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Abstract

Deficiency in biosynthesis of inner core of lipopolysaccharide (LPS) rendered a characteristic biofilm-forming phenotype in *E.coli*. The pathological implications of this new phenotype in *Shigella flexneri*, a highly contagious enteric Gram-negative bacteria that is closely related to *E.coli*, were investigated in this study. The *ΔrfaC* (also referred as *waaC*) mutant, with incomplete inner core of LPS due to deficiency in Hep biosynthesis, was characteristic of strong biofilm formation ability and exhibited much more pronounced adhesiveness and invasiveness to human epithelial cells than the parental strain and other LPS mutants, which also showed distinct pattern of F-actin recruitment. Failure to cause keratoconjunctivitis and colonize in the intestine in guinea pigs revealed that the fitness gain on host adhesion resulted from biofilm formation is not sufficient to offset the loss of fitness on survivability caused by LPS deletion. Our study suggests a clear positive relationship between increased surface hydrophobicity and adhesiveness of *Shigella flexneri*, which should be put into consideration of virulence of *Shigella*, especially when therapeutic strategy targeting the core oligosaccharide (OS) is considered as an alternative to deal with bacterial antibiotics-resistance.

Introduction

Shigella flexneri is a highly contagious facultative intracellular pathogen causing acute inflammatory enteritis in human. Following infection of epithelial cells and macrophages, *S. flexneri* escapes from the phagocytic vacuoles via Ipa-mediated vacuole membrane lysis and enters the cytoplasm, where active proliferation takes place(Philpott et al. 2000; Schroeder & Hilbi 2008). Bacteria then spread intra- and intercellularly by recruiting the cellular actin polymerization machinery with the help of the intra/intercellular spread factor, IcsA(Lett et al. 1989; Suzuki & Sasakawa 2001). While the invasion process and immune evasion of *S. flexneri* have been extensively studied, the early adhesion process has not been adequately understood since *Shigella flexneri* lacks general adhesion machinery and exhibits relatively poor adhesiveness compared with other pathogenic enterobacteria(Brotcke Zumsteg et al. 2014; Carayol & Tran Van Nhieu 2013; Phalipon & Sansonetti 2007; Pizarro-Cerdá & Cossart 2006) .

LPS is a glycolipid located in the outer membrane of Gram-negative bacteria. It is composed of three covalently linked domains: lipid A, which is embedded in the outer membrane; the oligosaccharide core including inner and outer parts; and repeats of the O-polysaccharide or O-antigen, which cover the bacterial surface. As an essential pathogenic component, LPS of gram-negative bacteria triggers strong immune responses, which are directly related to the adverse clinical outcomes(Alexander & Rietschel 2001; Wang & Quinn 2010). In contrast to the highly variable and antigenic O-antigen portion, the core oligosaccharide (OS), especially the inner (lipid A-proximal) core, composed of two 3-deoxy-Dmanno-oct-2-ulosonic acids (Kdo) and

three L-glycero-D-mannoheptose (Hep), called HepI, HepII, and HepIII, is conserved across *E. Coli, Shigella* and *Salmonella* and possesses limited structural variation. Therefore, targeting the core OS for general therapeutic application has been considered as an alternative strategy against antibiotics-resistant Gram-negative bacterial infection(Desroy et al. 2009; Di Padova et al. 1993; Moreau et al. 2008). Although inhibition of Kdo biosynthesis is usually lethal to bacteria, a defect in Hep biosynthesis results in a viable bacterial cell with a characteristic "deep rough" phenotype (Grizot et al. 2006; Klena et al. 2005).

Biofilm, a microorganism community formed on an environmental surface and by cell aggregation has been implicated in around 80% of microbial infections in vivo(Hall-Stoodley et al. 2004) (Davies 2003). Nevertheless, the relevance of biofilm formation to *Shigella* virulence has not been thoroughly interrogated. Recent studies have shown that LPS composition regulates biofilm formation besides the previously reported association with variations in salt concentration, starvation or changes in pH(Ellafi et al. 2012). For example, deficiency in Hep synthesis in *E.coli* resulted in dramatic biofilm formation on abiotic surface, which is caused by enhanced hydrophobicity of the bacteria surface due to the loss of oligosaccharide (Nakao et al. 2012). Although *Shigella* LPS has been extensively studied through deletion of a serious of genes involved in LPS synthesis, disruption of Hep biosynthesis has never been reported in *Shigella* so far (Hong & Payne 1997; Martini et al. 2011; Sandlin et al. 1995).

In this study, we characterized biofilm forming potentials and pathological behaviors of various

LPS-truncated *Shigella flexneri* strains. Analysis of the LPS mutants revealed that the autoaggregation and biofilm forming capacity of *Shigella* negatively correlated with the LPS chain length in general. The deep-rough LPS mutant *ΔrfaC*, characteristic of strong biofilm formation abilities, exhibited much more adhesive and invasive than the parental strain and other LPS mutants, albeit with undermined fitness. Immunofluorescence analysis revealed that the *ΔrfaC* strain exhibited distinct patterns of IcsA distribution and F-actin recruitment. Finally, using the guinea pig keratoconjunctivitis model, we showed that the biofilm-forming *Shigella* strain failed to colonize in vivo, indicating that the fitness gain on host adhesion resulted from biofilm formation is not sufficient to offset the loss of fitness on survivability caused by LPS deletion.

