Study on the Promotion of Bacterial Biofilm Formation by a \textit{Salmonella} Conjugative Plasmid and the Underlying Mechanism

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Abstract

To investigate the effect of the pR\textsubscript{ST98} plasmid, originally isolated from \textit{Salmonella enterica} serovar Typhi (\textit{S. Typhi}), on biofilm (BF) formation, we carried out \textit{in vitro} experiments using \textit{S. Typhi}, \textit{Salmonella enterica} serovar Typhimurium (\textit{S. Typhimurium}) and \textit{Escherichia coli} (\textit{E. coli}). We further explored the effects of pR\textsubscript{ST98} \textit{in vivo} by establishing two animal models, a tumor-bearing mouse model and a mouse urethral catheter model. Moreover, we examined the relationship between the quorum-sensing (QS) system and pR\textsubscript{ST98}-mediated BF formation. These studies showed that pR\textsubscript{ST98} enhanced BF formation in different bacteria \textit{in vitro}. In both animal models, pR\textsubscript{ST98} promoted BF formation and caused more severe pathological changes. It was previously reported that \textit{Salmonella} senses exogenous N-acylhomoserine lactones (AHLs) through the regulatory protein SdiA and regulates the expression of genes including the virulence gene rck, which is located on the virulence plasmid of some serotypes of \textit{Salmonella}. In this study, we confirmed the locus of the rck gene on pR\textsubscript{ST98} and found that AHLs increased rck expression in pR\textsubscript{ST98}-carrying strains, thereby enhancing bacterial adherence, serum resistance and bacterial BF formation. In conclusion, the \textit{Salmonella} conjugative plasmid pR\textsubscript{ST98} promotes bacterial BF formation both \textit{in vitro} and \textit{in vivo}, and the mechanism may relate to the AHL-SdiA-Rck signaling pathway.

Introduction

\textit{Salmonella}, a facultative anaerobic bacterium that has a broad range of hosts including humans, farm animals and plants, causes serious infection and thousands of deaths each year, posing a significant threat to humans.

A large outbreak of \textit{Salmonella enterica} serovar Typhi (\textit{S. Typhi}) infection occurred in the 1980s. Five hundred ninety-one strains were isolated from the blood of patients who had acute and severe clinical symptoms. It was shown that more than 80% of isolates were multi-drug resistant, which was attributed to a large plasmid (R plasmid) with a size of 139 kb, designated as p\textsubscript{RST98}, belonging to the Inc\textsubscript{C} group (Fig. 1) [1]. Our previous study showed that p\textsubscript{RST98} is a chimerical plasmid carrying genes responsible for drug resistance and virulence. The strains harboring p\textsubscript{RST98} were found resistant to trimethoprim, streptomycin, kanamycin, sulfonamide, neomycin, gentamicin, chloramphenicol, tetracycline, carbencilin, ampicillin, and cephalosporin. It was confirmed in our previous studies that p\textsubscript{RST98} contains a DNA sequence homologous to the \textit{Salmonella} plasmid virulence gene (\textit{sfr}), which was found in all pathogenic \textit{Salmonella} spp. except \textit{S. Typhi}. The sequence of the ORF (open reading frame) of \textit{sfr} and \textit{sfrB} on p\textsubscript{RST98} shared more than 99% similarity with that of \textit{sfr} and \textit{sfrB} on the virulence plasmid in \textit{Salmonella enterica} serovar Typhimurium (\textit{S. Typhimurium}) [2], indicating the presence and distribution of \textit{sfr} in \textit{Salmonella}. Later studies demonstrated that p\textsubscript{RST98} increased the serum resistance of \textit{Salmonella}, promoted \textit{S. Typhi} survival in macrophages \textit{in vitro} and decreased the LD\textsubscript{50} (50% lethal dose) values of \textit{S. Typhimurium} in infected mice [3]. Recent studies in our laboratory found that p\textsubscript{RST98} had inhibitory effects on autophagy in macrophages, thus weakening the innate immunity of host cells [4–5]. In addition, p\textsubscript{RST98} is a conjugative plasmid that spreads easily among \textit{S. Typhi}, \textit{S. Typhimurium}, \textit{Escherichia coli} (\textit{E. coli}) and \textit{Shigella flexneri} (\textit{S. flexneri}) \textit{in vitro}, and it was very easily transferred from \textit{S. Typhimurium} to \textit{E. coli} in mice [6]. Given these characteristics of p\textsubscript{RST98}, it is expected that this plasmid plays important roles in bacterial resistance against hostile immune factors and in causing aggravated infection.

