Mutation of a *Salmonella* Serogroup-C1-Specific Gene Abrogates O$_7$-Antigen Biosynthesis and Triggers NaCl-Dependent Motility Deficiency

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Abstract

Several molecular detection marker genes specific for a number of individual *Salmonella* serogroups have been recently identified in our lab by comparative genomics for the genotyping of diverse serogroups. To further understand the correlation between serotype and genotype, the function of a *Salmonella* serogroup-C1-specific gene (SC$_{2092}$) was analyzed in this study. It was indicated from the topological prediction using the deduced amino acid sequence of SC$_{2092}$ that this putative protein was highly similar to the confirmed Wzx flippases. Furthermore, SDS-PAGE revealed that lipopolysaccharide (LPS) biosynthesis, specifically O-antigen synthesis, was incomplete in an SC$_{2092}$ in-frame deletion mutant, and no agglutination reaction with the O$_7$ antibody was exhibited in this mutant. Therefore, it was revealed that this *Salmonella* serogroup-C1-specific gene SC$_{2092}$ encoded a putative flippase, which was required for O$_7$-polysaccharide biosynthesis, and was designated here as wzxC$_1$. Subsequently, the effects of the deletion of wzxC$_1$ on bacterial motility and sodium chloride (NaCl) tolerance were evaluated. The wzxC$_1$ mutant lacked swarming motility on solid surfaces and was impaired in swimming motility in soft agar. Moreover, microscopic examination and RT-qPCR exhibited that an increased auto-aggregation and a strong defect in flagella expression, respectively, were responsible for the reduced motility in this mutant. In addition, the wzxC$_1$ mutant was more sensitive than the wild-type strain to NaCl, and auto-aggregation of mutant cells was observed immediately upon the addition of 1% NaCl to the medium. Interestingly, the motility deficiency of the mutant strain, as well as the cell agglomeration and the decrease in flagellar expression, were relieved in a NaCl-free medium. This is the first study to experimentally demonstrate a connection between a *Salmonella* serogroup specific gene identified by comparative genomics with the synthesis of a specific O-antigen biosynthesis. Also, our results show that the mutation of wzxC$_1$ triggers a NaCl-dependent motility deficiency.

Citation: Zhou X, Liu B, Shi C, Shi X (2014) Mutation of a *Salmonella* Serogroup-C1-Specific Gene Abrogates O$_7$-Antigen Biosynthesis and Triggers NaCl-Dependent Motility Deficiency. PLoS ONE 9(9): e106708. doi:10.1371/journal.pone.0106708

Editor: Dipshikha Chakravortty, Indian Institute of Science, India

Received May 25, 2014; Accepted August 4, 2014; Published September 11, 2014

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: This study was supported by the National Natural Science Foundation of China (grant Nos. 31230058 and 31000779) (http://www.nsfc.gov.cn/); Ministry of Science and Technology of China (grant Nos. 2012AA101601, 2012BAC17B10, and 2012BAD29B02) (http://www.most.gov.cn/). The funders have no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The genus *Salmonella* is comprised of a heterogeneous group of Gram-negative bacteria, differentiable by biochemical and serological properties. The O-antigen (the main component of the bacterial cell wall LPS) contributes major antigenic and immunogenic characteristics, and is a basis of *Salmonella* serotype diversity. On the basis of the structural variation in O-antigens, *Salmonella* has been divided into 46 serogroups [1]. The most common O-antigen serogroups are A, B, C1, C2 and D, strains of which cause approximately 70% of *Salmonella* infections in humans and animals [2–5]. Each *Salmonella* serogroup is defined by an antigenic formula that indicates the specific O-antigens present in the LPS of strains belonging to that serogroup. For example, *Salmonella enterica* serovar Choleraesuis (S. Choleraesuis), which causes extra-intestinal infections or sepsis in humans [6,7] and has a higher mortality rate in humans than other *Salmonella* serovars [8], and strains of other sevovars within serogroup C1 have the antigenic formula O:6,7,14, indicating the presence of O$_6$–O$_7$– and, in some cases, O$_{14}$-antigens. The underline for the O$_{14}$-antigen in the formula indicates that this antigen is only present if the strain is lysogenized by a converting phage. Furthermore, the O$_6$– and O$_{14}$– antigens are present in the LPS of serogroups other than C1; thus, only the O$_2$-antigen is unique to serogroup C1. It has been reported that strains of serogroup C1 represented 20% to 35% of all *Salmonella* isolated from fecal samples of beef and dairy cows in the United States [9]. Also, the latest reports from the United States Centers for Disease Control and Prevention in 2012 showed that 12 acute salmonellosis cases (total 50 cases) were caused by strains from *Salmonella* serogroup C1 [10]. Although many highly virulent serovars are from *Salmonella* serogroup C1, this serogroup has not received sufficient attention from researchers, specifically the synthesis of their unique O-antigen.
Nearly all *E. coli* and *Salmonella* O-antigens are synthesized by the Wzx (flippase)/Wzy (polymerase)-dependent pathway [11]. Genes encoding enzymes involved in O-antigen biosynthesis are mostly grouped together on the chromosome in an O-antigen *rfb* gene cluster, and the structural difference of the O-antigens is generally mirrored by genetic distinction in these clusters [12]. *Salmonella* serogroup C1 has a different O-antigen structure than that from serogroups A, B, C2 and D, and genes in the *rfb* region of *Salmonella* serogroup C1 show no significant similarity to that of other *Salmonella* serogroups [13]. Although studies of the O-antigen biosynthesis in *Salmonella* serogroup C1 were initiated several years ago, the understanding of its genetic pathway still needs to be resolved.