Materials & Methods

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *Shigella* strains were cultured aerobically at 37°C in Tryptic Soy (TS) broth (Aoboxing, Beijing, China) or on TS agar plates with 0.1% Congo Red. Antibiotics (Sigma) were used as follows: ampicillin 100μg/ml; kanamycin 100μg/ml.

strain construction. Bacterial gene knockout was performed using the λ Red recombination system(Datsenko & Wanner 2000). Briefly, bacterial cells transformed with pKD46 were grown in the presence of L-arabinose to induce the expression of the lambda Red recombinase. A linear PCR product, amplified using the primers listed in Table 2, containing a kanamycin-resistance cassette (KRC) flanked by FLP and 50 bp of the 5'- and 3'-end homologous sequences of the

target gene was electroporated into the bacterial cells and kanamycin was used to select the transformants. The plasmid pKD46 was eliminated by incubation at 37°C. To cure the kanamycin maker, pCP20 was introduced into kanamycin-resistant cells to elicit the recombination of flanking FLP sequences at both ends of the kanamycin cassettes. PCR screening for cured colonies were performed using specific primers listed in Table 2.

LPS preparation and electrophoresis. LPS was prepared from *Shigella* strains as described by Hitchcock & Brown (1983). Bacteria grown overnight on TSA were resuspended in PBS to an OD600 of 0.8. The bacterial pellet from 1.5 ml of the suspension was then resuspended in 125ml lysis buffer (0.1 M Tris/HCl pH 6.8, 2% SDS, 4 % β-mercaptoethanol and 10 %, v/v, glycerol) and boiled for10 min. Proteinase K (50mg, Sigma) was added and the mixture incubated for 1 h at 60℃. Samples were run on a Tris-Tricine gel) and visualized by silver staining.(Hitchcock & Brown 1983)

Autoaggregation assay. Overnight-cultured bacteria were harvested by centrifugation (10,000 × g for 2 minutes) and two ml of whole cells standardized at OD_{600}= 1 after suspension in PBS were placed in a 14-ml polyethylene tube and incubated at 4℃ under a static condition. The OD_{600} of the phase above the sediment by aggregation was recorded at different time points.

Biofilm formation assays. Biofilm formation by *Shigella* strains was assayed as previously described with some modifications. For biofilm analysis on polystyrene surface, 10^7 CFU of *Shigella* in 100 µl of TSB broth was inoculated into the wells of a 96-well flat-bottom polystyrene microtiter plate. The bacterial strains were grown at 37℃ for 48 hours under a static condition and the planktonic cells in liquid medium were discarded. The plate or tube was

washed twice with distilled water and air-dried. Attached biofilms were stained with 0.1% crystal violet for 20 minutes. Then, the plates were rinsed twice with distilled water to remove excess stain and air-dried. In order to quantify the amount of biofilm on a 96-well plate, all stain associated with the attached biofilms was dissolved with 95% ethanol, then OD_{595}absorbance was measured using a microplate reader.

To prepare the biofilm sample for Confocal laser scanning microscopy (CLSM) analysis, GFP-expressing plasmid was electroporated into *Shigella* strains. 10^8 CFU of GFP-*Shigella* was added in 1 ml of TSB broth per well and incubated at 37℃under a static condition. Biofilms were grown on cover glass (φ=14mm) placed in 24-well polystyrene cell culture plates. After 48 hours, the cover glass was rinsed twice with PBS to remove any planktonic cells. After washing, the cells were fixed in 3% paraformaldehyde/PBS and mounted with Anti-Fade solution (Invitrogen) containing DAPI onto glass slides. After the preparation, the samples were examined under the confocal laser scanning microscope ZEISS LSM 710 (Carl-Zeiss), and images were processed by LSM software ZEN (Carl-Zeiss, Oberkochen, Germany).

To prepare the biofilm sample for scanning electron microscopy (SEM), the biofilms grown in the cover glass dehydrated in graded ethanol, critical point dried with CO2 and coated with gold-palladium beads with a diameter of 15 nm. Samples were photographed using a Philips XL-30 scanning electron microscope at 20 kV.

***In vitro* adhesion and invasion assays and microscopy.** One day before the assays, HeLa cells (ATCC CCL-2) were seeded into 24-well plates at a density of ~10^5 cells per well. One hour before the infection, cell culture medium were changed into serum-free medium and ~10^6 CFU

Sf301 or its LPS mutants from mid-exponential phase was added to the cells together. Bacteria were centrifuged (2000rpm, 10min, RT) onto HeLa cells (moi 10:1, or indicated moi) to synchronize the infection. For adhesion assay, after washing, the cells were lysed with distilled water and the CFU was enumerated after plating. For invasion and proliferation assays, bacteria/HeLa mixtures were incubated for 40min after centrifugation and then washed, treated with gentamycin-containing (25μg/ml) medium for another 1 hour (invasion) or 4 hours (proliferation) before lysed for plating. Adhesion was defined as the total number of HeLa cell-associated bacteria and is shown as the percentage of input. Invasion and proliferation was defined as the total number of intracellular bacteria in cells (extracellular bacteria was killed by gentamycin, a cell-impermeable antibiotic). Average results of three independent experiments are shown as mean±SD.