Due to their significance in the food industry and in public health, bacterial biofilms (BFs) have become the focus of studies...
in conjugative plasmid-enhanced BF production in \textit{E. Coli} [19–20]. However, very few factors conducted by conjugative plasmid were reported in \textit{Salmonella}. Because the \textit{pRST98} plasmid has the ability to conjugate, we explored the relationship between \textit{pRST98} and BF formation in different \textit{Salmonella} and \textit{E. coli} strains by multiple methods \textit{in vitro}, including violet dye staining, scanning electron microscopy (SEM), and confocal laser scanning microscopy (CLSM). Furthermore, two animal models were established to investigate the effects of \textit{pRST98} on BF formation \textit{in vivo}. One was a tumor-bearing mouse intravenously infected by \textit{S. Typhimurium} \textit{γ3337lux} and \textit{γ3337lux/pRST98} (by the conjugal transfer of \textit{pRST98} to \textit{γ3337lux}) [21]. Here \textit{S. Typhimurium} was used as a surrogate for \textit{S. Typhi} because \textit{S. Typhi} only causes human infections, and no suitable model has been established for investigation of \textit{S. Typhi} pathogenesis. \textit{S. Typhimurium} is a facultative anaerobic bacterium that can survive both in tumor active areas and necrosis areas. In addition, \textit{S. Typhimurium} is driven toward tumors through chemoattraction in infections. Three important receptors, the aspartate receptor, the serine receptor, and the ribose/galactose receptor, bind to compounds released by tumor and specifically attract \textit{S. Typhimurium} to preferentially migrate to the tumor [22]. The other animal model was a mouse with a urethral catheter infected by \textit{E. coli} \textit{K12W1485} and \textit{E. coli} \textit{K12W1485/pRST98} (by the conjugal transfer of \textit{pRST98} to \textit{E. coli K12W1485}) because \textit{E. coli} is one of the most common microbes in nosocomial infections.

N-acylhomoserine lactones (AHLs) are signaling molecules of the quorum sensing (QS) system, which responds to bacterial population density and triggers some gene expressions. APLs play an important role in BF formation. Though \textit{Salmonella} does not produce AHLs, it synthesizes the signal molecule receptor SdiA, which responds to AHLs released by other bacteria [12]. Lee found that SdiA binds extracellular signals and affects BF formation in \textit{E. coli}; however, no direct link has been found between AHLs and BF formation in \textit{Salmonella} [23]. Encoding an outer membrane protein, the \textit{rck} gene on the virulence plasmid of some serotypes of \textit{Salmonella} was regulated by SdiA. It was found that the \textit{rck} operon affects the expression of plasmid-encoded fimbiae, which were shown to be vital components of the extracellular matrix and to promote BF formation [24–25]. In this study, we investigate the effects of \textit{pRST98} on BF formation and its interactions with the AHLs-SdiA-Rck pathway.

**Materials and Methods**

**Bacteria and culture conditions**

The bacteria used in our study were listed in Table 1. Bioluminescent strains of \textit{S. Typhi} and \textit{S. Typhimurium} were constructed by electroporation of the pBEN276 plasmid containing a constitutive \textit{lux} expression cassette, and the \textit{lux} expression cassette recombinated within the bacterial chromosome according to reference [26]. The use of bioluminescent bacteria provides an effective tool in the detection of \textit{S. Typhimurium} BF formation \textit{in vivo}. These strains were grown to mid-logarithmic phase in Luria-Bertani (LB) medium at 37°C, with a shaking speed of 200 r.p.m. Ampicillin was added into the medium at a concentration of 100 μg/ml to maintain the stability of the \textit{pRST98} plasmid in some strains. The bacterial population density was determined by measuring \textit{OD}_{600} values with a spectrophotometer.

**Cell lines and animals**

CT26 colon carcinoma cells (ATCC CRL-2638) and HeLa cells purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences of Chinese Academy were cultured as a
monolayer in RPMI1640 Medium (Sigma, America) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Thermo Scientific, America). Six- to seven-week-old female BALB/c mice were purchased from the Experimental Animal Center of Soochow University.