In addition, O-antigens have been demonstrated to be involved in diverse interactions between bacterial cells and the environment. Several research groups have recently described an effect on motility in O-antigen mutant strains, however, the explanations for the role of O-antigen on motility behavior are diverse. O-antigen mutants in *Myxococcus xanthus* are defective in S (social) motility [14]; Mutants of *S. Typhimurium* lacking O-antigen synthesis showed normal swimming motility but conditional defects on swarming motility [15]; The deletion of O-antigen in *E. coli* showed significant defects in both swimming and swarming motilities [16]; Loss of O-antigen ligase in *Proteus mirabilis* inhibited swimming motility on solid surfaces [17]. At present, several mechanisms for O-antigen’s function in motility have been identified, affecting motility in diverse ways, including relief of RcsB-mediated repression of flagellin gene expression [16,17] and reducing surface friction by acting as a wettability agent [15]. However, it is thought that there are additional unknown roles for O-antigen involved. Therefore, further studies are needed to achieve a more comprehensive understanding of the role of O-antigen in bacterial motility.

Recently in our lab, we found seven genes that were conserved and specific for *Salmonella* serogroup C1 by comparative genomic analysis [18]. According to preliminary analyses, most of the C1-specific genes encode membrane proteins with high numbers of transmembrane segments (TMs). One conserved *Salmonella* serogroup C1-specific gene (locus SC_2092 in *S. Choleraesuis*; GenBank Accession # NC_006905) that putatively encodes a protein with 12 TMs is within the O7 antigen biosynthesis operon and specific for serogroup C1-specific gene (locus SC_2092 in *S. Choleraesuis*). The second PCR product containing a deletion of wzxC1-rev (5’-GGTCTAGACGTTGCTGCACTGCT-3’) plus wzxC1-int-for (5’-GGAAAAGAAAATGATTACGGTCCGCCTATTAC-3’) and wzxC1-rev (5’-CGAGGTCTGTATTTTTGTTTTTGGCTT-3’) plus wzxC1-int-for (5’-CTGGTTAACCTTTATGTTTGGAC-3’) using KOD Dash DNA polymerase (Toyobo, Japan). The products generated by the first PCR were a 593-bp fragment containing the DNA sequence upstream of wzxC1 and a 658-bp fragment containing the DNA sequence downstream of wzxC1. The 20-bp overlap sequences (underlined) allowed amplification of a 1251-bp product after a second PCR with the template of the mixture of two first PCR products and primers wzxC1-for and wzxC1-rev, which introduced XbaI and SacI restriction ends upon digestion (bold), respectively. The second PCR product containing a deletion of wzxC1 was cloned into the pMD18-T vector (TaKaRa, Japan) to generate pMID12ΔwzxC1. DNA sequencing was carried out to confirm the correct construction.

The marker-free in-frame deletion mutant of wzxC1 in *S. Choleraesuis* was screened by double selection in two steps. In the first step, the pMID12ΔwzxC1 plasmid with the deletion of wzxC1 was excised with *XbaI* and *SacI*, ligated into suicide vector pRE112 [24], which carries a chloramphenicol resistance gene and a sucrose-sensitivity gene *sacB*. The resulting plasmid pREΔwzxC1 was transformed by electroporation (2500 V, 5 ms) into *E. coli* SM10pir [25]. Then, 1 ml of *E. coli* SM10pir cells (ca.10^6 CFU/ml) containing the plasmid pREΔwzxC1 and 3 ml of the wild-type *Salmonella* cells (ca.10^6 CFU/ml) were mixed in a culture flask (quantities to 10-ml) for 8 h at 37°C to accomplish the conjugation process. Recipient cells were plated on LA (LB with 1.5% agar) supplemented with chloramphenicol (35 μg/ml) to select the trans-conjugant strain SC + pREΔwzxC1, containing the plasmid integrated into the *S. Choleraesuis* genome as single crossover. In the second step, a colony of SC + pREΔwzxC1 was grown in LB to allow for a second crossover. After overnight growth on LB at 37°C, the SC + pREΔwzxC1 culture was plated on LA containing 8% (w/v) sucrose, which selected for loss of the pRE112 vector (carrying sacB gene). Colonies that grew on this medium were tested for chloramphenicol sensitivity to ensure the
loss of plasmid by using LA containing chloramphenicol. The resulting strain, S. Choleraesuis ΔwzxC1, was confirmed by PCR with primers wzxC1-f and wzxC1-r, and sequencing of the resulting PCR-product using the same primers.

