A Novel LysR Family Factor STM0859 is Associated with The Responses of Salmonella Typhimurium to Environmental Stress and Biofilm Formation

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Submitted 27 August 2021, accepted 4 November 2021, published online 20 December 2021

A b s t r a c t

Salmonella enterica subsp. enterica serovar Typhimurium (ST) is an intracellularly parasitic bacterium. This zoonotic pathogen causes food poisoning and thus imposes a severe threat to food safety. Here, to understand the regulatory roles of the novel transcription factor STM0859 on the response of ST to environmental stress and biofilm formation, the STM0859 gene-deficient strain and the complementation strain ΔSTM0859/STM0859 were generated, respectively. Then, its capacity of responding to environmental stresses and biofilm (BF) formation ability under different stresses, including acid, alkali, high salt, cholate, and oxidative stresses was tested. We further analyzed the interaction between the STM0859 protein and the promoter of the acid stress response-related gene rcsB by performing an electrophoresis mobility shift assay (EMSA). The results showed that acid resistance and BF formation capacities of ST-ΔSTM0859 strain were significantly weaker, as compared with those of Salmonella Typhimurium SL1344 (ST-SL1344) wild strain (p<0.01). Quantitative qRT-PCR analysis showed that the expression levels of acid stress and BF formation-related genes, rcsB and rpoS, of ST-ΔSTM0859 strain were significantly reduced at the transcription levels, while the transcription levels of these genes were fully restored in complementation strain ST-ΔSTM0859/STM0859. The results of EMSA showed that STM0859 was capable of binding the promoter DNA fragments of the rcsB gene, suggesting that STM0859 can promote the transcription of the rcsB gene through interaction with its promoter, thereby exerting an indirectly regulatory role on the adaptive responses to acid stress and BF formation of ST. This study provided new insights into the regulatory mechanisms of the LysR family factors on the tolerances of ST under adverse environmental stresses.

K e y w o r d s: Salmonella Typhimurium, biofilm, environmental stress, STM0859 gene

Introduction

Salmonella is a gram-negative intracellular pathogenic bacterium (Richardson et al. 2018), widely present in contaminated animal-derived foods or drinking water. This bacterium frequently infects humans due to ingestion of contaminated foods (Jajere 2019). The infection of this zoonotic pathogen will result in various clinical symptoms, which are characterized by diarrhea, abdominal pain, and vomiting. Especially in immunocompromised individuals (e.g., infants, children, or the elderly), Salmonella can cause serious syndromes and
even death, leading to serious global food safety and public health issues (Zishiri et al. 2016).

As a representative strain of Salmonella enterica subsp. enterica, Salmonella Typhimurium (ST) can survive under unfavorable environmental conditions, such as dryness, extreme temperatures, antibiotics, and disinfectants. What is more, it can form a biofilm (BF) (Borges et al. 2018), which enables ST to acquire the capability of long-term survival in the contaminated eggs, meat, milk, and other animal-derived food sources, causing frequent occurrences of human food-borne salmonellosis (Lamas et al. 2018; Rohren et al. 2019). Currently available studies have shown that ST needs several transcriptional factors to regulate the expression of specific genes to adapt to the complex and changeable environments to withstand various adverse environmental stresses (Suar and Ryan 2015).

Among the transcriptional factors found in prokaryotes, LysR is an important transcription factor family, which was proved to be related to bacterial quorum sensing, response to environmental stress, biofilm (BF) formation, and regulation of virulence (Gebhardt et al. 2020). Through bioinformatics analyses, we have revealed that STM0859 protein is a novel member of LysR transcription factor family of ST. However, so far, the regulatory roles of STM0859 in ST response to environmental stress and BF formation remain unclear.

The main purpose of this study was to clarify the regulatory role of a novel transcription factor STM0859 of the LysR family in response to environmental stress and BF formation. Using λ-Red homologous recombination technology, we knocked out the STM0859 gene and carried out the detection of responses to environmental stress, BF formation capability, and the expression levels of related genes of STM0859 gene-deficient strains, aiming to provide new insights into the regulatory mechanism of STM0859 for the response to environmental stress in ST.

Experimental

Materials and Methods

Plasmids, strains and growth conditions. The plasmids pKD3, pKD46, pCP20, and pBR322 (The Key Laboratory of Preventive Veterinary, Shihezi University) were employed to construct recombinant vectors. Salmonella Typhimurium SL1344 was used as wild-type strain (The Key Laboratory of Preventive Veterinary, Shihezi University) to generate deletion and complementation strains of ST, while Escherichia coli TOP10 strain was used for all plasmid construction. These strains were routinely cultured in Luria-Bertani (LB) broth at 37°C with vigorous shaking or on LB agar plates containing 1.5% (WT/Vol) agar.

