibit 5OP dependent activation. We then show that in a L-proline transporter knockout mutant L-proline competition remain unaffected, implying sensing of 5OP is extracellular. Last, we identify a transcriptional effect of 5OP exposure, upregulation of the mgtCBR operon, known to be activated during host invasion. While mgtCBR is known to be regulated by both low pH and L-proline starvation, we show that 5OP regulation of mgtCBR is indirect through changes in pH and is not dependent on the 5OP chemical structure similarity to L-proline. We also show this response to be PhoPQ dependent. We further show that the aggregation response is independent of pH modulation, PhoPQ and MgtC and that the mgtCBR transcriptional response is independent of YfeA and BcsA. Thus, the two responses are mediated through two independent signaling pathways. To conclude, we show *Salmonella* responds to 5OP specifically to regulate aggregation and not specifically to regulate gene expression. When and where in the *Salmonella* life cycle does 5OP sensing takes place remains an open question. Furthermore, because 5OP inhibits c-di-GMP through the activation of an external sensor, and does not require an internalization step like many studied biofilm inhibitors, 5OP or derivatives might be developed into useful biofilm inhibitors.

Cyclic-di-GMP (c-di-GMP) is a bacterial second messenger which in *Salmonella enterica* serovar Typhimurium (*Salmonella*), as well as other bacteria, controls a behavioral switch regulating motility versus sessility, mainly in the form of biofilm formation. In *Salmonella*, high levels of c-di-GMP lead to protective cellulose secretion. As biofilms are a major problem in medical and industrial settings, for example by forming on catheters or on gallstones, there is a need for the identification and development of biofilm inhibitors.

*Salmonella*, like all organisms, has to sense changes in its environment in order to modify its behavior and optimize its nutrient acquisition and chances of survival. Indeed, c-di-GMP levels in bacteria are controlled by sensors which respond to extracellular cues. The *Salmonella* genome encodes 17 c-di-GMP enzymes that synthesize or degrade c-di-GMP, theoretically in response to diverse environmental signals. Thus, we have previously performed a screen for nutrient type compounds that are naturally sensed by *Salmonella* to modulate the levels of c-di-GMP. One of the compounds identified in our screen to negatively regulate c-di-GMP and cellulose dependent cellular aggregation, and a potential biofilm inhibitor, is 5-oxoproline (5OP).

5OP, also known as pyroglutamic acid and pidolic acid, is a ubiquitous but little studied natural amino acid derivative. It is an enzymatic intermediate in the eukaryotic γ-glutamyl cycle, converted into glutamate by 5-oxoprolinase, and is also an unavoidable damage product formed spontaneously from glutamine and other sources. Furthermore, human inborn errors of metabolism that lead to 5OP buildup result in metabolic acidosis, hemolytic anemia, and neurological problems. Thus, understanding if *Salmonella* indeed senses this specific molecule and characterizing how *Salmonella* responds to 5OP might shed light on the functions of this molecule.
Furthermore, as *Salmonella* is a pathogen, understanding if and how 5OP sensing integrates into host–pathogen interactions might lead to new methods to prevent or treat infection.

Of note, l-proline is known to regulate c-di-GMP and cellulose secretion, as well as *Salmonella* virulence factors including the *mgtCBR* operon11 during *Salmonella* residence inside of host macrophages12–14. Because l-proline and 5OP are very similar molecules (Fig. 1a), we hypothesized that it is possible that the effects of 5OP are dependent on its structural similarity with l-proline. Thus, this study was aimed at characterizing 5OP sensing in *Salmonella* including its specificity and relation to l-proline sensing.

**Materials and methods**

**Strain, plasmids and primers.** A list of strains and plasmids can be found in Supplementary Table S1, and a list of primers used for cloning, verifying knockout strains, and for quantitative PCR (qPCR) can be found in Supplementary Table S2. Most knockout mutants were a kind gift from Prof. Roi Avraham, originally obtained from the *Salmonella enterica* serovar Typhimurium SL14028 (*Salmonella*) knockout strain library15. All strains derived from this library were verified by us to hold the expected mutation by sequencing the knockout area. The *putP* knockout mutant strain used in this study was generated from the same SL14028 strain background utilizing the method described by Datsenko and Wanner16.