**Slide agglutination test, LPS extraction and SDS-PAGE analysis of O-antigen production**

The slide agglutination tests were performed using antisera (Ningbo Tianrun Bio-Pharmaceutical Co. Ltd., Zhejiang, China) on the basis of somatic O7 antigen according to the Kaufmann-White scheme. Test *Salmonella* in a drop of saline was placed on a slide, and a drop of antiserum was added and mixed. Then the slide was rocked gently for approx. 1 min. An LPS Extraction kit (NiRON Biotechnology, Korea) was used following the manufacturer’s instruction. LPS was then separated on Ready Gel Precast Tris-HCl polyacrylamide gels with 15% and 5% acrylamide in the separating and stacking gels, respectively (Bio-Rad Laboratories, USA) in buffer with 2% SDS, and fixed overnight in buffer with 10% acetic acid and 40% methanol. The gels were stained with a silver stain kit (Bio-Rad Laboratories, USA) following the manufacturer’s instruction. All the solutions were prepared fresh before use. The experiment was repeated three times.

**Topology prediction of this putative transmembrane protein**

Topology models were generated by the consensus web server TOPCONS [26] (http://topcons.chr.su.se/). Five TOPCONS algorithms (SCAMPI-seq, SCAMPI-msa, PRODIV, PRO and OCTOPUS) were used for the prediction of transmembrane helices. In addition, ZPRED was used to predict the Z-coordinate (the distance to the membrane center) of each amino acid, and ΔG prediction was used to calculate the free energy for membrane insertion of potential TMs. Given the amino-acid sequence of the test protein, the server provided the predicted topology. The prediction took 10–30 s, and the results were displayed graphically and downloaded in text format.

**Motility assays**

The bacterial swimming and swarming motility were assayed on media containing different concentrations of agar. With low agar concentrations (0.3%), the bacteria swim through water channels inside the media. With higher agar concentrations (0.5–0.7%), the bacteria swarm over the agar surface.

Swimming and swarming assays were performed as described previously [27]. Moreover, swimming and swarming assays were tested in both standard LA (containing 10 g NaCl per liter) and NA (LA without NaCl) media containing different concentrations of agar. Using sterile toothpicks, single colonies from streak plates were stabbed into the swimming plates (containing 0.3% agar) and incubated for 24 h. At least six independent colonies were checked for each strain tested. For swimming motility assays, 5 µl of an overnight culture grown in LB was spotted directly on each plate (containing 0.6% agar) and allowed to dry for 10 min with the lid removed. Plates were then covered and incubated at 37°C for 24 h before observation. The effect of bio-surfactin on swarming motility was also tested. Briefly, 5 µl of solution containing *Bacillus subtilis* surfactin (10 µg/ml, Sigma-Aldrich, USA) were spotted in the center of the swarming motility assay plate (LA media containing 0.6% agar), allowed to dry for 5 min, inoculated with the test strains on top of the surfactin spot, again allowed to dry for 10 minutes and then incubated for 24 h at 37°C.

**Flagella and bacterial morphological features examined by transmission electron microscopy (TEM)**

The morphological features of the mutant and the wild-type strains were examined by transmission electron microscopy (TEM). Samples were prepared for TEM by two different methods. In the first method a suspensions of the *Salmonella* strain was placed on copper grids, allowed to form a film on the grid for two minutes before the excess solution was removed using absorbent paper, and the grids were dried at room temperature [28]. For the second method, thin sections of the *Salmonella* strains were prepared for TEM as previously described [29]. Brieﬂy, ﬁxed bacteria cells embedded in agar were post-ﬁxed using 1% OsO4 in 0.05 M sodium cacodylate buffer for 1 h at room temperature. The cells were washed at least three times using sterile water and subsequently dehydrated in an increasing series of 15-minute ethanol rinses (30%, 50%, 70%, and 100%). The dehydrated cells were embedded in Embed-812 resin and sectioned to a thickness of 70 nm. The sectioned blocks were placed on carbon-coated 200 mesh copper grids, and stained with uranyl acetate for 10 min and Sato’s lead for 5 min. All samples

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**Table 1. Strains and plasmids used in this study.**

| Strain or plasmid | Comment(s) | Source/reference |
|------------------|------------|-----------------|
| S. Choleraesuis ATCC10708 | Wild-type, *Salmonella* serogroup C1, Type strain | Shanghai Entry-Exit Inspection and Quarantine Bureau of China |
| ΔwzxC1 | SC_2092 deletion mutant of S. Choleraesuis ATCC10708 by pRE112Δwzx | This study |
| ΔwzxC1-C | wzx deletion mutant complemented with plasmid pREwzxC1-C | This study |
| E. coli TG1 | supE, hisD, thrA1, lacD (lac-proAB), F’::traD36, proA8-, lacIq, lacZΔM15 | Laboratory stock |
| E. coli SM10 (pir) | thi-1 leuK2 proA2 his-4 argE2 lacY1 galK2, ara14xyl5 supE44, /pir | [25] |
| pMD18-T | Cloning vector, Amp’ | TaKaRa, Japan |
| pRE112 | pGPT04 suicide plasmid, pir dependent, oriT, oriV, sacB, Cm’ | [24] |
| pREΔwzx | pRE112 containing a 1251 bp wzx-deletion PCR product (593 bp of sequence upstream and 658 bp of sequence downstream of wzxC1, generated by overlap extension PCR); used to generate strain ΔwzxC1 | This study |
| pREΔwzxC | pRE112 containing a wild-type copy wzxC1 and its two flanks sequence; used to complement strain ΔwzxC1 | This study |