Ethics statement
All animal experiments were approved by the Animal Experimental Committee of the Soochow University (Grant 2111270) and were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Guidelines).

Comparison of BF by crystal violet staining
Bacteria cultured overnight in LB medium were diluted to \(OD_{600} = 0.4\). BF formation in polystyrene microtiter plates was assayed as described by O’Toole & Kolter [27] with modification. Briefly, cells were grown in the wells of the microtiter plates in 200 \(\mu \text{l}\) of LB medium supplemented with 1% glucose for 72 h at 30°C. The medium was then removed and replaced by 200 \(\mu \text{l}\) of 30% (v/v) acetic acid solution, and for 15 min, the dye was removed, and the wells were washed thoroughly with phosphate buffered saline (PBS). Following drying, BFs were observed with inverted microscopy and imaged. To quantify the attached bacteria, the crystal violet solution was solubilized with 200 \(\mu \text{l}\) of 30% (v/v) acetic acid solution, and the absorbance was measured at 570 nm (i.e., \(OD_{570}\)) in an ELISA reader (Biostek). The experiment was repeated three times with each sample in 4 wells.

Observation of BF structure with CLSM
Bacteria were cultured in the 24-well polystyrene plates at 30°C for 72 h. The pellets collected from the air-interface were placed on the microscope slides and stained with 0.01% Acridine Orange (AO). After scaled with 40% glycerine, the samples were observed with a Leica TCS-SP2 CLSM. Imaging was performed using the 40*/1.3 objective, and simulated three-dimensional images were generated with COMATAT software. The experiment was repeated three times with duplicate samples.

Detection of BF using SEM
The cultured pellets were transferred to cover slips pre-coated with lysine, followed by fixation with 4% glutaraldehyde and postfixation with 1% osmic acid before dehydration with a graded series of tert-butyl alcohol dilutions (30 to 100%). After the critical point in drying, the samples were observed with an XL-20 scanning electron microscope (Philip, Netherlands).

BF formation in two different animal models in vivo
For the tumor-bearing BALB/c mouse model, each group of six was subcutaneously inoculated with \(1 \times 10^6\) CT26 cells at the pre-abdominal site. When the tumor reached a diameter of 5–8 mm, the tumor-bearing mice were injected intravenously with \(1 \times 10^5\) CFU of \(S.\ Typhimurium\) \(\chi3337\/lux\) or \(\chi3337\/lux\)/pRST98 in PBS. In-vivo imaging was performed at 1 d, 2 d and 3 d post-infection (p.i.) using an FX Pro in-vivo imaging system (IVIS, DXS1000pro) to observe the injected bacteria in mice. Mice were sacrificed at 3 d p.i., and tumors, livers, and spleens were collected for SEM and colony forming unit (CFU) analysis.

The urethral catheter model, polyethylene tubes (PE10 with inside and outside diameter of 0.28 mm and 0.6 mm, respectively) pretreated with 75% ethanol and UV sterilized for 12 h, were incubated with \(E.\ coli\) K12 W1465 or with \(E.\ coli\) K12 W1465/pRST98 for 1 d. Female mice in each group of six were anesthetized by injecting 10% chloral hydrate in the enterocoelia. The periurethral area was sterilized with 75% ethanol, and the pre-incubated PE10 tubes were gently inserted transurethrally. PE10 tubes, livers, and kidneys were aseptically collected from sacrificed mice on 5 d and 8 d p.i., and washed with PBS. PE10 tubes were fixed in glutaraldehyde for SEM or stained with 0.01% AO staining.
solution for CLSM. In addition, PE10 tubes, as well as livers and kidneys, were sonicated for 20 min in PBS for CFU counting. For the preparation of paraffin sections, livers and kidneys fixed in 10% (v/v) paraformaldehyde were embedded in paraffin wax, sectioned with a thickness of 3–4 μm, placed on slides and stained with hematoxylin-eosin (H&E) staining solution.