**Bacterial growth conditions for flow cytometry and qPCR.** Bacteria were grown for 18 h at 37 °C with shaking in 2 ml of a minimal defined medium [21 mM K2HPO4, 11 mM KH2PO4, 3.8 mM (NH4)2SO4, ~ 3.8 mM KOH, 25 mM glycerol, 1 mM MgCl2, 10 μM FeCl3, 1 mM NaCl, 1 × MEM amino acid solution (Gibco), 1 × MEM nonessential amino acid solution (Gibco), pH 7.4]8. It should be noted, that unlike the growth experiments described in the next section, in experiments analyzing aggregation through flow cytometry or RNA expression by qPCR, glycerol as a carbon source was always present. If different strains were utilized in the same experiment, OD600 was measured and bacterial concentrations were normalized. Cultures were then diluted 1:100 or 1:20 for flow cytometry or qPCR, respectively, into 5 ml of the same medium lacking both amino acid solutions, and grown for an additional 2 h at 30 °C with shaking. Cultures were then pelleted by centrifugation for 3 min at 15,000g speed and resuspended in new minimal media without amino acids. 5-oxoproline (5OP), l-proline, or both were diluted in water and added in a volume of 1:5 of the bacteria, to a final concentration of 8 mM for 5OP, 8 mM for l-proline, and 8 mM for 5OP and 80 mM for l-proline in competition experiments in which both were present. For flow cytometry bacteria were incubated with the compounds at 30 °C standing in 96 well plates in a total volume of 100 μl for 1 h before flow cytometry analysis. For RNA extraction, bacteria were incubated at 30 °C shaking with compounds for 2 h in a total volume of 2 ml.

**Bacterial growth measurement.** Bacteria were directly inoculated from frozen stock into new minimal defined media without amino acids in which the glycerol was exchanged with 8 mM 5OP, or 8 mM l-proline. As a negative control we used minimal defined media without 5OP, l-proline, or glycerol. Bacteria were incubated for 3 days shaking at 37 °C. To measure growth, we utilized a flow cytometer (CytoFlex, Beckman Coulter Life Science company) and determined the number of events recorded by running 5 μl of culture. Supplementary Fig. S1a shows representative flow cytometer output scatter plots used for bacterial growth measurement. All events were counted. Control buffers prepared without bacteria inoculation were used to determine background
events. Thus, event counts reported below are the number of events above background. To verify that flow cytometry results indeed represent live cells, we also diluted the 3 day old cultures 1:100 into LB and followed growth kinetics by OD$_{600}$ using a plate reader. This resulted in very similar readings (Supplementary Fig. S1b) verifying flow cytometry analysis was indeed reporting growth.

**Measurement of cellular aggregation.** Aggregation was measured by flow cytometry as previously described. Briefly, a gate corresponding to high forward and side scatter events was established by comparing wild-type and a bcsA knockout mutant deficient in cellulose secretion and subsequent cellular aggregation (Supplementary Fig. S2). The same gate was used for all experiments. These high forward and side scatter events were considered aggregates. A second gate, encompassing all events above a certain threshold of forward and side scatter was established in order to count events which are likely bacterial cells but to omit events which are likely noise or buffer particles. We term this second gate "total events" and it encompasses within it the aggregate gate. The percentage of aggregate events out of the total events is reported. To make sure that high forward and side scatter events were indeed aggregates of bacteria and not a result of a change in size of single cells we imaged *Salmonella* cells by an imaging flow cytometer (image stream). We found indeed that high forward and side scatter events represented cellular aggregates (Supplementary Fig. S3).

**Measurement of mgtCBR expression.** OD600 of each sample was recorded for quality control. Then cultures were treated at a 1:1 ratio with Protect Bacteria reagent (Qiagen) to preserve RNA. Samples were incubated at room temperature for 5 min and then pelleted by centrifugation for 10 min at 5000g. Supernatants were removed. Pellets were either frozen or used directly for RNA extraction. RNA was extracted using the RNaseasy Protect bacteria mini kit (Qiagen) according to the manufacturer instructions. RNA quality and concentration was quantified using Nanodrop (Thermo Scientific). RNA was diluted to 50 ng/µl for cDNA reactions. cDNA was created with High-capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. For qPCR, the 16S ribosomal gene was used as a housekeeping gene of reference. Primers used to quantify mgtCBR expression were taken from Lee and Groisman, 2012. The 2(-ΔΔCT) method was used for analysis.