doi:10.1371/journal.pone.0106708.t001

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were observed with a Tecnai G2 spirit Biotwin microscope (FEI, Japan) operated at an accelerating voltage of 120 kV.

**NaCl tolerance measurements and auto-aggregation assay**

In order to determine differences in osmotolerance between the wild-type and mutant strains, cultures were grown in NB medium (LB medium without NaCl) with various salt concentrations (from 0% to 9%). Briefly, 50 µl of overnight culture in NB medium were inoculated into each 5-ml culture (NB medium containing diverse salt concentrations) in a 15-ml flask and incubated at 37°C shaking at 180 rpm for 24 h. The OD<sub>600</sub> was measured and photographs were taken by light microscopy (LM) at the same time.

The auto-aggregation assay was performed based on the method previously described by Shanks et al. [30] with slight modifications. Briefly, Salmonella cultures were statically grown in 5 ml LB medium at 37°C for 24 h in 16 × 150 mm test tubes. The upper 0.5 ml was carefully removed to measure its optical density (OD<sub>600</sub> (recorded as OD<sub>600 prevortex</sub>). The remaining culture in the test tube was then mixed by vortexing to re-suspend the aggregated cells, and 0.5 ml of the suspension was removed and its OD<sub>600</sub> was measured (recorded as OD<sub>600 postvortex</sub>). The "percent aggregation" was calculated using the formula: 100% × (OD<sub>600 postvortex</sub> - OD<sub>600 prevortex</sub>) / OD<sub>600 postvortex</sub>.

**RNA preparation and RT-qPCR analysis of gene expression**

Wild-type and mutant Salmonella cells were grown in LB or NB medium to an OD<sub>600</sub> around 1.2 for stationary phase samples. One milliliter of the cell culture was harvested by centrifuging at 12000 x g for 2 min. The cell pellet was re-suspended in 100 µl TE buffer containing 10 µg/ml of lysozyme (Roche Diagnostics, Penzburg, Germany), incubated at 37°C for 10 min, and the treated cells were once again harvested by centrifuging at 12000 x g for 1 min. The extraction of total RNA was performed with TRIzol reagent according to the manufacturer’s protocol (Invitrogen, Carlsbad, USA). DNase I treatment and reverse transcription (RT) were performed using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Japan).

Reverse transcription quantitative real-time PCR (RT-qPCR) assays were performed using an iQ Cycler (Bio-Rad, Watford, UK). The primers used are listed in Table 2 and were synthesized by Sangon Co. Ltd. (Shanghai, China). The reaction solution was as follows: ≤1 µg Power SYBR Green PCR Master Mix (2 x) (TaKaRa, Japan), 1 µM Forward Primer, 1 µM Reverse Primer, and 1–100 ng cDNA template, and nuclelease-free water to bring the solution to 25 µl. The amplification program was as follows: one cycle at 95°C for 4 min, 40 cycles at 95°C for 15 s, 58°C for 15 s and 68°C for 30 s. A melting-curve between 58°C and 95°C was analyzed to check the specificity of the amplification product after each PCR. The recA gene was used as a reference. All the samples, including no-template control, were analyzed in triplicate. The reaction efficiency (E) was calculated using the following equation: 

\[ E = \left[10^{\left(\frac{1}{C_{T}}\right)} - 1\right] \times 100. \]

Results were analyzed using the comparative critical threshold method (2^-ΔΔCT). Gene expression is generally regarded as up- or down-regulation as its relative expression (RE) level is increased or decreased by at least 2-fold, respectively, as previously described by Desroche et al. [31]. The RE for each gene was measured in triplicate. RE was significantly different when p-value was <0.05 using the T-test.

**Complementation of the wzx mutation**

To demonstrate that the wzx mutation alone was responsible for the observed phenotypes, a complemented strain was generated. Briefly, a 2778 bp DNA fragment containing an intact open reading frame (ORF) of wzx, including 593 bp sequence upstream of the translation start site and a 638 bp sequence downstream from the translation stop site, was amplified from the genomic DNA of S. Choleraesuis ATCC10708 using the primers wzx<sub>C1</sub>-for and wzx<sub>C1</sub>-rev. The PCR product was cloned into pMD<sub>18</sub>T (TaKaRa, Japan) to generate pMD<sub>18</sub>T<sub>Δwzx-C</sub>. The PCR product was conjugally transferred from E. coli SM10λpir into the S. Choleraesuis mutant strain Δwzx<sub>C1</sub>. The Δwzx<sub>C1</sub>-complemented strain ΔΔwzx<sub>C1-C1</sub> was then selected in two steps as described above. The resulting complemented strain, S. Choleraesuis ΔΔwzx<sub>C1-C1</sub>, was confirmed by PCR with primers wzx<sub>C1</sub>-for and wzx<sub>C1</sub>-rev, and DNA sequencing of the resulting PCR-product using the same primers.