Design of primers. Based on ST-STM0859 gene sequence (Accession number: FQ312003.1) deposited in GenBank, by using the Primer 5.0 software, we designed and constructed the STM0859 gene-deficient strain and complementation strain. The related specific primers are listed in Table I.

Analysis of molecular characterization of STM0859 protein. The amino acid sequence of STM0859 protein encoded by STM0859 gene was deduced, and then the molecular characterization of STM0859 was analyzed using online software such as Signal P 5.0 (http://www.cbs.dtu.dk/services/SignalP/), Blast, TMHMM 2.0, Prosite scan and Swiss-model software (http://www.expasy.org/), respectively.

Generation of ST-ΔSTM0859 gene deletion mutant and complementation strains. Briefly, ST-SL1344 was cultured with LB (Aoboxing, China) broth, and then pKD46 plasmid was introduced into ST-SL1344 competent cells via electroproporation. The positive transformants were screened out on LB culture containing Ampicillin (Amp) (100 mg/ml) (Sigma, Germany). The positive bacterial cells were verified by PCR with primer pair R1/R2. The positive bacteria were used as the homogenous recombinant bacterial host. With pKD3 as the template, the target fragment being used to knock out the STM0859 gene was amplified by PCR with primer pair R3/R4 targeting the left and the right arms of the STM0859 gene, which were homogeneous to right and left ends of the STM0859 gene and contains 50 bp in length. The amplified target fragment was recovered with DNA gel recovery kits (Omega, USA) and then introduced into the host bacterial SL1344-pKD46 competent cells via electroporation. The recombinant bacterial ST-ΔSTM0859 cat was screened on LB plates containing Amp and chloramphenicol (Cm) (Sigma, Germany) (100 mg/ml Amp and 34 mg/ml Cm), verified by PCR, and sequenced. The ST-ΔSTM0859 cat competent cells that were used to eliminate the pKD46 plasmid were prepared. After that, the pCP20 plasmid was introduced into these cells via electroporation. The transformed bacterial solution was sprayed evenly on a LB plate-containing Amp. The plate was placed in incubator at 30°C and cultured overnight. The positive clones were verified by PCR. The ST-ΔSTM0859 gene-deficient strain was obtained and verified by sequencing. For the construction of the ST-ΔSTM0859/STM0859, the full-length STM0859 gene with flank sequences was amplified using primers STM0859-F and STM0859-R and then cloned into pBR322 vector to generate pBR322-STM0859. Then, pBR322-STM0859 was transferred into the ST-ΔSTM0859 competent cells to obtain the complementation strain ST-ΔSTM0859/STM0859.

Effects of STM0859 gene deficiency on ST responses to environmental stresses. The tolerance capacities of ST-SL1344, ST-ΔSTM0859 and ST-ΔSTM0859/STM0859...
STM0859 in environmental stress were determined by reference to the method reported in the literature (Peng et al. 2016). Briefly, the bacterial solutions were transferred in 1:100 ratio into 50 ml of BHI liquid medium with pH values of 4 and 10, 4% NaCl, 0.3% cholate, and 1 mM H$_2$O$_2$ and incubated for 2 h, respectively. Then OD$_{600\text{ nm}}$ values of cultured bacterial solutions were measured hourly, and growth curves were plotted to analyze the effects of STM0859 gene deficiency on ST’s capacity to respond to environmental stresses. At the same time, the OD$_{600\text{ nm}}$ values of the overnight cultured (LB broth) bacterial solutions were adjusted to 1.0 and then sprayed at 0.3% semi-solid LB medium with pH values of 4, and 7, respectively, and cultured in a stationary culture system for 16 h at 30°C, each experiment was repeated three times. And then, the diameters of the bacterial colonies in the Petri dish were measured with a ruler, and its motion ability was analyzed under different pH conditions.