Analysis of the mechanism of pRST98 promoted BF formation by adherence assay

HeLa cells were seeded in 24-well tissue culture plates at 10^5 cells per well and incubated at 37°C and 5% CO2 for 12 h. Cells were infected with ST8, ST8-c-pRST98 or ST8-DpRST98 with an MOI of 100:1 in the presence of 1 μM C8-AHLs dissolved by DMSO (Sigma, America) or saline. The plates were incubated at 37°C with 5% CO2 for 60 min, and the cells were washed three times with PBS before lysing with 200 μl 0.2% Triton X-100 for 30 min at 37°C. The supernatant was collected for CFU counting. Each bacterial strain was assayed in triplicate, and experiments were repeated twice.

Serum resistance

Serum collected from 5 healthy rabbits and guinea pigs was filter-sterilized. S. Typhi were cultured in LB for 16 h at 37°C, gradual diluted OD600 value to 1×10^4 CFU/ml. Then, 20 μl bacterial cultures were incubated with 200 μl serum plus 1 μM C8-AHLs or saline 2 h at 37°C. CFUs were enumerated to count the surviving bacteria. The experiment was repeated twice with triplicate samples.

PCR and sequencing of rck gene

Genomic DNA was extracted from ST8, ST8-c-pRST98 and ST8-DpRST98 by boiling. PCR was performed using primers rck-F: 5’-GTTGTATCCCGGCGATCCTGA-3’ and rck-R: 5’-ATATGCCCAGAGCGGATAGAG-3’ [28], to detect the rck gene located on pRST98. Then, the gene was linked to the pEJT1.2 plasmid and transduced into E. coli TOP10. The rck gene was sequenced.

RT-PCR of rck gene

Total RNA extraction was performed using the Total RNA kit I (OMEGA bio-tek, America). The samples were centrifuged at 4000 r.p.m. for 10 min, and the supernatant was discarded. The pellet was resuspended in 100 μl lysis buffer (50 mg/ml lysozyme in Tris-EDTA buffer) and incubated at room temperature for 7 min. The subsequent steps of the RNA purification were performed according to the manufacturers’ instructions. The

![Figure 2. Comparison of BF developed by different bacteria. (A) Different bacteria cultured in vitro for 3 d in microtiter plates at 30°C and stained by crystal violet (400×). (B) Different bacteria cultured in vitro for 3 d in 96-well plates at 30°C and stained by crystal violet. (C) Optical density of cultures measured at a wavelength of 570 nm (OD570) after crystal violet staining (*p < 0.05). doi:10.1371/journal.pone.0109808.g002](image)
quality of the isolated RNA was assessed via gel electrophoresis (PowerPac Basic, America). RNA concentrations were determined using the NanoDrop System (Thermo Scientific, America). The expression of the rck gene was determined by SuperScript TM III platinum One-Step Quantitative RT-PCR System (Invitrogen, America) according to the manufacturers’ instructions. The reaction solution contained 2.5 μl of 2x reaction mixes, 1 μl of TaqMix, 0.2 μl of specific primers, 2 μl of mRNA, and 21.6 μl of DEPC water. Reactions were performed on a PCR system (MJ Research, America). cDNA was first produced in the RT step with 50°C for 15 min, followed by a DNA amplification step at 95°C, 5 min for denaturing, and 35 cycles (95°C for 40 s, 55°C for 30 s and 72°C for 115 s). The DNA product was observed and analyzed by gel electrophoresis and an automatic gel imaging analysis system (Syngene, UK). The primers used in this experiment were rck-F and rck-R.

C8-AHLs on BF formation

ST8/lux, ST8ΔpRST98/lux and ST8-c-pRST98lux were cultured in the 24-well polystyrene plates at 30°C for 24 h adding 1µM C8-AHLs in the experimental group and 1µM saline in the control group. The media were then removed and washed thoroughly with PBS for 3 times. BFs were observed with IVIS.

Statistical methods

Data among groups were compared by three independent analyses, using an unpaired two-tailed Student t test, a one-way

**Figure 3. Quantification of BF by CLSM.** Different bacteria were cultured in 24-well plates for 3 d, and the developed pellicles were harvested, placed on glass slides, and subjected to 3D image reconstruction by CLSM.

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ANOVA, and a SNK-q (Student-Newman-Keuls) analysis. Among all the analyses, a $p$ value $< 0.05$ was considered statistically significant. All the experiments were repeated three times with duplicate samples.