**Results**

The SC<sub>2092</sub> gene encodes a putative flipase Wzx required for O<sub>7</sub>-polysaccharide biosynthesis

The S. Choleraesuis gene SC<sub>2092</sub> is unique to strains of serogroup C1. The gene is located among a number of genes (O-antigen gene cluster) whose products appear to be associated with O<sub>7</sub>-antigen biosynthesis. The deduced amino acid sequence of the protein encoded by gene SC<sub>2092</sub> has 12 possible transmembrane segments distributed throughout its length, which is the most characteristic feature common to all Wzx proteins that function in transporting the pre-formed O-antigen units to the outside of the cell. Although gene SC<sub>2092</sub> and its deduced amino acid sequence share little sequence similarity with the wzx/Wzx<sub>g</sub> genes/proteins of S. Typhimurium (0.8% and 23%, respectively) and E. coli O157:H7 (4% and 24%, respectively), the topology prediction profile of the putative Wzx ortholog (the SC<sub>2092</sub> gene product) from S. Choleraesuis was highly similar to Wzx proteins from S. Typhimurium and E. coli O157:H7 (Fig. S1). All of the programs predicted that the N and C termini of the three above-mentioned proteins were located in the cytoplasm. The number of TMs and the orientation of the intervening loops for the three Wzx proteins predicted by different algorithms gave similar results. Based on these results we propose that the SC<sub>2092</sub> is a wzx gene encoding an O<sub>7</sub>-specific flipase required for O-antigen in strains of Salmonella of serogroup C1.

To provide experimental evidence that this putative wzx gene plays a role in LPS biosynthesis, we constructed a marker-free wzx deletion mutant of S. Choleraesuis ATCC10708, and a complemented strain using a double cross strategy. The strain constructs were confirmed by PCR. The results of DNA sequencing for these PCR products further verified the correct deletion and complementation of the allelic exchange mutagenesis (data not shown).

LPSs of the wild-type, wzx<sub>C1</sub> mutant and the complemented strains were isolated and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A). The LPS from the wild-type and the complemented strain exhibited a ladder pattern that is characteristic of LPS with a different number of O-repeat units in the O-antigen, and the upper ladder bands represent this portion of LPS containing O-antigen. In contrast, in the LPS profile of the mutant, the upper bands were completely absent. However, the other lower bands representing the LPS core...
showed no apparent changes (data not shown), suggesting that LPS O-antigen synthesis was defective in the \textit{wzx} mutant strain. This result was confirmed by the slide agglutination test with somatic O7 antiserum (Fig. 1B). Agglutination was obtained in both wild-type and complemented strains; moreover, the positive reactions appeared within 5 to 10 s. However, the negative reactions associated with mutant persisted even when slides were observed for an additional 5 to 10 min. Based on these data, this putative \textit{wzx} gene is designated as \textit{wzxC1}, which encodes a putative flippase that is required for O7-polysaccharide export in strains of \textit{Salmonella} of serogroup C1.

The \textit{wzxC1} deletion resulted in conditional defects on both swimming and swarming motility

The swimming motility of the wild-type and \textit{ΔwzxC1} mutant strains were determined using a conventional soft agar test (LB containing 0.3\% agar). Twenty-four hours after inoculation, the wild-type strain had produced an area of growth that completely filled the plate, indicating a high degree of swimming motility. However, the growth of the \textit{ΔwzxC1} mutant strain had not spread far from the inoculation point, indicating that the strain had reduced motility relative to the wild-type strain (Fig. 2A). In other words, an in-frame deletion in \textit{wzx} in \textit{S. Choleraesuis} strain \textit{ΔwzxC1} significantly reduced swimming motility. A similar result was observed when swimming motility was tested on LB plates containing 0.6\% agar (Fig. 2C). Interestingly, both the swimming and swimming motility defects of the \textit{ΔwzxC1} mutant strain were rescued in the absence of NaCl (Figs. 2B and 2D). In addition, to test if the hydrophilic O-antigen provides a surfactant function during swelling, the mutant strain was inoculated on swarming medium with a bio-surfactant from \textit{B. subtilis}. The swarming motility of the mutant was recovered by the addition of bio-surfactant (Fig. 2C MT and Fig. 2E MT). The addition of bio-surfactant had little effect on swimming motility (data not shown).

