STM2209-STM2208 (opvAB): A Phase Variation Locus of Salmonella enterica Involved in Control of O-Antigen Chain Length

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Abstract
STM2209 and STM2208 are contiguous loci annotated as putative protein-coding genes in the chromosome of Salmonella enterica. Lack of homologs in related Enterobacteria and low G+C content suggest that S. enterica may have acquired STM2209-STM2208 by horizontal transfer. STM2209 and STM2208 are co-transcribed from a promoter upstream STM2209, and their products are inner (cytoplasmic) membrane proteins. Analysis with the bacterial adenylate cyclase two-hybrid system suggests that STM2209 and STM2208 may interact. Expression of STM2209-STM2208 is subjected to phase variation in wild type Salmonella enterica serovar Typhimurium. Switching frequencies in LB medium are $6.1 \times 10^{-5}$ (ON→OFF) and $3.7 \times 10^{-7}$ (ON→OFF) per cell and generation. Lack of DNA adenine methylation locks STM2209-STM2208 in the ON state, and lack of the LysR-type factor Oxyc locks STM2209-STM2208 in the OFF state. Oxyc-dependent activation of STM2209-STM2208 expression is independent of the oxidation state of Oxyc. Salmonella cultures locked in the ON state show alteration of O-antigen length in the lipopolysaccharide, reduced absorption of bacteriophage P22, impaired resistance to serum, and reduced proliferation in macrophages. Phenotypic heterogeneity generated by STM2209-STM2208 phase variation may thus provide defense against phages. In turn, formation of a subpopulation unable to proliferate in macrophages may restrain Salmonella spread in animal organs, potentially contributing to successful infection.

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Introduction
Phase variation, the reversible switch of gene expression at high frequency (e. g., $>10^{-5}$ per cell and generation), is a common phenomenon in bacteria (reviewed in [1,2]). Switching turns gene expression from OFF to ON, or from low expression to high expression, and vice versa. A consequence of phase variation is phenotypic heterogeneity in clonal bacterial populations, a phenomenon of paramount relevance for bacterial survival in harsh environments. In bacterial pathogens, for instance, phenotypic heterogeneity in cell envelope components may facilitate immune evasion and modulation [2,3]. Classical examples of phase variation in pathogenic bacteria involve loci encoding surface-exposed proteins, cell appendices such as fimbriae, pili, and flagella, and lipopolysaccharide modification functions [1,2,4,5]. Phase variation, however, is not restricted to bacterial pathogens nor to loci that encode components of the cell surface [6,7].

Bacteria use a variety of mechanisms to produce phase variation [1], and a relatively common type of control involves switching between alternative epigenetic states. Each epigenetic state is propagated by a feedback loop, and reversed after a certain number of generations. Epigenetic regulation of phase variation systems is often controlled by DNA adenine methylation (reviewed in [8,9]). Paradigms of this kind of regulation are the pap operon of uropathogenic E. coli, which encodes fimbriae for attachment to the urinary epithelium [10], and the agn43 gene of E. coli, which encodes a non-fimbrial adhesin [11]. Other phase variation loci under Dam methylation control are the glycosyltransferase operon (gtr) of phage P22 [H], the clp operon of enterotoxigenic E. coli [12], the pef operon of the Salmonella virulence plasmid [13], and perhaps the S. enterica std fimbral operon [14].

This study describes a new phase variation locus in Salmonella enterica serovar Typhimurium. The locus, annotated as STM2209-STM2208 in the Salmonella genome database [15], is present in Salmonella enterica but not in Salmonella bongori nor in other enteric bacteria. Aside from the annotation of STM2209-STM2208 as putative protein-coding genes, the literature contains little information on STM2209-STM2208. An exception is a transcriptome analysis in Dam+ and Dam− strains of S. enterica which revealed that STM2209-STM2208 transcripts are more abundant in a Dam− background [16]. This observation tentatively classified STM2209-STM2208 as a locus repressed by Dam methylation [16]. However, we show that STM2209-STM2208 is actually a phase variation locus whose expression is locked in the ON state in Dam− mutants. We also show that lack of the LysR-like factor...
OxyR locks STM2209-STM2208 expression in the OFF state. STM2209 and STM2208 are part of a single transcriptional unit, and encode inner membrane proteins. Constitutive expression of STM2209-STM2208 alters lipopolysaccharide O chain length, reduces phage P22 adsorption, decreases resistance to serum, and impairs proliferation in macrophages. Altogether, our observations suggest that phase variation of STM2209-STM2208 may contribute to phenotypic heterogeneity in Salmonella populations, providing defense against phages and restraining Salmonella spread in animal organs.

**Methods**

**Bacterial strains, plasmids, bacteriophages, and strain construction**

All the strains of *Salmonella enterica* used in this study (Table 1) belong to serovar Typhimurium, and derive from ATCC 14028. For simplicity, *S. enterica* serovar Typhimurium is often abbreviated as *S. enterica*. *Escherichia coli* BTH101 (F' cya-99 adaD139 galE15 galK16 rpsL1 [Strr]; hsdR2 recA1 marB1) was used for bacterial two-hybrid assays. *E. coli* CC118 lambda pri [phaC20 thy-1 rpsE rpoB argE(氨) recA1 (氨 lambda pri)] and *E. coli* S17-1 lambda pri [recA pro