Effects of STM0859 gene deficiency on biofilm formation of ST. The BF was measured referencing the method described in the literature (Peng et al. 2016). Briefly, ST-SL1344, ST-ΔSTM0859, and ST-ΔSTM0859/STM0859 were cultured in LB broth overnight and inoculated 200 µl into the 96-well microplate and cultured for 30 h and 48 h, respectively. Each sample was divided into two groups. For each group, 9 parallel repetitions were set. The cells were stained with 1.0% crystal violet solution. After being dried at room temperature, the microplate was placed under an inverted microscope and visualized. 95% ethanol was added to each well and decolored for 30 min. The OD$_{595\text{ nm}}$ values were measured with a full-wave length microplate reader (Multiskan GO, USA).

| Primer name | Primer sequence (5’→3’) | Target gene/DNA fragment | Product size (bp) |
|-------------|-------------------------|--------------------------|------------------|
| R1          | TCTATGCAACAGGATGAAGCC   | pKD46                    | 1,046            |
| R2          | GTATTCTCCGCGCTTCTTG    | pKD3                     | 1,198            |
| R3          | ACTACGGCCTGAAGTGCCAACCCAAATACAGGGATGTAACGCTATTAGAGCCCTCTCAAAGCAAT | pKD3 | 1,198 |
| R4          | TCTATCAAAGGCAAAGGAGAC   | pCP20                    | 2,022            |
| R5          | CACCAAAAAACTCCTGTAAGAC  | pCP20                    | 2,022            |
| R6          | GACCTATACGGGAGATG        | ST-ΔSTM0859cat/ST-SL1344 | 220/1,146 |
| R7          | ATACGTTAATTGAGCCACA     | STM0859                  | 905              |
| R8          | GGAATTCTCGACTTGTATATATTAAGATTTA | STM0859 | 905 |
| R9          | CCTCGAGTCATCGACTGCTGCAGGTGAG | rcsB promoter | 102 |
| R10         | TCTTATTTATGTTAACCCAGCC  | rcsB promoter            | 102              |
| T6s RNA F   | GCCGTAATACGGGAGGTG       | 16s RNA                  | 167              |
| T6s RNA R   | CACCGCTCACACTGGGAAT      | 16s RNA                  | 167              |
| rcsB F      | AGCGTATTTCTCCGAGC        | rcsB                     | 193              |
| rcsB R      | TCCAGCAGCAGGAAGAC        | rcsB                     | 193              |
| rpoS F      | GGTGAGTTGGCGACAGAC       | rpoS                     | 132              |
| rpoS R      | CCGTCAGTTGATCCAGCC       | rpoS                     | 132              |
| Fur F       | TTTCTCGCAATTCAAGCG       | Fur                      | 158              |
| Fur R       | TGGGTAGAAGAAATCCGTC      | Fur                      | 158              |

Effects of STM0859 gene deficiency on expression of environmental stress related genes. Using the 16sRNA gene as an internal reference control, we measured the expression levels of genes rcsB, fur and rpoS related to acid stress and biofilm formation by real-time quantitative RT-PCR (qRT-PCR) with those primers (Table I) at the transcription levels. Briefly, ST-SL1344, ST-ΔSTM0859, and ST-ΔSTM0859/STM0859 were cultured in the LB medium to OD$_{600\text{ nm}}$ value of about 1.0, and the total RNA was extracted according to Trizol (Invitrogen, USA) instructions. Reverse transcription was performed with the AMV reverse transcription kit (TaKaRa, Japan) according to the operation procedure provided. Then, the qRT-PCR assay was carried out with the LightCycler 480 instrument (Roche, Switzerland) according to the SYBR Premix Ex TaqTM kit’s operation procedure (TaKaRa, Japan) with three repetitions per sample. The relative transcription level was calculated according to the method of $2^{-\Delta\Delta CT}$ for statistical analysis (Livak and Schmittgen 2001).
Expression and purification of recombinant protein STM0859. Recombinant protein STM0859 was prepared using a prokaryotic expression system. In brief, the STM0859 gene was cloned into pMD19-T (TaKaRa, Japan) to generate recombinant plasmid pT-STM0859. Then, pT-STM0859 and pET-32a (Invitrogen, USA) were simultaneously double digested with EcoRI and XhoI (TaKaRa, Japan). The digested products were separated via agarose gel electrophoresis. After gel electrophoresis, the target fragments and vectors were recovered and ligated with T4 ligase (TaKaRa, Japan) to generate pET-STM0859, which was correctly identified by PCR and restriction enzyme digestion and transformed into BL21 (DE3) strain of E. coli (Invitrogen, USA). After being induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma, Germany) for 8 h, the expressed protein was analyzed by SDS-PAGE and western blot. Then, the recombinant protein was purified according to the Ni-NTA protein purification system (Invitrogen, USA).

Analysis of the interaction between STM0859 and the promoter of rcsB gene. The interaction between the STM0859 protein and the rcsB promoter was analyzed using electrophoresis mobility shift assay (EMSA). Briefly, the rcsB promoter fragment was obtained by using the PCR method. After that, the rcsB promoter DNA fragment and STM0859 protein were co-incubated at room temperature for 30 min in the gel-shift buffer (Beyotime, China), with bovine serum protein (BSA) (Sigma, Germany) as the control protein. The rcsB promoter DNA fragment-STM0859 protein mixture was subjected to electrophoresis with 8% non-polyacrylamide gel, and the migration of rcsB-promoter DNA complex was observed in the gel imaging system after being stained.