The mutant had reduced osmotolerance to sodium chloride, and increased auto-aggregation

To determine whether the mutation of \textit{wzxC1} affected the morphological features of the mutant strain, transmission electron microscopy (TEM) images of intact wild-type and the mutant cells were collected (Fig. 3A–F). Relative to the wild-type cells, cells of the \textit{wzxC1} mutant formed multicellular aggregates (Figs. 3A and 3C). Nevertheless, the aggregation was alleviated when NaCl was removed from the medium (Figs. 3E and 3F). In addition, TEM of thin sections showed that the mutant strain had no remarkable difference with the wild-type strain in the morphology of bacterial cells and the integrity of cell wall (membrane) (Figs. 3G and 3H). However, unknown intracellular crystals (marked with arrow in Fig. 3H) around the inner membrane were present in most of the mutant cells.

The tolerance of the mutant and wild-type strains to NaCl, an osmotic agent, was evaluated, and results showed that the mutant

| Target mRNA | Gene product function | Sequences (5'–3') |
|-------------|-----------------------|-------------------|
| \textit{flgA} | flagellar basal body P-ring biosynthesis protein FlgA | F:CTGGCTTCAGCGACGAGGTG R:GCAGCAACGCCGACGACATAA |
| \textit{motA} | forms the ion channels that couple flagellar rotation to proton/sodium | F:CCCTGCTGTGGTTGTAAG R:GCTGCAGCTGCGTAC |
| \textit{motB} | motive force forms the ion channels that couple flagellar rotation to proton/sodium | F:CGCATAGCCGTCGTTAC R:GGGACCTGCTGCTGAT |
| \textit{cheA} | sensory histidine protein kinase, transduces signal between chemo- signal receptors and CheB | F:CTACTCATCATGTACGTCGTCAT R:GTTCGAGCGCTGTCGAT |
| \textit{cheB} | methyl esterase, response regulator for chemotaxis (cheA sensor) | F:GCATCTGGTGGCTACCCG T:GTTCGAGCGCTGTCGAT |
| \textit{recA} | DNA strand exchange and recombination protein | F:GATGAGGGCTGATGGTGCG R:GTTCGAGCGCTGTCGAT |

doi:10.1371/journal.pone.0106708.t002

\textbf{Figure 1.} The O7-antigen synthesis was blocked in the \textit{Δwzx} deletion mutant. A) The presence of O-antigen was determined by SDS-PAGE separation of LPS preparations followed by silver staining. Lane 1; wild-type \textit{S. Choleraesuis} strain ATCC10708; Lane 2, \textit{Δwzx} mutant strain; Lane 3, the \textit{Δwzx} complemented strain. B) Agglutination was examined with somatic O7 antiserum and pictures were taken within 5 min. Spot 1, wild-type \textit{S. Choleraesuis} strain ATCC10708; Spot 2, the \textit{Δwzx} mutant strain; Spot 3, the \textit{Δwzx} complemented strain. doi:10.1371/journal.pone.0106708.g001
was more susceptible to NaCl. It was observed that while the wild-type and the complemented strain were able to tolerate salt levels as high as 7%, the mutant was sensitive to salt concentrations above 4% (Fig. 4A). Also, cell aggregation, which did not occur in the wild-type or \( \Delta wzxC_1 \)-C complemented strain at NaCl concentrations as high as 7%, occurred in the \( \Delta wzxC_1 \) mutant at all salt concentrations tested (as low as 1% NaCl) (Fig. 4B). The auto-aggregation was tested with a culture medium containing 1% NaCl (Fig. 5A) and the “percent aggregation” was calculated using OD\(_{600}\) measurements from these cultures. While the wild-type showed 8.37% aggregation, the mutant demonstrated 59.62% aggregation (Fig. 5B). A swimming video recorded by light microscopy (LM) was consistent with conditional defects in motility and aggregation of the mutant strain (data not shown).

The \( WzxC_1 \) mutation showed a defect in flagellar gene expression

To distinguish whether the above-observed defect in motility was due to a deficiency in flagellar expression or a restriction in flagellar motion, we first examined flagellar formation by flagella silver staining (data not shown) and TEM observation of the wild-type and the \( \Delta wzxC_1 \) mutant cells. These TEM observations showed that the mutant strain appeared to exhibit reduced flagellation (Fig. 3D), while the wild-type strain had intact peritrichous flagella (Fig. 3B). In addition, the aggregate of mutant cells appears even more diminished of flagella (Fig. 3C).

To further investigate the nature of the observed decrease in motility and flagellation, the expression levels of five genes (\( flgA, motA, motB, cheA \) and \( cheB \)) required for flagella-mediated swimming motility and chemotaxis were determined by RT-qPCR for the wild-type and the \( \Delta wzxC_1 \) mutant cells grown in LB or NB medium (Fig. 6). Compared to the wild-type, the \( flgA \) gene expression was down-regulated by 5.3-fold in the mutant when the cultures were grown in LB (1% NaCl). In addition, the expression of two chemotaxis genes tested was effected differently (\( cheA \) decreased and \( cheB \) increased) in the \( \Delta wzxC_1 \) mutant relative to the wild-type when they were grown in the medium containing NaCl. However, the wild-type and mutant strains examined under a light microscope did not show detectable chemotaxis defect (biased running or tumbling behavior) (data not shown). Nevertheless, the expression of \( motA \) and \( motB \) did not change significantly, so it is hard to conclude that the motility deficiency in the mutant had relationship with its flagella rotation (the function of the two \( mot \) genes, Table 2). In a NaCl-free medium the five genes showed a modest, but insignificant, increase in expression in the \( \Delta wzxC_1 \) mutant strain compared to the wild-type strain. This indicates that the deficiency in \( flgA \) expression in the presence of NaCl may contribute to the observed salt-dependent decrease of motility.