**Table 1. Strains of *Salmonella enterica* serovar Typhimurium.**

| Strain | Genotype |
|--------|----------|
| ATCC 14028 | wild type |
| SV4536 | Δdam-230 |
| SV5573 | STM2209:3xFLAG |
| SV5574 | Δdam-230 STM2208:3xFLAG |
| SV5676 | ΔSTM2209::lac (transcriptional) |
| SV5677 | ΔSTM2208::lac (transcriptional) |
| SV5679 | ΔSTM2208::lac (translational) |
| SV5680 | Δdam-230 ΔSTM2209::lac (transcriptional) |
| SV5681 | Δdam-230 STM2208::lac (transcriptional) |
| SV5683 | Δdam-230 STM2208::lac (translational) |
| SV5734 | ΔSTM2209::lac (transcriptional) |
| SV5735 | Δdam-230 ΔSTM2209::lac (translational) |
| SV5812 | STM2209:3xFLAG |
| SV5813 | Δdam-230 STM2209:3xFLAG |
| SV5925 | ΔoxyR::Cm' |
| SV5989 | ΔoxyR::Cm' ΔSTM2208::lac (transcriptional) |
| SV5990 | Δdam-230 ΔoxyR::Cm' ΔSTM2208::lac (transcriptional) |
| SV6001 | ΔoxyR::Cm' STM2208::3xFLAG |
| SV6002 | Δdam-230 ΔoxyR::Cm' STM2208::3xFLAG |
| SV6004 | Δdam-230 ΔoxyR::Cm' STM2209:3xFLAG |
| SV6005 | Δdam-230 ΔoxyR::Cm' STM2209:3xFLAG |
| SV6013 | ΔSTM2209-2208 |
| SV6397 | oxyR::Tn9 |
| SV6401 | mut. GATC |
| SV6976 | mut. GATC ΔSTM2209-2208 |
| SV7031 | mut. GATC ΔSTM2208::lac (translational) |
| SV7032 | Δdam-230 mut. GATC ΔSTM2208::lac (translational) |
| SV7232 | ΔoxyR::Cm' mut. GATC ΔSTM2208::lac (translational) |
| SV7233 | Δdam-230 ΔoxyR::Cm' mut. GATC ΔSTM2208::lac (translational) |

RNA extraction

RNA was extracted from *S. enterica* stationary phase cultures (OD<sub>600</sub> ~ 3), using the SV total RNA isolation system (Promega) as described at http://www.ifr.ac.uk/safety/microarrays/protocols.html. The quantity and quality of the extracted RNA were determined using an ND-1000 spectrophotometer (NanoDrop Technologies). To diminish genomic DNA contamination, the preparation was treated with DNase I (Turf DNA free; Applied Biosystems).

**Quantitative reverse transcriptase PCR and calculation of relative expression levels**

An aliquot of 0.6 μg of DNase I-treated RNA was used for cDNA synthesis using the High-Capacity cDNA Archive kit (Applied Biosystems). Quantitative reverse transcriptase (RT)-PCR reactions were performed in an Applied Biosystems 7500 Fast

**Table 2. Plasmids constructed for this study.**

| Plasmid number | Description |
|----------------|-------------|
| pZ1758 | pGEMT::[PE5-PE2209] |
| pZ1759 | pGEMT::[PE5-PE2208] |
| pZ1812 | pKT25::STM2209 |
| pZ1905 | pUT18C::STM2208 |
| pZ1906 | pUT18C::STM2209 |
| pZ1907 | pKT25::STM2208 |

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Real-Time PCR System. Each reaction was carried out in a total volume of 25 µl on a 96-well optical reaction plate (Applied Biosystems) containing 12.5 µl Power SYBR Green PCR Master Mix (Applied Biosystems), 11.5 µl cDNA (1/10 dilution), and two gene-specific primers (RT2209-5 and RT2209-3 for STM2209, RT2208-5 and RT2208-3 for STM2208) at a final concentration of 0.2 µM each. Real-time cycling conditions were as follows: (i) 95°C for 10 min and (ii) 40 cycles at 95°C for 15 sec, and 60°C for 1 min. A no-template control was included for each primer set. Melting curve analysis verified that each reaction contained a single PCR product. Gene expression levels were normalized to transcripts of ospA, a housekeeping gene that served as an internal control. The Student’s t test was used to determine if the differences in retrotranscribed mRNA content observed in different backgrounds were statistically significant.

β-galactosidase assays

Levels of β-galactosidase activity were assayed as described previously [24], using the CHCl₃-sodium dodecyl sulfate permeabilization procedure. The Student’s t test was used to determine if the differences in β-galactosidase activities observed in different backgrounds were statistically significant.

Protein extracts and Western blotting analysis

Total protein extracts were prepared from bacterial cultures grown at 37°C in LB medium until stationary phase (OD₆₀₀ ~3). Bacterial cells contained in 0.25 ml of culture were collected by centrifugation and suspended in 50 µl of Laemmli sample buffer [1.3% SDS, 10% (v/v) glycerol, 50 mM Tris-HCl, 1.8% β-mercaptoethanol, 0.02% bromophenol blue, pH 6.8]. Proteins were resolved by Tris-Glycine-PAGE using 12% gels (for STM2208) or Tris-Tricine-PAGE 15% gels (for STM2209). The sample was heated (100°C for 10 min and (ii) 40 cycles at 95°C for 15 sec, and 60°C for 1 min. A no-template control was included for each primer set. Melting curve analysis verified that each reaction contained a single PCR product. Gene expression levels were normalized to transcripts of ospA, a housekeeping gene that served as an internal control. The Student’s t test was used to determine if the differences in retrotranscribed mRNA content observed in different backgrounds were statistically significant.