For fluorescent microscopy of bacterial adhesion to HeLa cells, cells were plated onto glass coverslips and adhesion assays were performed as described using Sf301 harboring the GFP_{UV}-expressing plasmid (moi 100:1). Cells were fixed in 3% paraformaldehyde/PBS at room temperature for 15 min, washed in PBS and mounted with Anti-Fade solution (Invitrogen) containing DAPI onto glass slides and visualized under Zeiss confocal microscope. For scanning electron microscopy (SEM), the cover glass was dehydrated in graded ethanol, critical point dried with CO2 and coated with gold-palladium beads with a diameter of 15 nm. Samples were photographed using a Philips XL-30 scanning electron microscope at 20 kV.

Lactate dehydrogenase (LDH) activity assay. The LDH assay was performed using the LDH cytotoxicity assay detection kit (Beyotime, China), according to the manufacturer's instructions.

The assay measures the conversion of a tetrazolium salt to a red formazan product, detectable by absorbance measurement at 490 nm. For this, a SpectraMax 190 Microplate Reader (Molecular Devices) was used.

Phalloidin staining assay. For phalloidin staining of F-actin in *Shigella*-infected HeLa cells, cells were plated onto glass coverslips and invasion assay was performed as described using Sf301 harboring the GFP_{UV}-expressing plasmid (moi 100:1) for two hours. Cells were fixed in 3% paraformaldehyde/PBS at room temperature for 15 min, washed in PBS and permeabilized in 0.1% Triton-X100 in PBS for 1 min. F-actin were stained with 80nM TRITC-phalloidin (Yeasen, Shanghai, China) for 30min. The coverslips was mounted with Anti-Fade solution (Invitrogen) containing DAPI onto glass slides and visualized under Zeiss confocal microscope.

Plaque assay. This was carried out according to Oaks et al. (1985) using confluent HeLa cell monolayers(Oaks et al. 1985). Briefly, HeLa cells to be used in the plaque assay were grown to 100% confluency in 6-well polystyrene plates (Corning) in DMEM supplemented with 10% FBS. One hour before the infection, cell culture medium were changed into fresh medium and Sf301 or its LPS mutants from mid-exponential phase (m.o.i 100:1) was added to the cells and subsequently incubated at 37°C for 90 min. During this adsorption or attachment phase, the plates were rocked every 30 min to assure uniform distribution of the plaque-forming bacteria. Next, an agarose overlay (5 ml) consisting of DMEM, 5%FBS, 25μg of gentamicin per ml, and 0.5% agarose was added to each plate. The plates were incubated at 37°C in a humidified 5% CO_2 and examined daily for up to 3 days for plaque formation. The agar was carefully removed

three days later and the HeLa monolayers were stained by Giemsa staining kit (Yeasen, Shanghai, China) in order to visualize the plaques.

Sereny test. Female Hartley guinea pigs, aged 6–8 weeks, weighing 200–300 g, were inoculated with 10^7 CFU/eye of mid-log phase Sf301 and its LPS mutants via conjunctival route as described, with 3 animals in each group. The protocol has been approved by the Animal Research Ethical Committee of the Xi'an Jiaotong University. Inoculated animals were observed and scored for 7 consecutive days for development of the conjunctivitis. Eyes were blindly scored by three individuals (DX, YPS, YC) on a scale of 0-3 defined as follows: grade 0 (no disease or mild irritation), grade 1 (mild conjunctivitis or late development and/or rapid clearing of symptoms), grade 2 (keratoconjunctivitis without purulence), grade 3 (fully developed keratoconjunctivitis with purulence).

Guinea pigs were anesthetized using chloral hydrate (10 mg/kg of body weight) before being inoculated via an intrarectal (i.r.) route with 10^8 CFU of Sf301 and its LPS mutants in 100 µl PBS, with 3 animals in each group. The protocol has been approved by the Animal Research Ethical Committee of the Xi'an Jiaotong University. Three pieces of feces of each inoculated animal were collected at the same time of the day for 18 days. Each piece of the feces were dispersed and 1ml PBS. After centrifuge, 100µl of the supernatant was plated onto MaConkey plates. *Shigella* bacteria were recognized as smooth, white colonies on MaConkey plates and further confirmed by PCR of *spa33* gene.

Results

201 **Generation and characterization of the LPS mutants of *Shigella flexneri***

202 To generate Shigella LPS mutants of different chain length, we knocked out *wzy*, *waaL* and *rfaC*
203 individually using the lambda red system (Fig. S1). As shown in Fig. 1A, the *wzy* mutant
204 contains a complete core and one copy of the O-antigen; the *waaL* mutant has a complete core
205 but no O-antigen; the *rfaC* mutant is deficient of Hep but retains the Kdo. SDS-PAGE analysis
206 confirmed that the all mutant strains produced proper LPS variants as expected (Fig. 1A, bottom
207 panel). The *Δwzy* and *ΔwaaL* strains formed WT-like colonies on Tryptone Soya Agar, while the
208 *ΔrfaC* strain grew round and smooth colonies with smaller size. In accordance with this, the
209 *ΔrfaC* mutant grew slightly slower in liquid tryptone soya broth medium than other strains
210 (Fig.1B). LPS truncation had no apparent impact on the morphology of individual bacteria cells
211 as illustrated by transmission electron microscopy (TEM) (Fig. 1C).