**pH modulation.** NaOH and HCl were used to modify the pH levels of cultures. NaOH was added to SOP exposed cultures until the pH was equal to that of DDW control cultures. Similarly, HCl was added to DDW control cultures until the pH reached the levels of SOP treated cultures. pH was quantified with pH strips (Macherey-Nagel).

**Statistics.** GraphPad Prism 9.3.1 was used for statistical analysis. One-way ANOVA was performed when comparing multiple samples. T-test was conducted when just two samples were compared.

**Results**

**L-Proline but not SOP is utilized by *Salmonella* as a nutrient source.** Responses to various nutrient type compounds could be an indirect response to their utilization in metabolism or a direct response to the activation of receptors that utilizes specific compounds as signals representing a certain environment. Furthermore, as SOP is very similar chemically to l-proline, we theorized this was a good control for specificity in our assays. To determine if SOP is utilized for growth in our experimental conditions we exchanged glycerol, found in our growth media, with either SOP or l-proline. We found that l-proline but not SOP was able to substitute glycerol for *Salmonella* growth (Fig. 1b). Of note, growth in SOP was somewhat higher than the no carbon control, but this was not statistically significant and negligible compared to growth with l-proline and glycerol. To make sure this was not because SOP was inhibiting growth, growth in a glycerol containing media with or without SOP was compared. SOP did not inhibit the growth of *Salmonella* (Supplementary Fig. S4). Thus, while *Salmonella* is able to efficiently internalize and utilize l-proline for growth, it is either not able to efficiently transport SOP into the cell or not able to incorporate it into its metabolism. In any case, external SOP is not a nutrient source *Salmonella* is able to efficiently utilize in our growth conditions. Thus, SOP is a signal to which *Salmonella* directly responds to.

**SOP but not l-proline regulates aggregation in a YfeA and BcsA dependent manner.** To examine the specificity of SOP signaling we first confirmed and extended earlier results. As previously published, exposure to SOP resulted in a reduction in cellular aggregation (Fig. 2a). A dilution analysis showed that the aggregation phenotype is dependent on the concentration of SOP (Supplementary Fig. S5). As expected, this phenotype was dependent on the YfeA (also called STM2410) phosphodiesterase (Fig. 2b), shown previously to be required for c-di-GMP modulation by SOP, and BcsA (Fig. 2b), a part of the cellulose secretion machinery.

As l-proline is very similar structurally to SOP (Fig. 1a) it is possible that l-proline is the molecule which is sensed rather than SOP. To determine if this is the case we measured the ability of l-proline to modulate aggregation. However, l-proline was not able to modulate aggregation (Fig. 2a). Thus, the receptor activated by SOP can differentiate between SOP and l-proline.

**L-Proline is a competitor for the SOP effect on aggregation.** Because l-proline and SOP are so similar in their chemical structure, it is possible that even though l-proline does not modulate aggregation by itself, it would compete with SOP over binding of a hypothetical SOP sensor. Thus, we compared the aggregation of wild type *Salmonella* exposed to just SOP or to both SOP and l-proline added in excess, at a 10 times ratio of SOP to l-proline.
higher concentration than 5OP. We found that adding l-proline in excess resulted in an attenuated aggregation response to 5OP (Fig. 2a). Thus, l-proline is an inhibitor of the 5OP aggregation response likely because of the structural resemblance between the two molecules.

**PutP is required for l-proline transport.** Because l-proline acts as an inhibitor, identifying the l-proline transporter active in our experimental conditions will be a useful tool to determine if 5OP is sensed inside the bacterial cell or externally. *Salmonella* is thought to encode at least three transporters for l-proline, the ProVWX glycine betaine/l-proline ABC system, the ProP proline/betaine transporter, and the PutP sodium/l-proline symporter. To identify which of these is required for l-proline transport in our assay conditions, we measured the growth of the corresponding knockout mutants in a growth medium in which we exchanged glycerol with l-proline as described above. The *putP* knockout mutant completely lost the ability to grow on the l-proline based media (Fig. 3a) while the other two knockout mutants grew just as well as the wild type (Supplementary Fig. S6). Thus, in our conditions the PutP transporter was required for l-proline internalization.