Statistical analysis of data. The data were analyzed by SPSS 18 software (IBM SPSS, USA). The continuous variables were compared using the analysis of variance (ANOVA), while the Chi-square test was used to analyze categorical variables. p-Value less than 0.05 was considered significantly different, while p value less than 0.01 was considered highly significantly different.

Results

As shown in Fig. 1, STM0859 protein had a characteristic helix-turn-helix (HTH) DNA-binding and LysR substrate-binding domains (Fig. 1A). Besides, STM0859 protein was composed of α-helices, β-turns, and random coils, which formed a cage-like shape in 3D structure (Fig. 1B).

PCR showed that the homogeneous recombination strain SL1344-pKD46 was successfully constructed (Fig. S1A). The target fragment was amplified with R3/R4 primer and transferred into SL1344-pKD46 to
A novel LysR family factor STM0859 of ST can generate ST-∆STM0859 strain (Fig. S1B). PCR verification of ST-∆STM0859 strain carrying pCP20 plasmid was performed with the primer pair R7/R8 (Fig. S2A). The fragments of 1,146 bp and 220 bp were amplified from ST-SL1344 and ST-∆STM0859 strains respectively (Fig. S2B). The constructed ST-∆STM0859 gene-deficient strain was verified by DNA sequencing, and the complementation strain ST-∆STM0859/STM0859 was confirmed by PCR (Fig. S3).

The growth of ST-SL1344, ST-∆STM0859 and ST-∆STM0859/STM0859 were consistent under 37°C culture condition. There was no significant difference in growth rate between the three strains in the stress environment of 4% NaCl, 1 mM H₂O₂, 0.3% cholate, and pH 10 (Fig. 2), indicating that the deficiency of STM0859 gene did not affect ST growth under the stressful environmental conditions, such as alkaline, high salt, oxidation and ethanol stresses. However, as
compared with that of ST-SL1344, the growth rate of ST-\(\Delta STM0859\) in the acidic stress environment of pH 4 (Fig. 2B), and the movement ability (Fig. S4) of the semi-solid culture base were significantly reduced (\(p<0.01\), Fig. 3), indicating that the STM0859 gene deficiency can lead to a decrease in ST’s acid resistance.

BF detection indicated that ST-SL1344, ST-\(\Delta STM0859\), and ST-\(\Delta STM0859/STM0859\) strains could form BF at 30 h and 48 h. However, the BF growth ability of ST-\(\Delta STM0859\) was significantly declined than that of ST-SL1344 and ST-\(\Delta STM0859/STM0859\) when observed under the inverted microscope (LEICA, Germany) (Fig. 4A). There was a significant difference in BF formation between ST-\(\Delta STM0859\) and ST-SL1344 (\(p<0.01\), Fig. 4B), indicating that STM0859 can promote BF formation.

The transcription levels of the genes rcsB and rpoS in ST-\(\Delta STM0859\) were significantly reduced when compared with those of ST-SL1344, while the transcription levels of these genes mentioned above were restored in ST-\(\Delta STM0859/STM0859\) (Fig. 5), indicating that the STM0859 gene deficiency reduced the expression levels of genes (rcsB and rpoS) related acid stress and BF formation in ST.

The pET-STM0859 recombinant vector was successfully constructed by double enzymatic digestion.
A novel LysR family factor STM0859 of ST

with EcoRI and Xho I (Fig. S5). After being induced by IPTG, SDS-PAGE and Western blot confirmed that the recombination protein STM0859 with a molecular weight of 48.6 kDa was obtained (Fig. S6). The results of the gel retardation assay (EMSA) showed that the DNA fragment of the rcsB gene promoter (Fig. 6A and 6B) displayed apparent band retardation after being bound by STM0859 protein (Fig. 6C). In contrast, the migration of the DNA fragment of the rcsB gene promoter with the unrelated protein BSA (control group) was not retarded (Fig. 6D), indicating that the STM0859 protein could specifically bind with the promoter of rcsB gene.