Results

1. The promotion effects of pRST98 on BF formation in different bacteria in vitro

To study the effect of the plasmid pRST98 on BF formation in different strains, several methods were employed, including crystal violet staining, CLSM, and SEM. Including S. Typhi ST8, S. Typhimurium χ3306 (the bioluminescent S. Typhimurium strains were also studied), E. coli K12 W1405 and their derivatives, three groups of bacteria were used in the crystal violet staining method to compare their ability to form BFs. For the intra-group comparison in the ST8 group, ST8 and ST8-c-pRST98 were found to develop thicker BFs than ST8 ΔpRST98 ($p < 0.05$) (Fig. 2A).

Consistently, BFs formed by S. Typhimurium carrying pRST98 were significantly more robust compared with those without pRST98 in the χ3306lux, χ3337lux and χ3337lux/pRST98 strains ($p < 0.05$) (Fig. 2A). Similarly, E. coli K12 W1405/pRST98 had a stronger ability to form BFs than E. coli K12 W1405 ($p < 0.05$). These results indicate that pRST98 plays an important role in promoting BF formation. For the inter-group comparison, Salmonella developed thicker BFs than E. coli did, and the difference was even more significant when both Salmonella and E. coli harbored pRST98, suggesting that pRST98 might enhance BF formation in Salmonella more strongly than in E. coli. Meanwhile, the lux gene was shown to have no effect on BF formation (data not shown), and there was no difference observed between χ3306 and χ3337 (Fig. 2A to C).

Bacteria harboring pRST98 developed slimy and viscous pellicles, while pRST98-free bacteria formed loose and less coherent BFs [13]. Tomography and three-dimensional reconstruction by CLSM showed that BFs in S. typhi ST8 and ST8-c-pRST98 were

Figure 4. Observation of BF by SEM. Different bacteria were cultured in 24-well plates for 3 d, and the developed pellicles were harvested, placed on glass slides, and subjected to SEM. doi:10.1371/journal.pone.0109808.g004
developed with 43.23 μm and 47.62 μm thicknesses, respectively, which were much thicker than that in ST2pRST98 with a thickness of 21.74 μm; S. Typhimurium harboring pRST98 was a stronger BF developer (χ3337: 24.22 μm vs χ3337/pRST98: 44.33 μm; χ3337lux: 25.89 μm vs χ3337lux/pRST98: 40.30 μm); E. coli K12W1485 produced a BF of 9.1 μm in thickness, while the BF developed by E. coli K12W1485/pRST98 had a thickness of 45.06 μm. The BF thicknesses of S. Typhimurium χ3306 and χ3306lux were 28.23 and 27.98 μm, which were not significantly different from S. Typhimurium χ3337 and χ3337lux (Fig. 3).

SEM provides a detailed view of the connections in a bacterial community. Bacteria harboring pRST98 significantly promoted BF formation as indicated by SEM, which showed that bacteria forming three-dimensional BF structures were embedded within denser matrices. However, the BFs of bacteria that did not harbor pRST98 were discontinuous and discretely patchy (Fig. 4). These results corroborate those from violet staining and CLSM, suggesting that pRST98 promotes BF formation in all of the tested bacteria, including S. typhi, S. Typhimurium, and E. coli.

2. pRST98 promotes BF formation in different bacteria in vivo

To study the effect of the pRST98 plasmid on bacterial proliferation and BF formation in vivo, we established two animal models, a tumor-bearing mouse model and a mouse urethral catheter model. Electrotransfoming the bacteria with the lux gene made it possible to detect dissemination in tumor-bearing mice by a non-invasive method, and lux was shown to have no effect on bacterial growth. After intravenously infecting mice, S. Typhimurium quickly circulated within the blood in the bodies of the mice. It was found that χ3337lux and χ3337lux/pRST98 accumulated preferentially in tumors detected by IVIS at 3 d p.i., and χ3337lux/pRST98 in tumor emitted stronger bioluminescence signals than χ3337lux did, indicating that χ3337lux/pRST98 formed thicker BFs. The same load of χ3337lux/pRST98 was used to infect normal mice as a control, but no signal was observed at the desired sites (Fig. 5A), most likely due to the quick dissemination in the blood that was beyond the detection limit of IVIS. To further analyze the histological changes in infected mice and bacterial load, the tumor, livers and spleens were sterilely recovered based on the IVIS images at 3 d p.i. for SEM and CFU.
Metastasis in livers and spleens by tumor cells, along with swelling organs, were found. The inflammation was more severe in the \( \chi_{3337}^{lux}/pRST98 \)-infected group. Consistent with the results from IVIS, SEM showed that more \( \chi_{3337}^{lux}/pRST98 \) was accumulated in tumor. The livers and spleens from mice infected with \( \chi_{3337}^{lux}/pRST98 \) were loaded with more bacteria as well, indicating that the \( pRST98 \) plasmid promoted bacterial spread and proliferation as well as enhancing virulence (Fig. 5B and C).