212

213 **Biofilm formation is enhanced in the *rfaC*-deleted *Shigella* strain**

214 The aggregation tendency of LPS mutants was initially evaluated in PBS and the results showed
215 that LPS truncation led to enhanced bacterial aggregation (Fig. 2A). Next, we examined the
216 biofilm formation of the LPS mutants on the polystyrene surface under a static culture condition.
217 While the WT strain had a poor ability to form biofilm, the LPS mutants showed improved
218 potential to do so (Fig. 2B). Moreover, the ability to form biofilm appeared to be negatively
219 correlated with the LPS length with the *ΔrfaC* strain (shortest LPS) being the most effective one
220 (Fig.2B). Fluorescence microscopy and scanning electron microscopy (SEM) further confirmed
221 the enhanced biofilm formation by the *ΔrfaC* strain on glass surface (Fig.2C-D). To test the

involvement of extracellular DNA in the biofilm formation by the *ΔrfaC* strain, DNase I was added when the bacteria were seeded into the polystyrene plates. Disruption of extracellular DNA significantly abolished the formation of biofilm without affecting the viability of the bacteria (Fig. 2E and Fig. S2). In addition, SEM analysis demonstrated that there were fibrous connections among bacterial cells in the biofilm (Fig.2F).

In vitro adhesiveness and thus invasiveness is promoted in biofilm-forming *rfaC*-deleted *Shigella flexneri* strain independent of TTSS

Since biofilm formation is often associated with bacterial virulence, we set out to evaluate the pathogenic activities of the biofilm-forming LPS mutants using the gentamicin protection assay. The *Δwzy* and *ΔwaaL* strains showed almost identical host invasion efficiency to the WT strain. By contrast, the *ΔrfaC* strain showed a much stronger invasiveness and intracellular proliferation than wild type (Fig.3A). Further dissection of time-dependent infection process revealed that the improved invasiveness came from enhanced adhesion during the initial host cell-*Shigella* contact (within 10 minutes), which was further confirmed by fluorescence microscopy and scanning electron microscopy (Fig.3B-D). To be specific, *ΔrfaC* mutant exhibited pronounced adhesiveness even when the type III secretion system (T3SS), responsible for bacterial invasion and virulence, was inactivated transcriptionally at low growth temperature (28-30℃). Of note, the invasion of low temperature-cultured bacteria was totally abolished as expected since no intracellular bacteria were recovered. To verify the cell-lysing ability of *ΔrfaC* mutant, we assayed the level of LDH in the cell culture medium 90 min post-infection. LDH is a stable

243 cytoplasmic enzyme that is only released on loss of membrane integrity. The LDH level in the
244 supernatant of *ΔrfaC*-infected HeLa cells was more than twice that of the M90TS-infected cell
245 supernatant (Fig.3E).
246
247 **_rfaC_-deleted strain showed different actin-based motility from other LPS-truncated strains**
248 To examine the distribution of IcsA in the LPS-truncated mutants, intracellular F-actin of the
249 infected host cells were visualized by fluorescent phalloidin, which binds to F-actin of
250 mammalian cells. Although F-actins could be recruited by all the *Shigella* strains except the
251 *icsA*-deleted strain (Fig.4A and Fig. S3)，their cellular distribution varies between WT and LPS
252 mutants. Typical “actin comets” and long protrusion were observed with the wild type strain
253 (Fig.4A), indicating a normal polar distribution of IcsA, which was further confirmed by the
254 competence to form regular plaques on HeLa cell monolayer in a plaque assay (Fig.4B). By
255 contrast, the *Δwzy* and *ΔwaaL* mutants assembled F-actins all around the bacteria cell (Fig.S3),
256 implying a circumferential distribution of IcsA, which caused aberrant actin-based motility
257 (ABM) and thus failure to form plaques on HeLa cells monolayer (Fig.4B). The *ΔrfaC* bacteria
258 also recruited F-actin in a circumferential pattern as other LPS-truncated strains do.
259 Nevertheless, a small amount of bacteria could assemble short and curly “actin-tail” behind the
260 bacteria (as arrows indicated in Fig.4A). Although *ΔrfaC* mutant could not form classical plaques
261 on HeLa cell monolayer in 3 days, the integrity of the monolayer were destroyed due to massive
262 cell detachment (Fig.4B).
263