Subcellular fractionation

Subcellular fractionation was performed as previously described [25], with some modifications. Briefly, bacteria were grown in LB medium at 37°C and spun down by centrifugation at 15,000 × g for 5 min at 4°C, then resuspended twice in cold phosphate-buffered saline (PBS, pH 7.4). The bacterial suspension was either mixed with Laemmli buffer (total protein extract) or disrupted by sonication. Unbroken cells were further removed by low-speed centrifugation (5,000 × g, 5 min, 4°C). The supernatant was centrifuged at high speed (100,000 × g, 30 min, 4°C) and the new supernatant was recovered as the cytosol fraction. The pellet containing envelope material was suspended in PBS with 0.4% Triton X-100 and incubated for 2 h at 4°C. The sample was centrifuged again (100,000 × g, 30 min, 4°C) and divided into the supernatant containing mostly inner membrane proteins and the insoluble fraction corresponding to the outer membrane fraction. An appropriate volume of Laemmli buffer was added to each fraction. After heating (100°C, 5 min) and clearing by centrifugation (15,000 × g, 5 min, room temperature), the samples were analyzed for protein content by SDS-PAGE.

Directed construction of point mutations

Mutation of the 4 GATC sites contained in the promoter region of STM2209-STM2208 was achieved using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). Briefly, a ~1.3 Kb fragment of the STM2209-STM2208 region containing the 4 GATC sites was cloned into the pGEMT plasmid using the oligonucleotides Clo2208-5 and Clo-2208-3. Mutations in every GATC were then introduced using oligonucleotides harboring CACGTG changes (labeled as DIR). The resulting plasmid containing the fragment with 4 CATG sites was then digested with XbaI and SacI, cloned onto the suicide plasmid pDMS197 [27] and propagated in E. coli C1118 lambda pr. Plasmids derived from pDMS197 were transformed into E. coli S17-1 lambda pr. The resulting strains were used as donors in matings with S. enterica 14028 harboring a Cm⁺ cassette in place of the 4 GATC sites (constructed using oligonucleotides delGATCG1 and delGATCG2) as recipients. Tet⁺ transconjugants were selected on E plates supplemented with tetracycline. Several Tet⁺ transconjugants were grown in nutrient broth (without NaCl) containing 5% sucrose. Individual tetracycline-sensitive segregants were then screened for cloramphenicol sensitivity and examined for the incorporation of the mutant allelle by S1AI digestion and DNA sequencing using external oligonucleotides. Construction of the oxyR₁₉₉C mutation was achieved in the same way, using the oligonucleotides ClooxyR5 and ClooxyR3 for cloning onto pGEMT, and the oligonucleotides oxyRC199SDIR and oxyRC199SDIR for cloning onto oxyRC199SDIR and oxyRC199SDIR for site-directed mutagenesis. A strain with a Cm⁺ cassette in place of the oxyR gene (constructed using oligonucleotides deloxyR199PS1 and deloxyR199PS2) was used as a recipient in this case.

Measurement of the efficiency of phage adsorption

The efficiency of phage adsorption was calculated as described by Gabig et al. [28]. Briefly, P22 bacteriophages were added to S. enterica cells from an LB liquid overnight culture at a multiplicity of infection of 0.1, and the mixture was incubated at 37°C. Samples were taken every 2 min, centrifuged for 1 min at 13,000 rpm in a microcentrifuge, and the supernatant was titrated on the S. enterica wild-type strain ATCC 14028. The sample obtained at time zero (a sample taken immediately after addition of bacteriophages to the cell suspension) was considered to correspond to 100% unadsorbed phages, and the remaining numbers were calculated relative to this number. The Student’s t test was used to determine if the differences in adsorption were statistically significant.

Electrophoretic visualization of lipopolysaccharide profiles

To investigate lipopolysaccharide (LPS) profiles, bacterial cultures were grown overnight in LB. Bacterial cells were harvested and washed three times with 0.9% NaCl. The
O.D.₆₀₀ of the washed bacterial suspension was measured to calculate cell concentration. A bacterial mass containing about 3.14 x 10⁶ cells was pelleted by centrifugation. Treatments applied to the bacterial pellet, electrophoresis of crude bacterial extracts, and silver staining procedures were performed as described by Buendia-ClaVERia et al. [29].

Calculation of phase transition frequencies

Phase transition rates were estimated as described by Eisenstein [30]. Briefly, a strain harboring an STM2208::lac fusion was plated on LB + X-gal and colonies displaying an ON or OFF phenotype after 16 h growth at 37 °C were selected, resuspended in PBS and respread on new plates. Phase transition frequencies were calculated using the formula (M/N)/g where M is the number of cells that underwent a phase transition, N the total number of cells, and g the total number of generations that gave rise to the colony.

Macrophage infection experiments

The rate of intramacrophage replication after 18 h infection was performed in J774 mouse macrophages as described in [31]. Briefly, macrophages were seeded at a density of 5 x 10⁵ in 24-well plates and grown in DMEM medium supplemented with 10% (v/v) fetal bovine serum at 37 °C, 5% CO₂. Bacteria were added to the wells at macrophage-to-bacteria ratio of 1:10. Phagocytosis was allowed to proceed for 30 min before washing three times with PBS and adding fresh DMEM media supplemented with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). β-galactosidase assays were carried out as described above. A level of β-galactosidase activity at least five fold higher than that measured for vectors alone indicates a positive interaction.