**L-Proline is a competitor also in a putP knockout mutant background.** Because l-proline likely competes with 5OP on its binding site in an unknown sensor and because PutP is required for l-proline internalization, the ability of l-proline to act as a competitor in a *putP* knockout mutant would allow to elucidate if 5OP sensing is internal or external. We found that in the *putP* knockout mutant background l-proline still functions as a competitor (Fig. 3b). Likewise, responses to 5OP and l-proline competition in the *proP* and *proV* mutants were similar (Supplementary Fig. S7). Thus, 5OP is likely sensed externally by *Salmonella*.

**5OP regulates mgtCBR transcription.** L-Proline starvation is known to regulate the expression of the pathogenicity related *mgtCBR* operon which also regulates c-di-GMP levels and cellulose secretion. As 5OP and l-proline are very similar molecules (Fig. 1a), and because we have previously shown 5OP regulates c-di-GMP and cellulose levels, we hypothesized it is possible that the mechanism of action of 5OP is to induce l-proline starvation by competitively inhibiting l-proline binding to unknown receptors or enzymes. As shown later, this hypothesis turned out to be wrong. However, this implied 5OP might also regulate *mgtC* transcription. Thus, we exposed *Salmonella* to 5OP, purified RNA and measured the level of transcript using three sets of primers. One set of primers targeted the mRNA leader sequence, the second set targeted *mgtC* itself, and the third set targeted *mgtB*. All three sets showed an increase in transcription in response to 5OP exposure in comparison with the unexposed control, with the *mgtB* area showing the highest increase (Fig. 4). Thus, 5OP exposure indeed regulates also *mgtCBR* as hypothesized.
L-Proline does not regulate mgtCBR expression. Because our hypothesis for the 5OP mechanism of action included l-proline starvation, and because we found l-proline to be a competitor for 5OP dependent aggregation, we examined if l-proline would affect mgtCBR expression by itself or as a competitor to 5OP. Surprisingly, we found l-proline had no effect on mgtCBR expression (Fig. 4). Furthermore, because of large variation it was not clear if l-proline was a competitor for 5OP dependent mgtCBR regulation. To conclude, it seemed unlikely that the 5OP mechanism of action included l-proline starvation as we originally hypothesized.

5OP regulation of mgtCBR is pH and PhoPQ dependent. The mgtCBR operon is also known to be regulated by pH through the two component system PhoPQ. Notably, 5OP (pKa = −1.76) is a stronger acid than l-proline (pKa = 1.99). Thus, it might regulate mgtCBR nonspecifically by modulating pH levels. To determine if this mechanism of action is responsible for the mgtCBR regulation phenotype, we exposed Salmonella to 5OP but countered the acidity by adding NaOH. This resulted in a loss of mgtCBR expression (Fig. 5a). Furthermore, exposing Salmonella to acidic pH without 5OP produced a similar upregulation of the mgtCBR operon as during exposure to 5OP (Fig. 5a). Last, a Salmonella phoP knockout mutant did not modulate mgtCBR expres-
mgtCBR regulation by 5OP was non-direct through pH modulation and not because of its similarity to L-proline. 5OP regulation of cellular aggregation is pH, PhoPQ and MgtC independent. The results described in the previous section show that 5OP regulates pH levels to increase mgtCBR operon expression through PhoPQ activation. The results also show that 5OP regulated c-di-GMP, and resultant cellulose dependent cellular aggregation through YfeA and BcsA. Because MgtC is known to regulate c-di-GMP and cellulose by itself, it was possible these were all parts of one signaling pathway starting with 5OP, going through MgtC and ending with cellular aggregation. To determine if this is the case, we characterized the effect of pH on cellular aggregation: we either added NaOH to counter 5OP's acidity, or used HCl to reduce the pH independently of 5OP. We found that even when countering 5OP's acidity, the 5OP aggregation effect was maintained (Fig. 5c). Concurrently, changing the pH in the absence of 5OP did not result in a modulation of cellular aggregation (Fig. 5c). Furthermore, we tested if the reduction in aggregation in response to 5OP will also occur in a phoP knockout mutant. We found that cellular aggregation was still modulated in this mutant similarly to wild type (Fig. 2b). Thus, aggregation was independent of both pH and PhoPQ. To conclude our analysis, we also tested