Truncation of LPS rendered bacteria susceptible in vitro and in vivo

LPS, especially long-chain LPS of gram-negative bacteria provides protection for the bacteria against unfavorable environment. While loss of LPS may contribute to biofilm formation and pathogenic activities such as adhesion and invasion, the adverse effects associated with LPS deletion must also be taken into consideration to accurately gauge the overall influences on bacterial fitness. To do this, we measured the susceptibility of the LPS mutants to distilled water (to mimic the low osmotic shock in environment) and 10% pooled human serum (to mimic the adverse environment *in vivo*). While WT bacteria well survived the low osmotic shock (water) during the experiment, all three LPS mutants showed severely compromised viabilities after two hours in water. The *ΔrfaC* mutant in particular, even begun to exhibit significantly reduced viability within 30min (Fig. 5A). Human serum showed much stronger killing activity than water toward *Δwzy*, *ΔwaaL* and *ΔrfaC* strains (Table 3). It is interesting to note that although most of the bacteria were killed by the serum, a small number of colonies were recovered for the *ΔrfaC* strain versus no colony for *Δwzy* and *ΔwaaL* strains (Fig. 5B). Whether this improved survivability of the *ΔrfaC* strain against serum is related to its biofilm-forming ability warrants further investigation.

Given the dramatic virulence-boosting phenotype of the *ΔrfaC* mutant in vitro, we next examined the in vivo effects of LPS mutants on virulence using two independent animal models. The Sereny test (guinea pig keratoconjunctivitis) indicated that *Δwzy, ΔwaaL* and *Δrfac* strains failed to cause any manifestation of conjunctivitis during a period of 2 weeks' infection. Wild type, on the other hand, provoked a typical keratoconjunctivitis 72 h post-infection (Table. 4). To

285 test the colonizing ability of the "super-adhesive" *ΔrfaC* mutant in the intestine, guniea pigs were
286 inoculated with 10^8 CFU of *Shigella* via intrarectal route. Feces analysis revealed that *Δwzy,*
287 *ΔwaaL* and *ΔrfaC* failed to colonize the intestines of guinea pigs, and bacteria were evacuated in
288 three days without any exhibited symptoms. Nevertheless, animals inoculated with wild type
289 stably excreted bacteria in 18 tested days, albeit without observed symptoms either (Fig. 5B).
290

291 **Discussion**

292 In the present study, we described a new and unusual phenotype of *Shigella flexneri* that is
293 characteristic of enhanced biofilm formation. Deficiency in Hep synthesis in LPS due to deletion
294 of *rfaC* gene resulted in "deep-rough" LPS with an incomplete inner core containing only the
295 Kdo moieties. Although various LPS truncations have been studied for their effects on the
296 biological activities of *Shigella*, the deep-rough mutant has never been tested. In this work, we
297 found that the deep-rough *Shigella* (*ΔrfaC*) exhibited significantly enhanced biofilm-forming
298 ability and strong adhesiveness toward host cells, which is in sharp contrast to the WT strain
299 (Brotcke Zumsteg et al. 2014; Kline et al. 2009; Mahmoud et al. 2015; Schroeder & Hilbi 2008).
300 Besides, this deep-rough mutant could efficiently proliferate in the host cells and cause extensive
301 cell lysis in few hours after infection. However, the pronounced *in vitro* virulence observed with
302 this mutant was not recapitulated in vivo, most likely due to its increased vulnerability to
303 environmental stress caused by LPS loss.
304

305 The biofilm of *ΔrfaC Shigella* showed a typical mesh-like structure and its formation is

306 dependent on extracellular DNA as proven by DNase I treatment experiment. Previous studies
307 have shown that LPS truncations in *E. coli* and *Porphyromonas gingivalis* also promoted biofilm
308 formation (Nakao et al. 2012; Nakao et al. 2006), suggesting that the correlation between LPS
309 loss and biofilm-forming capacity might be a common feature in gram-negative bacteria.
310 Bacterial surface hydrophobicity is a major determinant for biofilm formation(Donlan 2002;
311 Mitzel et al. 2016). LPS truncation reduced the hydrophilic sugar moieties from bacteria surface
312 and increased the exposure of the hydrophopic outer membrane lipid layer, thus enhancing the
313 hydrophobicity on bacteria surface and facilitating biofilm formation.
314
315 WT *Shigella flexneri* adheres to host cells much less efficiently *in vitro* than other enterobacteria
316 due to the lack of general adhesion apparatus like fimbriae (Edwards & Puente 1998; Pizarro-
317 Cerdá & Cossart 2006; Snellings et al. 1997). To our surprise, the *ΔrfaC* mutant exhibited
318 extraordinary adhesion to host cells and manifested normal invasion and proliferation
319 capabilities as well. The molecular mechanism of the hyperadhesiveness of *ΔrfaC* mutant
320 remains unclear. Interestingly, we noticed that the *ΔrfaC Shigella* tended to form clusters on
321 abiotic surface or host cells. Whether this cell-cell clustering is also a result of the improved
322 hydrophobicity caused by complete LPS shedding requires further investigation. Nevertheless, it
323 is conceivable that the clustered *Shigella* may adhere to host cells as one entity, in which
324 numerous weak contacts contributed by each individual bacterium are pooled to improve the
325 overall avidity of the bacterial cluster toward the host cell.
326

Previous studies have shown that the long-chain LPS was essential for maintaining polar distribution of IcsA(Doyle et al. 2015; Morona et al. 2003; Sandlin et al. 1995). Indeed, the *Δwzy* and *ΔwaaL* mutants with shortened LPS displayed circumferential distribution of IcsA. Interestingly, a small amount of *ΔrfaC* bacteria could assemble atypical, short and curly actin-tail, implying the heterogeneous distribution of IcsA in this new mutant. Results from plaque assay showed that *ΔrfaC* bacteria failed to form typical plaque on HeLa monolayer as the WT does, although it did caused mass host cell death eventually. This could be caused by a limited number of correctly assembled ABM in function or by premature killing of the host cell before the actin protrusions could reach adjacent cells due to much enhanced intracellular proliferation. How the *ΔrfaC* mutant retains certain level of functional IcsA warrants further investigation.