PE10 tubes pre-incubated with \( E. coli \) were inserted into the mouse urethras. The mice were still active at 5 d post-insertion. Stable BF of \( E. coli K_{12}^W_{1485}/pRST98 \) or \( E. coli K_{12}^W_{1485} \) developed on the surface of PE10 tubes were detected by CLSM after 5 d post-insertion under bright light. SEM, CLSM and CFU counting showed that the BF formed by \( E. coli K_{12}^W_{1485}/pRST98 \) were thicker and had denser extracellular matrices compared with those in the control strain \( E. coli K_{12}^W_{1485} \) (Fig. 6A to C). However, the livers and kidneys recovered from mice showed no pathological changes at 5 d post-insertion. When the insertion was extended to 8 d, sluggish behavior appeared in all mice, and more severe symptoms were observed in the \( E. coli K_{12}^W_{1485}/pRST98 \) group.

![Figure 6](image-url)

Figure 6. PE10 tubes recovered from the mouse urethral catheter model and histological changes of livers and kidneys. (A) Analysis of \( K_{12}^W_{1485} \) and \( K_{12}^W_{1485}/pRST98 \) BF on PE10 tubes at 5 d p.i. by SEM. (B) Quantification of \( K_{12}^W_{1485} \) and \( K_{12}^W_{1485}/pRST98 \) colonizing on PE10 tubes at 5 d p.i. \( (p<0.05) \). Dots and dashes indicate the cfu of \( K_{12}^W_{1485} \) and \( K_{12}^W_{1485}/pRST98 \), respectively, recovered from BF on PE10 tubes. The middle long horizontal line represents the mean cfu, and the short line represents the SD. \( (**p<0.01) \). (C) The tubes recovered from mice after urethral catheter at 5 d p.i. were washed with PBS and stained with AO, and bacteria were detected by CLSM. (D) H&E staining of livers and kidneys at 8 d after application of urethral catheter. (a and b), Livers of mice infected with \( K_{12}^W_{1485} \). (c and d), Kidneys of mice infected with \( K_{12}^W_{1485}/pRST98 \). (g and h), Kidneys of mice infected with \( K_{12}^W_{1485}/pRST98 \). doi:10.1371/journal.pone.0109808.g006
pRST98 group demonstrated by abdominal dropsy, swelling in livers and kidneys, and punctate lesions. The symptoms induced by *E. coli* K12 W1485/pRST98 BFs showed further histological changes in livers and kidneys by H&E staining, including inflammatory cell infiltration and severe damage in the hepatic lobule and the glomerular structure (Fig. 6D). At 12 d post-insertion, most of the mice infected with *E. coli* K12 W1485/pRST98 died, while the mice with *E. coli* K12 W1485 infection survived longer than 17 d after insertion.

3. C8-AHLS enhances bacterial adherence, resistance, *rck* locus and transcription and bacterial BF formation

AHLS, signaling molecule of the QS system, were shown to affect the BF formation in *E. coli* and the bacterial adherence [16]. To determine whether AHLS have similar effects on the BF formation in *Salmonella*, bacterial adherence assays were performed in the ST8 group treated with C8-AHLS. It was found that ST8 ΔApRST98 and ST8-c-pRST98 displayed higher adherence rate than ST8 ΔApRST98 (*p < 0.05), while no difference was observed for the adherence rate between ST8 and ST8-c-pRST98 (*p > 0.05). As for the control group (treated with saline), the adherence of the three strains to HeLa cells was similar (*p > 0.05). ST8 and ST8-c-pRST98 incubated with C8-AHLs showed more adherence than with saline (Fig. 7A). This result indicates that AHLS promoted bacterial adherence, on which pRST98 may have an effect.