Results

STM2209-STM2208 is a Salmonella-specific locus

STM2209 and STM2208 are contiguous loci annotated as putative protein-coding genes in the chromosome of *Salmonella enterica*. The STM2209 and STM2208 ORFs are conserved in *Salmonella enterica* serovar Typhimurium strains ATCC 14028, SL1344, and LT2 (GenBank accession numbers CP001363.1, FQ312003.1 and AE006481.1, respectively), in the vicinity of the sugar transport gene *setB* [34]. The STM2209 and STM2208 ORFs are also conserved in other *Salmonella enterica* serovars but not in *Salmonella bongori* nor in the genera Escherichia and Shigella. Alignment of the predicted amino acid sequences of STM2209 and STM2208 using BLASTP [35] detected no obvious homologs of STM2209-STM2208 outside *Salmonella enterica*. A diagram of the chromosome region in *Salmonella enterica* and related Enterobacteriaceae is shown in Fig. 1.

Both STM2209 and STM2208 have low G+C content (37% for STM2209 and 38% for STM2208) compared to both the average of the region (53%) and that of the *Salmonella enterica* genome (52%) [36]. Because horizontally acquired genes often have distinctive base composition, specifically low G+C content [37,38], these observations suggest that STM2209-STM2208 may have been acquired by horizontal gene transfer. The organization of the STM2209 and STM2208 ORFs suggests that they may be part of a single transcriptional unit: both coding sequences are on the same DNA strand, and are separated by only one nucleotide. Genome sequence analysis in *silico* predicts that STM2209 may encode a small peptide of 40 amino acids, while STM2208 may be a larger protein product of 221 amino acids. In *silico* analysis of protein structure using the TMHMM transmembrane prediction software [39] predicts the existence of one transmembrane domain in STM2209, and two transmembrane domains in STM2208 (data not shown). In *silico* analysis also indicates that STM2208 shares a domain with proteins belonging to the Wzz superfamily of O-antigen chain length regulators. This family includes proteins involved in lipopolysaccharide biosynthesis that confer a modal distribution of chain length on the O-antigen component of lipopolysaccharide [40]. This domain is also found in bacterial tyrosine kinases [41].

Expression of the STM2209-STM2208 locus is regulated by Dam methylation

A previous study showed that STM2209 and STM2208 are expressed at higher levels (13 fold for STM2209 and 8 fold for STM2208) in a *S. enterica* Dam⁻ mutant [16]. These observations suggested that expression of the putative STM2209-STM2208 transcriptional unit might be repressed by Dam methylation. To confirm Dam-dependent regulation, transcriptional and translational *lac* fusions were constructed in both loci. Protein variants tagged with the 3xFLAG epitope were also constructed. The effect of Dam methylation on STM2209-STM2208 expression was monitored by β-galactosidase assays, qRT-PCR, and Western blotting in isogenic Dam⁺ and Dam⁻ strains. Higher level of β-galactosidase activity, higher amount of retrotranscribed STM2209-STM2208 mRNA, and increased level of the STM2208-3xFLAG product were detected in the Dam⁻ back-
The extent of derepression differed slightly depending on the method, expression of STM2209-STM2208 was significantly higher in a Dam− background in all experiments. These results confirm that Dam methylation represses STM2209-STM2208. Furthermore, our ability to detect Dam-dependent regulation with both transcriptional lac fusions and qRT-PCR suggests that Dam-dependent regulation of STM2209-STM2208 may be transcriptional.

Identification of OxyR as a regulator of STM2209-STM2208

A genetic screen based on the T-POP3 transposon [43] was used to search for positive regulators of STM2209-STM2208. For this purpose, a Dam− strain carrying a lac translational fusion in STM2208 (SV5683) was used. This strain forms deep blue colonies on LB plates [44], thus explaining the small colony size of the isolate. However, the isolate formed large colonies on LB supplemented with X-gal, and white colonies were sought. Only a small white colony was obtained in the screen. Cloning and sequencing of T-POP3 obtained in the screen. Cloning and sequencing of T-POP3 indicated that T-POP3 had inserted in the oxyR gene. OxyR− mutants are severely impaired to form colonies on LB plates [44], thus explaining the small colony size of the isolate. However, the isolated formed large colonies on LB + catalase, a standard procedure that permits colony formation by OxyR mutants [44]. To confirm that oxyR loss-of-function abolished STM2209-STM2208 expression in a Dam− background, the oxyR gene was disrupted using lambda Red recombinase. The resulting strain (SV5925), which carries a null oxyR allele, was used in further experiments.

Figure 1. Diagram of the region containing STM2209-STM2208 on the Salmonella enterica chromosome. The homologous regions of Salmonella bongori, E. coli, and Shigella flexneri are also shown. The STM2209-STM2208 operon is shown in yellow. Black arrows represent conserved genes. White arrows represent non conserved genes. Grey arrows represent genes found at a different chromosome location on the S. enterica chromosome.
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Analyses of β-galactosidase activity and Western blotting showed that expression of STM2209-STM2208 is virtually abolished in an OxyR background (Fig. 4). As above (Fig. 2), high levels of β-galactosidase and of the STM2209-3xFLAG and STM2208-3xFLAG products were detected in the Dam2 background only. These experiments indicate that OxyR is essential for the expression of STM2209-STM2208. Interestingly, putative OxyR binding sites are found in the promoter region of STM2209-STM2208 (see below).