Although the *ΔrfaC* mutant showed a significantly boosted virulence in vitro, it failed to colonize in vivo. Susceptibility test against low osmotic shock or serum revealed that this mutant is highly sensitive to environmental stress. Clearly, the virulence benefit gained from biofilm formation and/or improved adhesion is not enough to compensate for the loss of fitness on survivability in vivo.

In conclusion，this study characterized a new *Shigella flexneri* mutant with deficiency in Hep synthesis of LPS. This mutant was capable of forming biofilm on abiotic surface and manifested extraordinary adhesiveness to host cells. Our study established a clear positive relationship between increased surface hydrophobicity and adhesiveness of *Shigella flexneri*. Although it is

not realistic for a pathogenic bacteria to shed off LPS to gain hydrophobicity in vivo, our study raised a possibility that the host adhesiveness of Shigella may be modulated by altering the hydrophobicity of the bacteria.

Acknowledgments We thank Dr. J Yu from Strathclyde Institute of Pharmacy and BioScience, University of Strathclyde (Glasgow, UK) for providing the *Shigella flexneri* 2a strain Sf301 and plasmids for λ Red recombination system. We thank P. Wang from the Laboratory of Electron Microscopy of School of Medicine and J. Yan from the Center for Biomedical Engineering and Regenerative Medicine of Frontier Institute of Science and Technology, Xi'an Jiaotong University, for their technical assistance with microscopic analysis.

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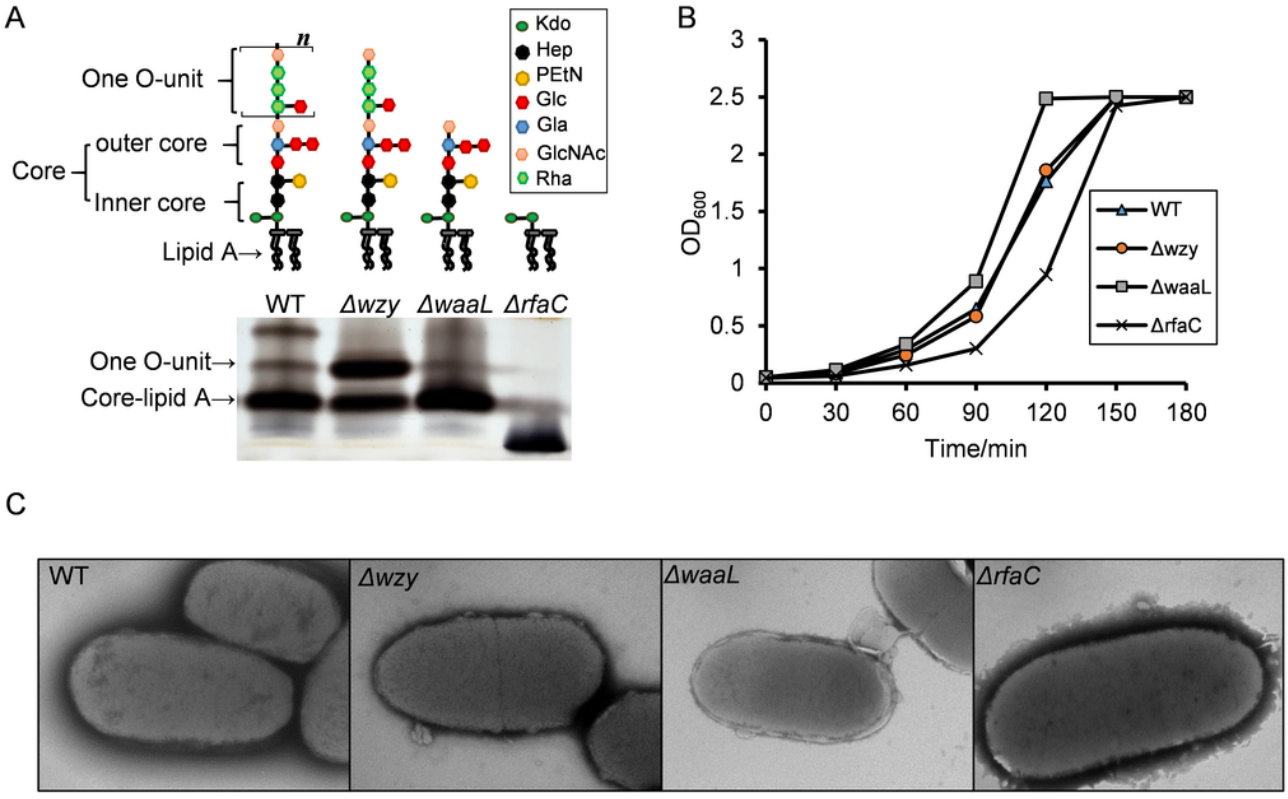
441