**5OP regulation of cellular aggregation is pH, PhoPQ and MgtC independent.** The results described in the previous section show that 5OP regulates pH levels to increase mgtCBR operon expression through PhoPQ activation. The results also show that 5OP regulated c-di-GMP, and resultant cellulose dependent cellular aggregation through YfeA and BcsA. Because MgtC is known to regulate c-di-GMP and cellulose by itself, it was possible these were all parts of one signaling pathway starting with 5OP, going through MgtC and ending with cellular aggregation. To determine if this is the case, we characterized the effect of pH on cellular aggregation: we either added NaOH to counter 5OP's acidity, or used HCl to reduce the pH independently of 5OP. We found that even when countering 5OP's acidity, the 5OP aggregation effect was maintained (Fig. 5c). Concurrently, changing the pH in the absence of 5OP did not result in a modulation of cellular aggregation (Fig. 5c). Furthermore, we tested if the reduction in aggregation in response to 5OP will also occur in a phoP knockout mutant. We found that cellular aggregation was still modulated in this mutant similarly to wild type (Fig. 2b). Thus, aggregation was independent of both pH and PhoPQ. To conclude our analysis, we also tested
an mgtC knockout mutant. Aggregation in response to 5OP was equally reduced in a mgtC knockout mutant and wild type bacteria (Fig. 2b). To conclude, the cellular aggregation effect in response to 5OP is independent of the mgtCBR pathway.

5OP regulation of mgtCBR is independent of YfeA and BcsA. While the fact that modulation of pH affected the 5OP mgtCBR transcriptional response but not cellular aggregation already indicated these were separate pathways, we also tested if the transcriptional response was dependent on YfeA and BcsA. We thus exposed yfeA and bcsA knockout mutants to 5OP and measured mgtCBR operon levels. We found that both of these mutants had increased levels of mgtCBR in response to 5OP just like wild type bacteria (Fig. 6a,b). Thus, 5OP dependent mgtCBR transcription and cellular aggregation are two independent pathways.

Discussion

We have previously shown that 5OP is sensed by Salmonella to regulate c-di-GMP and cellulose dependent cellular aggregation levels8. Because 5OP is not a well-studied metabolite22, and because it is very similar to l-proline, as the two molecules differ by just one oxygen atom, it was possible that l-proline rather than 5OP was the molecule being sensed. Here we show this is not the case. 5OP rather than l-proline is sensed to regulate cellulose dependent cellular aggregation. Furthermore, we also show that modulating the pH by 5OP does not affect this specific pathway, thus the chemical structure of 5OP and not its effect on pH is sensed. Additionally, many molecules are sensed because they are nutrients utilized by the cells sensing them. In fact, at times it is difficult to determine if a molecule is actively sensed, or if physiological effects stem from the utilization of a molecule as a nutrient. Here we show that unlike l-proline, 5OP cannot be efficiently utilized as a nutrient source in our growth conditions. Finally, we show that l-proline is a competitive inhibitor for this pathway. This further proves that the specific chemical structure of 5OP is a signature sensed by a specific sensor. Thus, 5OP is specifically sensed by Salmonella. Presumably, it represents a specific environment for Salmonella. Further work identifying the environment in which 5OP is sensed is required to understand the importance of 5OP as a signal for Salmonella.

We also show that another result of 5OP sensing is the upregulation of the mgtCBR operon. As the mgtCBR operon is known to contribute to phagosome residence11, it implies a connection of 5OP sensing to pathogenicity. Furthermore, as the mgtCBR operon is known to be regulated by l-proline starvation13, it was possible that 5OP was a competitive inhibitor for l-proline binding to an unknown receptor or enzyme, possibly emulating l-proline starvation. However, mgtCBR is also regulated by pH21. Indeed, we found that it was modulation of pH and not l-proline starvation by 5OP which resulted in upregulation of the mgtCBR operon. Thus, regulation of mgtCBR transcription by 5OP, unlike the c-di-GMP/cellulose sensing pathway, is nonspecific and dependent on changes in pH levels rather than on the specific chemical structure of 5OP.