OxyR is a global transcription factor that can sense oxidative stress by direct oxidation. In the oxidized state, OxyR activates the expression of oxidative-stress-responding genes [45]. However, OxyR also acts as a transcriptional regulator irrespective of its oxidative state. In the absence of oxidative stress, OxyR remains mostly in the reduced form due to the reducing environment of the cell [46]. Several observations suggested that the oxidative state of OxyR is not relevant for STM2209-STM2208 regulation. One was that an H₂O₂ concentration sufficient to promote the expression of genes belonging to the classical OxyR regulon (genes activated by oxidative damage) showed no effect on the expression of STM2209-STM2208 (data not shown). Furthermore, the spacing between the half sites in the putative OxyR binding sites described below is consistent with specific binding of the reduced form of OxyR [47]. To determine the effect of oxidation of OxyR upon STM2209-STM2208 expression, we constructed a point mutant version of the oxyR gene (strain SV6397). The resulting OxyRC199S protein is locked in the reduced form as it cannot form the disulfide bond required for oxidation [46,47]. Dam+ and Dam2 strains harboring this mutation showed levels of STM2209-STM2208 expression similar to those described above for strains carrying the wild type oxyR allele (data not shown). These observations suggest that oxidation of OxyR is not necessary for STM2209-STM2208 expression.

STM2209-STM2208 expression undergoes phase variation under the control of Dam methylation and OxyR

In the course of our experiments with strains carrying STM2209::lac or STM2208::lac fusions in a wild type background, we detected phenotypic heterogeneity when culture aliquots were spread on plates containing X-gal. These strains formed white colonies that later turned pale blue, indicating low expression of STM2209 and STM2208. However, deep blue colonies were also

**Figure 2. Regulation of STM2209-STM2208 by Dam methylation.** A. Levels of STM2209 and STM2208 mRNAs, measured by qRT-PCR (Dam⁺: white histograms; Dam⁻: black histograms). Level of STM2209 mRNA in Dam⁻ background is considered 100%. Values are averages and standard deviations from 7 independent experiments. B. β-galactosidase activity of transcriptional STM2209::lac and STM2208::lac fusions in Dam⁺ and Dam⁻ backgrounds (white and black histograms, respectively). Values are averages and standard deviations from 3 independent experiments. C. β-galactosidase activities of translational STM2209::lac and STM2208::lac fusions in Dam⁺ and Dam⁻ backgrounds (white and black histograms, respectively). Values are averages and standard deviations from 3 independent experiments. D. Western blot analysis of STM2209-3xFLAG and STM2208-3xFLAG proteins in Dam⁺ and Dam⁻ backgrounds.

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seen, especially on plates that contained high numbers of colonies (e.g., $\geq 1,000$ colonies). Whenever a blue colony was isolated and streaked out for single colonies, a mixture of white and blue colonies was obtained. This observation suggested that \textit{STM2209-STM2208} expression might undergo phase variation, and that switching from OFF to ON might occur at lower frequencies than switching from ON to OFF.

Phase variation frequencies in the \textit{STM2209-STM2208} locus were calculated using the formula \( \frac{M}{N} \cdot g \) where \( M \) is the number of cells that underwent a phase transition, \( N \) the total number of cells, and \( g \) the total number of generations that gave rise to the colony [30]. An \textit{STM2208::lac} translational fusion was used for these experiments. The frequency of \( \text{OFF} \rightarrow \text{ON} \) transition was estimated to be \( 6.1 \pm 0.2 \) $10^{-2}$ per cell and generation. The \( \text{ON} \rightarrow \text{OFF} \) switching rate was around 1,000-fold higher: \( 3.7 \pm 0.1 \) $10^{-2}$ per cell and generation. Phase variation of \textit{STM2209-STM2208} expression was also unaffected by the oxidation state of \textit{OxyR} (data not shown).

Phase variation was abolished in both \textit{Dam}\textsuperscript{−} and \textit{OxyR}\textsuperscript{−} mutants (Fig. 5). Lack of Dam methylation locks \textit{STM2209-STM2208} expression in the ON state, and lack of \textit{OxyR} locks \textit{STM2209-STM2208} expression in the OFF state. An \textit{oxyR} mutation is epistatic over a \textit{dam} mutation, an observation that may indicate that activation of \textit{STM2209-STM2208} transcription by \textit{OxyR} is Dam-methylation sensitive. However, both Dam methylation and \textit{OxyR} are needed to establish phase-variable expression of \textit{STM2209-STM2208}.

\textit{In silico} analysis of the DNA sequence upstream the \textit{STM2209-STM2208} promoter revealed the existence of 4 GATC sites arranged in a symmetrical pattern (Fig. 6). Phase variation was abolished by site-directed mutagenesis of the GATC sites present in the promoter region of \textit{STM2209-STM2208} (strain SV6401). Furthermore, the four base pair substitutions introduced in the \textit{STM2209-STM2208} UAS do not destroy known critical regions of the \textit{OxyR} binding sequence [47].

\textit{B-galactosidase} activity assays and Western blotting analysis proved that regulation by Dam methylation was abolished when the GATC sites were eliminated (Fig. 6). Expression of \textit{STM2209-STM2208} was $\approx 2$ fold higher in the \textit{GATC-less} mutant (SV7031) than the \textit{Dam}\textsuperscript{−} mutant (SV5683) (Fig. 6), but \textit{STM2209-STM2208} expression was abolished in both \textit{Dam}\textsuperscript{−} and \textit{OxyR}\textsuperscript{−} mutants (Fig. 5). Lack of Dam methylation locks \textit{STM2209-STM2208} expression in the ON state, and lack of \textit{OxyR} locks \textit{STM2209-STM2208} expression in the OFF state. An \textit{oxyR} mutation is epistatic over a \textit{dam} mutation, an observation that may indicate that activation of \textit{STM2209-STM2208} transcription by \textit{OxyR} is Dam-methylation sensitive. However, both Dam methylation and \textit{OxyR} are needed to establish phase-variable expression of \textit{STM2209-STM2208}.