1

LPS structures, growth and TEM analysis of the *Shigella flexneri* LPS mutants in this study

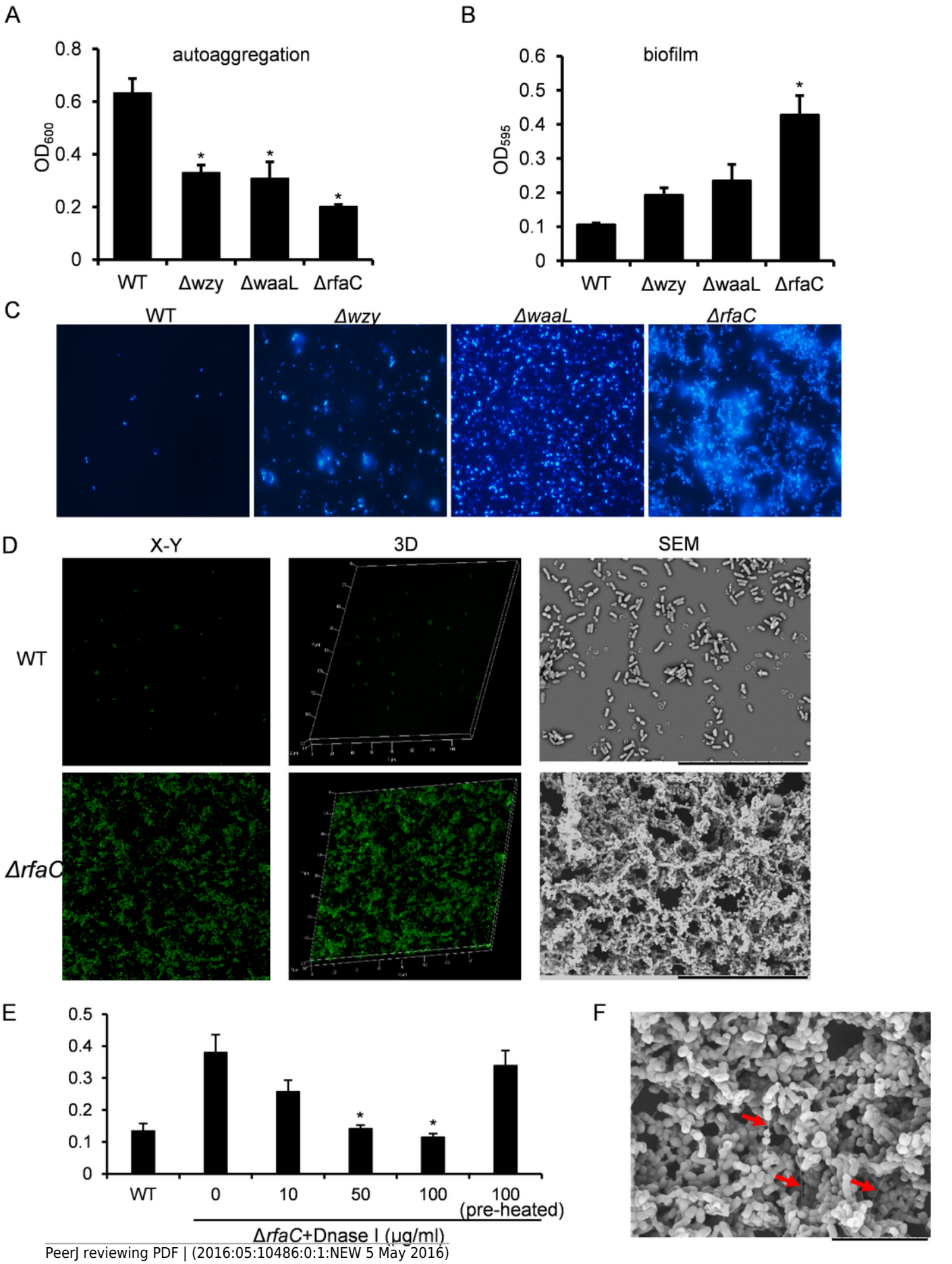
(A) Illustrative drawing of LPS structures of Sf301, *Δwzy*, *ΔwaaL* and *ΔrfaC* strains is showed in the upper panel. Kdo: 3-deoxy-D-mannooct-2-ulosonic acid; Hep, L-glycero-D-manno-heptose phosphate; PEtN, O-phosphoryl-ethanolamine; Glc, D-glucose; Gla, d-galactose; GlcNAc, N-acetyl-D-glucosamine; Rha, L-rhamnose. In the lower panel, LPS of these strains was analyzed by silver staining of polyacrylamide gel after SDS-PAGE. (**B**) Growth of Sf301 and the LPS mutants. Each strain was grown in TSB broth under shaking conditions at 37℃. Absorbance at OD600 was measured at different time points. (**C**) Representative transmission electron microphotographs (TEM) of each strain are shown. Overnight culture of each strains were collected and stained by with 1.5% phosphotungstic acid for 90s and examined under TEM. Arrows indicate the dark spots on the surface of *ΔrfaC* strain.