AHLS promote BF formation in *E. coli*, which subsequently increases bacterial resistance against hostile factors including serum. To investigate whether AHLS enhanced *Salmonella* resistance, a complement-mediated killing assay was performed. When incubating with rabbit and guinea pig serum, the survival

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**Figure 7. The effect of AHLS on *rck* expression and its related function.** (A) The adherence rate of *S. Typhi* to HeLa cells in the presence of AHLS (*p < 0.05). (B and C) Quantification by CFU of surviving bacteria after incubation with sera from rabbits (B) and guinea pigs (C) in the presence of AHLS and saline (*p < 0.05).

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Figure 8. The locus of rck and its expression. (A) PCR of rck gene in pRST98. M: 1000 bp DNA ladder; Lane 1: S. Typhi ST8; Lane 2: S. Typhi ST8ΔpRST98; Lane 3: S. Typhi ST8-c-pRST98. (B) The effect of AHLs on the expression of the rck gene. M: 1000 bp DNA ladder; Lane 1: S. Typhi ST8 treated with AHLs; Lane 2: S. Typhi ST8 treated with saline; Lane 3: S. typhi ST8-c-pRST98 treated with AHLs; Lane 4: S. Typhi ST8-c-pRST98 treated with saline; Lane 5: S. Typhi ST8ΔpRST98 treated with AHLs; Lane 6: S. Typhi ST8ΔpRST98 treated with saline.

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Discussion

In response to limited nutrients and stressful conditions, many microorganisms form BFs by secreting polymeric matrices to interweave individual cells and build structural communities on abiotic or living surfaces. Due to the significance of BF formation in increasing the resistance of bacteria against hostile environments, BFs have become a significant research interest in the medical, food, and environmental fields.

Jean-Marc Ghigo first found that natural conjugative plasmids have the capability of promoting BF formation in E. coli [15]. In addition, bacteria harboring conjugative plasmids developed thicker BFs than those not harboring such plasmids. However, the relationship between the conjugative plasmids in Salmonella and BF formation has not been studied.

The effects of pRST98 on BF formation were explored in this study. Crystal violet staining, SEM and CLSM revealed that S. Typhi, S. Typhimurium and E. coli harboring pRST98 formed thicker BF in vitro, compared with the isogenic strains not carrying pRST98. It was also observed that S. Typhimurium χ3306 and χ3337 had similar abilities to form BFs, which is inconsistent with the study of Teodóso JS et al [29]. We speculated the different plasmids and GF-producing systems may contribute to this inconsistency. We noticed that E. coli K12W1485/pRST98 had a weak ability to form BFs compared with Salmonella strains harboring pRST98. This heterogeneity in BF formation may arise because the synthesis of extracellular polymeric substances (EPS) in Salmonella outcompetes that in E. coli in medium, as reported by Rong Wang et al. Regarding the heterogeneity in the promotion of BFs by conjugative plasmids, Roeder HL et al. proposed that the different genetic backgrounds of the plasmid-harboring hosts may account for different BF formation when the same plasmid was used [30]. Our previous study demonstrated that in different genera, the conjugal transfer conditions of the pRST98 plasmid were different in vitro or in mice, and the resistance markers encoded by the same plasmid varied in different strains, which showed the diversity and complexity of the gene expression from the plasmid. Thus, the effects of BF formation by different plasmids in various hosts may demand specific analysis.

In animal experiments, a tumor bearing mouse model was used to study the effects of pRST98 on BF formation in S. Typhimurium, which was used as a surrogate of S. Typhi because no animal model is available for S. Typhi infection. In the tumor-bearing mouse model, χ3337lux/pRST98 was found preferentially in tumors with a considerably larger amount than χ3337lux. The observation that solid tumors are treatable via bacterial infection...
was made previously [31–32]. Colonization of bacteria on solid tumors could cause growth retardation or even the complete elimination of the tumors [33]. pRST98 promoting host bacterial BF formation may have a therapeutic potential in fighting against tumors. Furthermore, our invasion study demonstrated that pRST98 aggravates the tumors. Furthermore, our invasion study may have a therapeutic potential in fighting against tumors. However, our invasion study has not been performed in vivo after they established BFs. The intracellular invasion by Salmonella may be due to the differential expression of invasive genes on Salmonella pathogenicity island 1 (SPI-1) induced by BF formation [34].