Figure 3. Identification of the transcription initiation site of \textit{STM2209-STM2208} by primer extension. Putative -35 and -10 promoter modules and the +1 site are shown in boldface. The transcription initiation site is indicated by an arrow. doi:10.1371/journal.pone.0036863.g003

Figure 4. Regulation of \textit{STM2209-STM2208} expression by Dam methylation and \textit{OxyR}. A. Effect of an \textit{oxyR} null mutation on the $\beta$-galactosidase activity of translational \textit{STM2209::lac} and \textit{STM2208::lac} fusions in \textit{Dam}\textsuperscript{−} and \textit{Dam}\textsuperscript{+} backgrounds (white and black histograms, respectively). Values are averages and standard deviations from 3 independent experiments. B. Western blot analysis of the effect of an \textit{oxyR} null mutation on the levels of \textit{STM2209-3xFLAG} and \textit{STM2208-3xFLAG} proteins in \textit{Dam}\textsuperscript{−} and \textit{Dam}\textsuperscript{+} backgrounds. doi:10.1371/journal.pone.0036863.g004
STM2208 expression was locked in the ON state in both strains (Fig. 5). Construction of strain SV6401 thus permitted to analyze the consequences of STM2209-STM2208 constitutive expression avoiding the pleiotropic effects of dam mutations (see below).

The STM2209 and STM2208 gene products are proteins located in the inner (cytoplasmic) membrane of Salmonella enterica

The subcellular location of STM2209 and STM2208 was investigated using 3xFLAG-tagged variants. Electrophoretic separation of cell fractions (cytosol, cytoplasmic membrane and outer membrane) was performed, and Western analysis of the separated protein preparations was carried out with a commercial anti-FLAG antibody. The results unambiguously showed that STM2209 and STM2208 are located in the S. enterica inner (cytoplasmic) membrane (Fig. 7).

Evidence for interaction between STM2209 and STM2208 in the Salmonella cytoplasmic membrane

STM2209 may represent a novel example of a membrane peptide, an emerging class of functional molecules [49]. Because certain membrane peptides have been shown to interact with membrane protein partners, we investigated whether STM2209 interacts with the inner-membrane protein STM2208. To test interaction between STM2209 and STM2208 in vivo, we used the Bacterial Adenylate Cyclase Two-Hybrid (BACTH) assay, a procedure that permits the detection of specific interactions between inner membrane proteins [50]. STM2209 and STM2208 were independently cloned on plasmids pUT18C and pKT25. Four plasmid constructs were obtained (pUT18C-STM2209, pKT25-STM2209, pUT18C-STM2208, and pKT25-STM2208), and their interaction was tested in an E. coli CyaA− mutant (BTH101). Functional complementation was determined by measuring β-galactosidase activity. High levels of β-galactosidase activity were obtained with both plasmid pairs, compared with the basal activities of the plasmid vectors or with the activity of one fusion protein only (Fig. 8). These results suggest that STM2209 and STM2208 may interact indeed.

Constitutive expression of STM2209-STM2208 reduces P22 adsorption to S. enterica

During strain construction experiments by P22 HT transduction, we obtained reduced numbers of transductants whenever the strain that constitutively expresses STM2209-STM2208 (SV6401) was used as a recipient. This observation, combined with the fact that STM2209 and STM2208 are components of the cell envelope, raised the possibility that constitutive synthesis of STM2209 and STM2208 might impair adsorption of bacteriophage P22. To test this hypothesis, we compared the kinetics of P22 adsorption to the wild type strain, to a strain that constitutively expresses STM2209-STM2208 (SV6401), and to a strain that harbors a deletion of STM2209-STM2208 (SV6013). Suspensions of P22 bacteriophage and S. enterica were mixed, and samples were taken every two minutes, and centrifuged. The supernatant was subsequently titrated to monitor the presence of unattached phages (Fig. 9). Adsorption of P22 to S. enterica cells was found to be severely impaired in the strain that constitutively expresses STM2209-STM2208 (SV6401), which proved to be largely refractory to phage P22 attachment. In contrast, P22 adsorption remained unaltered in a strain carrying a STM2209-STM2208 deletion (SV6013) regardless of the presence of the mutated GATCs (SV6976). These experiments suggest that phase variation of STM2209-STM2208 may split clonal populations of S. enterica into two subpopulations, one of which is P22-sensitive while the other is P22-resistant.

Constitutive expression of STM2209-STM2208 alters chain length distribution in the lipopolysaccharide O-antigen of S. enterica

Because phage P22 is known to attach to the LPS of Salmonella enterica to initiate infection [51], we examined whether the strain that constitutively expresses STM2209-STM2208 (SV6401) showed LPS alterations. Migration of the LPS in polyacrylamide
Constitutive expression of STM2209-STM2208 reduces S. enterica resistance to guinea pig serum

O-antigen chain length has been described to be crucial for serum resistance in Salmonella [54,56–59]. Survival in serum was analyzed by treating exponentially growing cells with 30% non-immune guinea pig serum. Constitutive expression of STM2209-STM2208 caused increased killing by serum (Fig. 11). This is likely to be complement-mediated, since heat-inactivated serum did not impair growth of strain SV6401 (data not shown).