Discussions

As an important foodborne pathogen, ST can infect humans through animal food and cause poisoning, posing a serious threat to food safety and public health. Moreover, ST can survive in stressful environments such as low temperature, hyperosmolarity, and acidity, and form biofilms under certain conditions to resist unfavorable internal and external environments (Kim et al. 2013; Ramachandran et al. 2016). It has been proven that many factors are associated with the responses of Salmonella Typhimurium to environmental stress and biofilm formation (Eran et al. 2020). Available studies have confirmed that the transcription factor is an essential regulatory molecule in ST, regulating virulence and environmental stress-related gene expression (Song et al. 2020). Among them, the members of LysR family are the transcription factors widely found in prokaryotes (Yang et al. 2020), playing important regulatory roles in the life activities of bacteria. As a LysR transcription factor family member, ST-STM0859 contains a DNA binding domain and

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Fig. 5. Determination of the relative transcriptional levels of rpoS, rcsB and fur gene; ns – indicates not significant, * – p < 0.05, while ** – p < 0.01.

Fig. 6. Analysis of the interaction between STM0859 and the promoter sequence of rcsB gene using EMSA; A) the relevant features and localization in genomic DNA of ST, B) the promoter sequence of rcsB gene used in this study, C) interaction between STM0859 and the promoter of rcsB gene, D) interaction between BSA and the promoter of rcsB gene (negative control).
an HTH-18 domain consisting of a helix-turn-helix in molecular structure, similar to that of E. coli and Acinetobacter. Moreover, sequence analysis of the STM0859 gene showed that this gene is highly conservative among ST strains (data not shown).

It has been shown that there are significant differences in the expression levels of LysR family proteins in different growth environments, suggesting that they are involved in the regulation of the bacterial responses to environmental stresses (Zhang et al. 2019). Srinivasan et al. (2013) revealed that oxyKRP was involved in regulating the response to oxidative stresses, drug resistance, and virulence of Klebsiella pneumoniae. Frigel et al. (2019) confirmed that ST LysR transcription factors OxyR, SpvR, LeuO, CysB were involved in the regulation of oxidative stress and toxicity. Fu et al. (2019) revealed that four LysR-type transcriptional regulator family proteins (LTTRs) were related to antibiotic resistance in Aeromonas hydrophila. Herein, we confirmed that the acid stress tolerance and BF formation ability were significantly reduced after deleting the ST STM0859 gene, suggesting that STM0859 was involved in regulating acid stress tolerance and BF formation of ST.

The current researches have found that Salmonella has evolved a complex acid-resistant mechanism involving the regulation of transcription factors and the synthesis of acid shock proteins in the acid stress environments (Lang et al. 2020). When ST infects host cells, the first pressure is the host's stomach acid (Lee and Kim 2017). Therefore, sensing and responding to pH changes is critical to the survival of ST. It has been proven that fur, rcsB and rpoS play important roles in modulating the adaptability of Salmonella Typhimurium to environmental stresses such as low temperature, hyperosmolarity or acidity, and biofilm formation (Knudsen et al. 2014; Leclerc et al. 2017). Here, we confirmed that the transcription levels of genes rcsB were significantly reduced in the STM0859 gene deficiency strain.

Based on the analysis of the transcription levels of those genes related to acid response and biofilm formation using qRT-PCR, we further explored the interaction between the STM0859 protein and the promoter DNA fragment of rcsB gene whose expression was significantly reduced. It was confirmed that the STM0859 protein could modulate the mRNA level of the rcsB gene by binding with its promoter. Given that rcsB plays an important role in the adaptive response to acid stress (Lehti et al. 2012); thus, it is reasonable to speculate that the STM0859 protein can indirectly regulate the response to acid stress and BF formation through the interaction with the promoter of the rcsB gene.

However, the acid stress response is a highly complex process in Salmonella, which is involved acid resistance systems and a complicated regulation network (Kenney 2019). The mechanism insights into the regulation of acid stress response at the molecular level will contribute to developing novel antibiotics to deal with Salmonella Typhimurium infection (Zhao and Houry 2010).

Taken together, this study confirmed that the novel LysR family factor STM0859 was involved in the acid stress tolerance and BF formation of ST. Moreover, the STM0859 could bind the promoter of the rcsB gene and alter its expression, thus indirectly modulating the response to acid stress and BF formation of ST.

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**Ethical statement**

The experiments were carried out in accordance with the guidelines issued by the Ethical Committee of Shihezi University.

**Acknowledgments**

The authors thank the field staff for providing the materials for this study.

**Funding**

This work was supported by a grant from the National Key Research and Development Program (No. 2016YFD0500900), the Grant from Youth Science and Technology Innovation Leader of Xinjiang Production and Construction Corps (No. 2016BC01), the Key Scientific and Technological Projects in Agriculture of Xinjiang Production and Construction Corps (No. 2019GC026), and Natural Science Foundation of Xinjiang Uygur Autonomous Region (No.: 2021D101A40).

**Conflict of interest**

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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Supplementary materials are available on the journal’s website.