**Discussion**

The \( wzxC_1 \) gene, which is located in the O-antigen gene cluster, was identified in our previous work as being specific for *Salmonella* serogroup C1 [18]. In the present study, we provide experimental evidence to reveal the relationship between this gene and the O-polysaccharide biosynthesis of serogroup C1. We also found that...
the ΔwzxC1 mutant displayed NaCl-dependent motility deficiency, decreased flagellar expression and increased auto-aggregation.

Our results provide several lines of evidence that wzxC1 encodes a putative flippase required for O-polysaccharide biosynthesis. Since structural knowledge of TM proteins is difficult to attain experimentally, the in silico prediction of membrane protein topology, such as the positions and orientation of the membrane-spanning regions, serves as a facile means to quickly obtain fundamental structural data of TM proteins. TOPCONS is a freely available web server for consensus prediction of membrane protein topology, and the topological prediction algorithms used in TOPCONS are sufficient in almost 70% of instances to correctly predict the overall number of putative TMs, as well as the orientation of the protein in the IM [32]. Wzx proteins share very little amino acid sequence similarity, and their genes also have low nucleotide sequence homology. A high level of variation in amino acid sequence identity of the flippases is observed between species [33]. In this study, mutation of wzxC1 resulted in negative reactions with the O7 slide agglutination test, while, positive reactions appeared in the wild-type and the complemented mutant strain (Fig. 1A), an observation that is consistent with the results described by Liu et al. [35] in S. Typhimurium P9351 and Burrows and Lam [36] in Pseudomonas aeruginosa. Since O7 is present in all strains of the C1 serogroup, an O2 antibody agglutination test is the most commonly used method for serological characterization for identification of Salmonella serogroups C1. In this study, mutation of wzxC1 resulted in negative reactions with the O2 slide agglutination test, while, positive reactions appeared in the wild-type and the complemented mutant strain (Fig. 1B).

Our results demonstrated that wzxC1 encodes a flippase required for synthesis of the O7 antigen common to strains within the C1 serogroup. Therefore, this Salmonella serogroup-specific gene was shown to be involved in O-antigen biosynthesis. In this study, loss of O-antigen flippase in S. Choleraesuis resulted in significant defects in both swimming and swarming motilities (Figs. 2A and 2C). This result differs from the previous report in which deletion of the S. Typhimurium wzxS2 had little effect on swimming motility [15]. In addition, the mutation of wzxC1 resulted in an aggregative phenotype (Fig. 3C and Fig. 4B).

This propensity was also observed in LPS synthesis defective mutants of Citrobacter freundii swarming on agar surfaces [37] and Stenotrophomonas maltophilia swimming in TS broth [38]. Moreover, it is speculated from the TEM observations that deletion of wzxC1 resulted in an observable reduction in flagellation, with most of the cells aggregating into bunches of three or more cells nearly devoid of flagella (Fig. 3D) with a smaller number of individual cells that appear only partially flagellated (Fig. 3F). Also, our results with the RT-qPCR assay

Figure 3. Morphological features in the wild-type and ΔwzxC1 deletion mutant strains revealed by TEM. TEM images of intact cells of wild-type S. Choleraesuis strain ATCC10708 (A and B) and the ΔwzxC1 deletion strain grown in medium with NaCl (C and D) or in NaCl-free medium (E and F). TEM thin sections of wild-type S. Choleraesuis strain ATCC10708 (G) and ΔwzxC1 deletion strain (H). The unknown intracellular crystals in the mutant cells were marked with the arrows.

doi:10.1371/journal.pone.0106708.g003
Figure 4. The effect of NaCl concentration on final culture density and cell morphology. The wild-type (WT), Δ*wzx*C1 mutant (MT) and complemented mutant (MT-C) strains were grown in various concentrations of NaCl for 24 h. (A) The final OD600 for cultures grown in different NaCl concentrations (0%–9%) was measured and (B) Morphologies at selected NaCl concentrations were recorded by light microscopy. CK: NB medium as a negative control. The data in (A) are from triplicate samples and images in (B) are representative of the three replicate samples. White bar = 10 μm. doi:10.1371/journal.pone.0106708.g004

Figure 5. Auto-aggregation of the wild-type and the Δ*wzx*C1 mutant. Visual aggregation (A) on and percent aggregation (B) of wild-type (WT) and Δ*wzx*C1 mutant (MT) cultures grown statically for 24 h at 37°C. doi:10.1371/journal.pone.0106708.g005
compared with those of the wild-type are shown. The agar [15], thus the addition of a bio-surfactant can restore normally acts to increase "wettability" by extracting water from aggregation and motility.