Constitutive expression of STM2209-STM2208 reduces S. enterica proliferation in macrophages

Additional screens and phenotypic assays were performed in search for functions of STM2209-STM2208 phase variation besides the formation of a P22-resistant subpopulation with reduced resistance to serum. The trials included: (i) growth in various media at different temperatures and different osmolarities; (ii) resistance to acidic pH, cationic peptides, bile, and hydrogen peroxide; (iii) motility; (iv) biofilm formation; (v) and invasion of epithelial and macrophage cell lines. Most trials did not show differences associated either to loss or constitutive expression of STM2209-STM2208. A remarkable exception was that constitutive expression of STM2209-STM2208 impaired intracellular proliferation within macrophages (Fig. 12).

On the other hand, a strain carrying a STM2209-STM2208 deletion showed intramacrophage proliferation at a level similar to that of the wild-type strain. These observations suggest that repression of STM2209-STM2208 expression may be required to permit Salmonella proliferation within macrophages. However, a nonproliferating S. enterica population may be also generated by switching STM2209-STM2208 to the ON state.

Discussion

STM2209 and STM2208, hitherto annotated as putative genes of unknown function in the genome of Salmonella enterica serovar Typhimurium, are absent in Salmonella bongori and in other species of enteric bacteria (Fig. 1). This assortment, combined with G+C content lower than the core Salmonella genome (38% vs. 52%, approximately), suggests acquisition by horizontal transfer.

STM2209 and STM2208 are part of a single transcriptional unit, and are transcribed from a promoter upstream STM2209 (Fig. 3). The STM2209 gene product is a small hydrophobic peptide (putatively, 34 amino acids) while STM2208 encodes a larger hydrophobic protein (putatively, 221 amino acids). Both...
STM2209 and STM2208 are located in the cytoplasmic membrane (Fig. 7). Certain structural features of STM2209 and STM2208 are reminiscent of those found in interacting peptide-protein pairs located in the bacterial cytoplasmic membrane [49]. For instance, the putative transmembrane domain of STM2209 and the putative N-terminus-proximal transmembrane domain of STM2208 are rich in phenylalanine and share additional amino acid sequence features. STM2209 and STM2208, however, lack common packing motifs described elsewhere for transmembrane-helix interactions, such as GxxxG, Ala-coil or motifs of serine and threonine [60–62]. Small regulatory peptides often interact with

Figure 8. Analysis of the in vivo interaction between STM2209 and STM2208 using the BACTH system. The E. coli BTH101 strain was co-transformed with plasmids encoding fusion proteins or empty. The basal level of β-galactosidase activity measured with empty vectors was approximately 90 Miller units. Values are averages and standard deviations from 3 independent experiments. doi:10.1371/journal.pone.0036863.g008

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Figure 9. Effect of constitutive expression of STM2209-STM2208 on adsorption of bacteriophage P22 to S. enterica. The efficiency of P22 attachment to S. enterica is shown as the percentage of non-adsorbed phages relative to the initial number. Strains are represented by black squares (wild type), white squares (SV6013, ΔSTM2209-STM2208), black circles (SV6401, mut. GATC) and white circles (SV6976, mut. GATC ΔSTM2209-STM2208). Values are averages and standard deviations from 6 independent experiments. doi:10.1371/journal.pone.0036863.g009

Figure 10. Lipopolysaccharide profiles of the wild type strain (lane 1), SV6013 (ΔSTM2209-STM2208) (lane 2), SV6401 (mut. GATC) (lane 3) and SV6976 (mut. GATC ΔSTM2209-STM2208) (lane 4), as observed by electrophoresis and silver staining. doi:10.1371/journal.pone.0036863.g010
larger proteins encoded in the same transcriptional unit, modulating their activity or stability [49]. This study presents evidence that STM2209 and STM2208 interact indeed (Fig. 8). The functional significance of STM2209-STM2208 interaction remains unknown; a tentative analogy with other peptide-protein pairs [49,63] permits the speculation that the STM2209 peptide might modulate the function of the STM2208 protein or act as a subunit in a larger complex.

Expression of the STM2209-STM2208 locus is subjected to phase variation (Fig. 5), and the OFF→ON switching frequency in LB medium is 3 orders of magnitude lower than ON→OFF switching (6.1×10^{-6} vs. 3.7×10^{-2} per cell and generation). Skewed frequencies of switching are also found in other phase variation loci: for instance, in the E. coli pap operon, the OFF→ON switching frequency is 5.54×10^{-4} per cell and generation, while the ON→OFF switching frequency is 2.34×10^{-2} per cell and generation [61]. Hence, like in pap, the subpopulation of cells that express STM2209-STM2208 in LB is smaller than the population of cells that do not express STM2209-STM2208. However, the switching frequencies detected under laboratory conditions can be different from those occurring in natural environments [8,65]. In the pap operon, for instance, the switching frequencies are skewed by environmental inputs involving global regulators like Crc and H-NS and the stress-responsive system CpxRA [66-68].