Another animal model, a mouse urethral catheter model, was established to study the effects of pRST98 in E. coli on BF formation in vivo. E. coli K12W1485/pRST98 was found to form only discrete patchy BFs at 3 d post-implantation, while E. coli K12W1485 was not detected in tubes until 5 d post-implantation (data not shown). E. coli K12W1485/pRST98 developed denser BFs at 5 d post-implantation, in line with bacterial titers recovered from established BFs on tubes. No histological changes were observed in the livers and kidneys of either group. When the implantation with tubes pre-incubated with E. coli was extended to 8 d or beyond, more severe inflammation was observed. Significantly, S. Typhimurium χ3337 and E. coli K12W1485/pRST98 caused more severe inflammation in organs than χ3337ltxd. A similar phenomenon was observed for E. coli K12W1485/pRST98 and K12W1485. These results indicate that pRST98 aggravates the infection by promoting BF formation. Recently Rong Wang and Victoria J. Savage et al. demonstrated that the BF increases horizontal transfer of multi-resistant conjugative plasmids to plasmid-free bacteria compared to planktonic bacteria [35–36]. Therefore, it seems that conjugative plasmids facilitate BF formation, and vice versa. Therefore, given the intestinal origin and the conjugative transfer of pRST98, interaction between pRST98 and BF may make Salmonella infections worsen.

QS, a bacterial communication system, has been implicated in BF formation. To date, three types of Salmonella-associated QS signals have been described as AHLs, autoinducer-2 (AI-2) and autoinducer-3 (AI-3). However, the study on AI-2 and AI-3 revealed their minor roles in Salmonella BF formation in some conditions. While Salmonella does not produce AHLs, the AHL receptor SdiA was found in Salmonella to sense exogenous AHL signals to influence BF formation. A recent study revealed that the presence of SdiA enhances E. coli O157:H7 (O157) colonization and persistence in fecal shedding of the bovine large intestine, the prerequisites for developing a BF. Rck is a 17-kDa outer-membrane protein encoded by the rck gene located on the virulence plasmid of Salmonella enterica serovars Enteritidis and Typhimurium. The expression of rck in both E. coli and S. Typhimurium confers bacterial resistance against complement-mediated killing [37]. Rck is homologous to Yersinia enterocolitica Aii, which is capable of influencing bacterial adherence to epithelial cell lines [38]. We hypothesized that rck may influence BF formation. In the present study, it was proven that the rck gene was located on pRST98, and rck-containing pRST98 and C8-AHLs enhanced the cellular adherence of bacteria harboring pRST98 and increased bacterial resistance against serum by activating transcription of rck. In addition, C8-AHLs promoted BF formation in bacteria containing pRST98. These results partially explained the pRST98-mediated BF promotion.

The mechanism of the effects of conjugative plasmids on BF formation is certainly complex and reciprocal. It is not clear whether the reported explanations could be applied to this study, although the studies on the mechanism may provide some clues. Further investigations will be focused on the factors that contribute to pRST98-mediated BF formation and the mechanisms associated with the heterogeneity in BF formation.

Takken and others, demonstrated that the conjugative plasmid pRST98, which was isolated from S. typhi, can promote BF formation in intestinal bacteria such as S. Typhi, S. Typhimurium, and E. coli. Animal models showed that pRST98 promotes BF formation in S. Typhimurium and E. coli. In attempting to investigate the underlying mechanism, we found that the transcription of rck located on pRST98 is activated by C8-AHLs. Therefore, it is reasonable to conclude that pRST98 promotes BF formation in its host bacteria through the AHL-SdiA-Rck pathway. The relationship between the conjugative plasmid pRST98 and BF formation could provide insights into the prevention and treatment of Salmonella BF-related disease and intestinal infection.

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Author Contributions

Conceived and designed the experiments: SW RH. Performed the experiments: ZL FY LZQ YL. Analyzed the data: ZL FQ SW RH. Wrote the paper: FQ ZL YL HN.

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