pathway/factors involved in flagellation balance between auto- and motility are antagonistic phenotypes. It is likely that regulatory our results together with these reports show that auto-aggregation interfere with motility in the presence of low levels of flagella [41]. The auto-aggregation mediated by aggregation protein Ag43 can the mutant strain was rescued by the addition of bio-surfactant (Fig. 2E), which indicates that O-antigen also acted as a bio-surfactant and was used as an internal control. Errol bars indicate standard deviations. Stars indicate that the fold-changes in gene expression were significantly different (p-value, 0.05).

doit:10.1371/journal.pone.0106708.g006 (Fig. 6) demonstrated that reduced flagellar expression of genes involved in synthesis of flagella contributed to this swimming defect. Previous studies with Campylobacter jejuni have correlated the expression of flagella with auto-aggregation [39,40]. In E. coli, the auto-aggregation mediated by aggregation protein Ag43 can interfere with motility in the presence of low levels of flagella [41]. Our results together with these reports show that auto-aggregation and motility are antagonistic phenotypes. It is likely that regulatory pathway/factors involved in flagellation balance between auto-aggregation and motility.

The loss of surface O-antigen prevented swarming because it normally acts to increase “wettability” by extracting water from the agar [15], thus the addition of a bio-surfactant can restore swarming motility. In this study, the swimming defect in the ΔwzxC1 mutant strain was rescued by the addition of bio-surfactant (Fig. 2E), which indicates that O-antigen also acted as a bio-surfactant, aiding in motility, in S. Choleraesuis. Flagellar upregulation was regarded as a marker for swarmer cell differentiation [17]. FlhDC is a well-characterized flagellar master regulator of S. enterica, and the up-regulation of flhDC expression is required for the high-level expression of flagella [42]. Nevertheless, neither the up-regulation of flhDC expression nor the down-regulation of resB (the response regulator of flhDC) was observed in this study (data not shown) during swarming, which was consistent with the report from Partridge and Harshey [43] that Salmonella cells do not upregulate flagellar gene expression to increase flagellar numbers during swarming.

In addition, we found that the removal of NaCl in the medium restored both swimming and swarming motilities of the ΔwzxC1 defective mutant (Figs. 2B and 2D). Moreover, auto-aggregation (Figs. 3F and 4B) and reduced flagellar gene expression (Fig. 6) were also alleviated when ΔwzxC1 mutant cells were grown in the absence of NaCl. These recovery phenomena might be related with the alteration in osmotolerance to NaCl of the ΔwzxC1 mutant (Fig. 4A). Wzx flipases belong to the polysaccharide transporter (PST) family of proteins, which is classified as one of four members of the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily. Recently, it has been observed that Wzx forms cationic lumen channel that mediates anionic O-antigen subunit translocation in P. aeruginosa [44,45]. Another member of the MOP exporter superfamily, multidrug and toxin extrusion (MATE) proteins, have been extensively found to utilize Na+ as the coupling ion during extrusion of hydrophobic compounds from the cytoplasmic side via antiport [46]. Our recovery results following removal of the NaCl in the medium showed that this cationic lumen channel formed by Wzx is may be indicative of Na+-dependent transport. Furthermore, it has been reported that changes in external osmolality (achieved by varying NaCl concentration in growth media) regulate flagellar expression by the EnvZ pathway in E. coli [47] and the RcsB-RcsC signaling pathway in S. Typhi [48]. Therefore, further research is needed to reveal why a deletion of Wzx flipase may mediate a NaCl-dependent motility deficiency, increased cell-cell aggregation, and changes in flagellation in S. Choleraesuis.

In conclusion, a Salmonella serogroup C1-specific gene, designated as ΔwzxC1, encodes a putative flipase required for O2-polysaccharide biosynthesis. Auto-aggregation and a defect in flagellar expression were responsible for the reduced swimming motility in a ΔwzxC1 mutant strain. The O-antigens in S. Choleraesuis may act as a surfactant to regulate swarming motility. Motility deficiency, a cell aggregation phenomenon, and decreases in cell flagellation could be relieved by the removal of NaCl in the medium, which suggests that this serogroup C1-specific gene ΔwzxC1 is involved in salt tolerance and the Wzx flipase probably displays Na+-dependent antiport activity.

Supporting Information
Figure S1 Topology prediction profile of Wzx proteins. The topology prediction profile of the putative Wzx open reading frames from S. Choleraesuis (A), S. Typhimurium (B), and E. coli O157:H7 (C). The consensus prediction of these membrane protein topologies were generated by TOPCONS (http://topcons.chr.su.se/).

(TIF)

Author Contributions
Conceived and designed the experiments: XZ CS XS. Performed the experiments: XZ BL. Analyzed the data: XZ BL. Contributed reagents/materials/analysis tools: XZ BL. Contributed to the writing of the manuscript: XZ CS.

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