Lack of Dam methylation locks STM2209-STM2208 in the ON state (Fig. 5), thus explaining why STM2209-STM2208 was initially considered a locus repressed by Dam methylation [16]. Dam methylation has been previously shown to control phase variation systems along with a variety of transcriptional regulators [8]. However, Dam methylation can also regulate gene expression indirectly, either as a consequence of lack of DNA mismatch repair or by controlling expression of postranscriptional regulators [48,69]. In the case of STM2209-STM2208, the observation that site-directed mutagenesis of GATC sites located upstream the STM2209-STM2208 promoter locks expression in the ON state (Fig. 6) provides preliminary evidence that Dam methylation may regulate STM2209-STM2208 transcription. Evidence that STM2209-STM2208 is a new locus under the control of a Dam-sensitive transcriptional regulator is further supported by the identification of the LysR-like factor OxyR as a positive regulator of STM2209-STM2208 expression (Fig. 4). OxyR is a well known transcriptional regulator [45], and has been previously shown to control phase variation of other Dam methylation-sensitive loci: the E. coli ags43 gene [70,71] and the P22 gtr operon [4]. Unlike ags43, which is repressed by OxyR [70], and gtr, which is both activated and repressed by OxyR [4], STM2209-STM2208 is under positive control by OxyR (Fig. 4). Like in ags43 and in gtr, however, the oxidation state of OxyR is irrelevant for control of STM2209-STM2208 expression.

When STM2209-STM2208 expression is locked in the ON state, Salmonella cells become resistant to phage P22 (Fig. 9), presumably by alteration of O-antigen chain length in the lipopolysaccharide (Fig. 10). Hence, phase variation of STM2209-STM2208 expression in wild type populations of Salmonella can be expected to generate a subpopulation of P22-resistant cells. Resistance might be potentially extended to other Salmonella-specific lambdoid bacteriophages [72]. Phase variation in mechanisms of defense against bacteriophage infection has been previously described [6]. A phase variation system that controls Salmonella lipopolysaccharide modification has been also described in phage P22 [4]. However, to our knowledge, STM2209-STM2208 may be the first example of a phase variation system that confers phage resistance through alteration of O-antigen chain length.

O-antigen alteration may be also the cause of two infection-related traits associated to STM2209-STM2208 expression. One is increased sensitivity to serum (Fig. 11), which may be explained by the involvement of O-antigen chain length in serum resistance [32,54,56,58]. Reduced capacity to proliferate in macrophages (Fig. 12) could also be attributed to modification of the structure of LPS [73,74], although the relevance of O-antigen chain length in the Salmonella-macrophage interaction has been questioned [75,76]. On the other hand, LPS-containing outer membrane vesicles have been shown to mediate delivery of Salmonella virulence effectors to macrophages [77], suggesting that constitutive synthesis of STM2209 and STM2208 might impair the secretion process. Current evidence suggests that diversity in the structure and distribution of O-antigen length permits a balance between resistance to antimicrobial compounds and the ability to interact with different cell types [75]. Indeed, it has been described that O-antigen length is reduced upon growth inside murine...
macrophages [78] in a way reminiscent of the effect of constitutive expression of STM2209-STM2208 (Fig. 10). Interestingly, expression of STM2209-STM2208 is upregulated inside epithelial cells and macrophages [79]. STM2209-STM2208 displays features typical of Gram-negative O-antigen chain length regulators such as WzzPE (16–35 repeats) and WzzPE-Pf (>100 repeats): a common protein structure consisting of two transmembrane domains and a hydrophilic periplasmic domain, relative richness in proline residues in the second transmembrane segment [80], and a particular set of conserved amino acid residues near the N-terminal end [81]. STM2209 lacks, however, a predicted coiled-coil periplasmic domain typical of many O-antigen chain length regulators [80]. However, other O-antigen chain length regulators show little or no potential for coiled-coil formation. Furthermore, there is a correlation between coiled-coil potential of the periplasmic domain and the modal length conferred on the LPS O-antigen chains [80]. Because constitutive expression of STM2209-STM2208 leads to short modal length of the O-antigen (Fig. 10), lack of coiled-coil potential is not surprising.

Formation of a phage-resistant subpopulation upon STM2209-STM2208 phase variation may have obvious selective value. In contrast, the potential advantage of forming a less virulent bacterial subpopulation may be at the first sight intriguing. However, subpopulation formation has been described at several stages of host colonization by Salmonella, and a tentative interpretation is that reduction or arrest of bacterial growth is part of a stealthy strategy that increases the chances of successful infection. For instance, bistability in the synthesis of flagellin helps Salmonella to evade the host caspase-1 inflammatory response [3]. Another example is found upon Salmonella entry into macrophages: the population splits into two subpopulations, one of which replicates while the other enters a dormant-like state [82]. It has also been suggested that a successful infection strategy might involve the sacrifice of a fraction of the total population [83]. It might be argued that the rate of STM2209-STM2208 switching to the ON state (approximately, 6 × 10⁻⁷ per cell and generation) may be too low to produce a bacterial subpopulation of relevant size in animal tissues, especially in macrophages which typically host very low numbers of Salmonella cells [84]. However, as discussed above, the switching rates observed in the laboratory may not apply to other growth conditions [1]. Actually, the introduction of deterministic elements in stochastic gene regulation may be a common feature of phase variation systems [85]. Phase variation of STM2209-STM2208 might thus occur at different rates in different environments. Formation of a phage-resistant subpopulation, however, can be expected to have selective value regardless of the subpopulation size.

We propose that the STM2209-STM2208 locus is renamed opf (for O-antigen phase variation) so that the STM2209 gene is henceforth known as opfA, and the STM2208 gene as opfB.

Supporting Information

Table S1 Oligonucleotides.

(DOC)

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Author Contributions

Conceived and designed the experiments: IC ABBP JC. Performed the experiments: IC. Analyzed the data: IC ABBP JC. Contributed reagents/materials/analysis tools: IC ABBP JC. Wrote the paper: IC ABBP JC.
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