Because we have shown 5OP to regulate c-di-GMP and cellulose dependent cellular aggregation8 and because here we show that 5OP regulates the mgtCBR operon, and finally because this operon is known to regulate c-di-GMP levels and cellulose14, we initially theorized that these two pathways were connected. According to this hypothesis, 5OP would signal through pH modulation to activate PhoPQ, upregulating the mgtCBR operon, which would then regulate c-di-GMP, cellulose and cellular aggregation. However, multiple results showed this was not the case. mgtCBR regulation, but not aggregation, depended on the two-component system PhoPQ. Inversely, aggregation, but not mgtCBR regulation, depended on YfeA and BcsA. Moreover, the aggregation pathway was also independent of MgtC itself. Thus, these two pathways are completely separate. Furthermore,
because mgtCBR regulation, but not aggregation, depended on pH modulation rather than on the specific chemical structure of SOP, these are two separate signaling pathways already at the sensor level. The fact that the aggregation pathway is independent of MgtC is somewhat surprising, as previous work showed that MgtC regulates c-di-GMP and cellulose through a pathway requiring ATP synthase. However, an important difference in the two described pathways is that the MgtC pathway described is indirect, relaying on long term changes in ATP levels, with phenotypes identified 6 h into growth. Likely, our experiments were too short to identify the described effects of a mgtC knockout mutant. Moreover, it is important to note that Salmonella encodes 17 c-di-GMP modulating enzymes, further emphasizing the fact that many signaling pathways in Salmonella regulate c-di-GMP, cellulose secretion, and cellular aggregation.

Sensing can be of the internal environment or the external environment. Indeed, Salmonella senses both internal l-arginine levels through ArgR and external l-arginine levels through ArtI. Here we show 5OP regulates mgtCBR expression through modulation of pH levels utilizing the PhoPQ two component system. Thus, mgtCBR is modulated by external SOP. To determine if SOP dependent aggregation was controlled by internal or external SOP we utilized the putP mutant, deficient in l-proline import. We show l-proline continues to ameliorate 5OP aggregation also in the putP knockout mutant, which we have shown does not utilize l-proline as a nutrient source likely because l-proline is not internalized. Therefore, 5OP is sensed externally and not internally.

As discussed above, PhoPQ is the sensor system responding to SOP pH modulation to regulate mgtCBR. The SOP sensor which regulates c-di-GMP, cellulose secretion, and cellular aggregation is likely YfeA itself. YfeA includes an EAL cytoplasmic domain with a phosphodiesterase activity as well as a seven transmembrane domain termed MASE1 which is its sensor domain. Thus, the external parts of YfeA’s MASE1 domain either bind SOP directly or indirectly. We have previously shown that the case of l-arginine, the direct sensor was a periplasmic l-arginine binding protein, ArtI, which activates the diguanylate cyclase, YedQ (also called STM1987). Thus, it is also possible that YfeA is not the direct SOP sensor, but that another protein, upstream in the signaling cascade, binds SOP. One possibility for such a protein is ProX, the substrate binding periplasmic protein of the ProVWX glycine betaine ABC transporter, which in Escherichia coli is known to bind l-proline. However, numerous additional substrate binding periplasmic proteins are encoded by Salmonella with unknown substrate specificities.

To conclude, in this work we show that SOP sensing has two effects on Salmonella (Fig. 7). The first is a non-direct effect through modulation of pH on the expression of the mgtCBR operon through PhoPQ. The second effect is the result of specific sensing of SOP through the activation of the YfeA phosphodiesterase to reduce c-di-GMP levels. While we quantified cellular aggregation, a second phenotype, harder to characterize at short time scales, is YcgR dependent regulation of flagella based swimming. Thus, while in our laboratory conditions we measured BcsA dependent cellulose secretion and subsequent cellular aggregation, the relevant physiological phenotype may be motility, which is enhanced in low c-di-GMP levels. Only upon identification of the relevant environment in which Salmonella senses SOP it would be possible to conclude which of the possible phenotypes, mgtCBR expression, cellular aggregation or motility is the natural target of SOP sensing.

**Data availability**
All data generated or analysed during this study are included in this published article and its supplementary information file.
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Author contributions

E.S. and E.M. designed the research. E.S. performed the research. N.S. and E.M. mentored E.S. All authors wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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