2

Analysis of biofilm formation of LPS mutants of Sf301

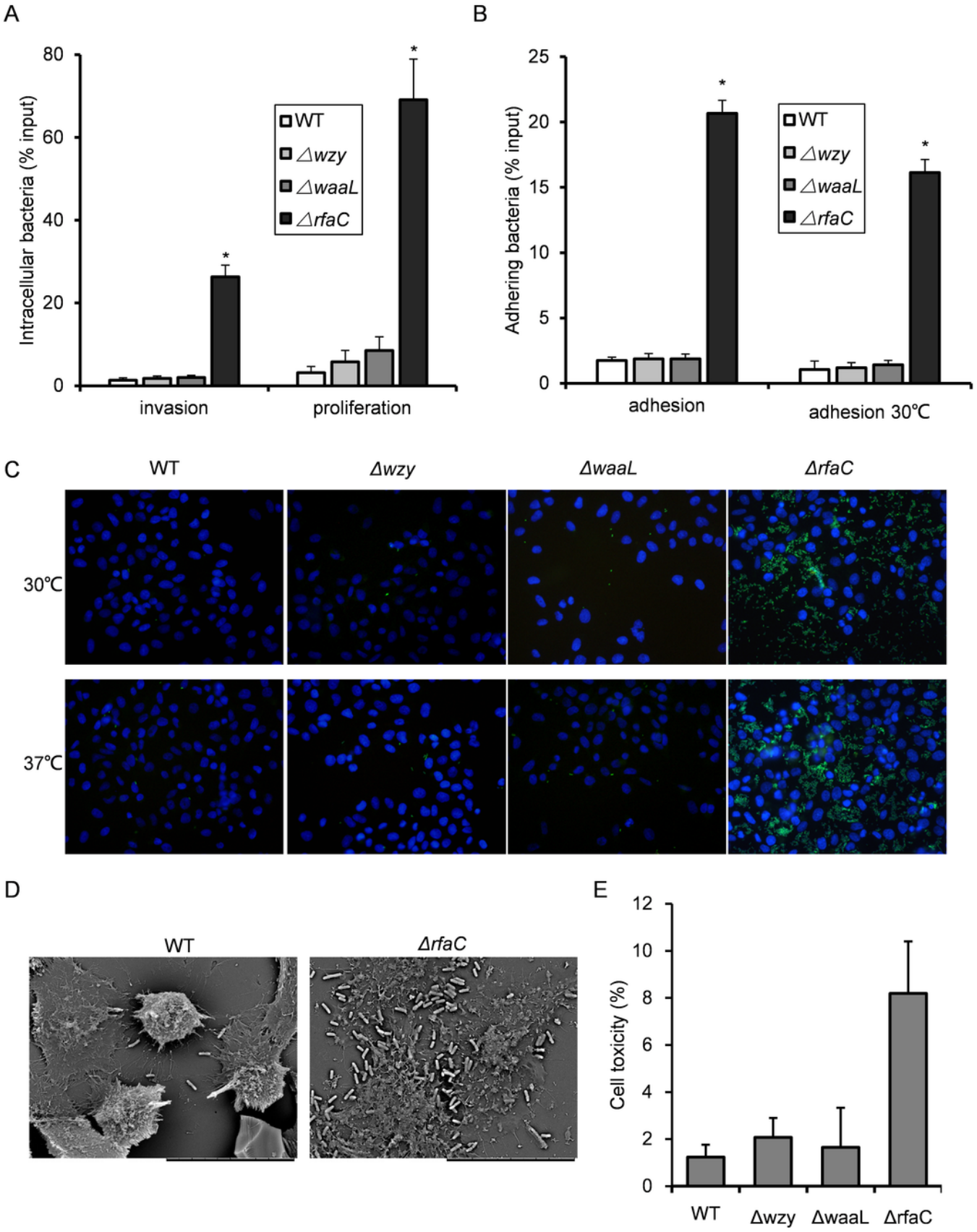
(A) Autoaggregation phenotype by LPS mutants. Each strain standardized at OD_{600}= 1.0 in PBS was used for autoaggregation assay. The value at OD_{600} after an18-hour incubation is shown as the mean ± SD of results from three independent experiments. Statistical analysis was performed using student t-test. *, P 0.01 against autoaggregation level of strain Sf301. (**B**) Biofilm formation by *Shigella flexneri* LPS mutants in 96-well flat-bottom polystyrene microtiter plate when compared to the parental strain. The mean ± SD of results from three independent experiments are shown. Statistical analysis was performed using student t-test. *, P 0.01, against biofilm formation level of strain Sf301. (**C**) Biofilms on glass cover slides of LPS mutants stained by DAPI. (**D**) CLSM and SEM analysis of biofilms formed by *ΔrfaC* strain. A section which has representative signals in the defined area is shown in the left column (X–Y). The overview of biofilms in the same area of each X–Y section is shown as 3D image in the middle column (3D). Biofilms under SEM are shown in the right column. (**E**) Influence of DNase I treatment on the biofilm formation of *ΔrfaC* strain. *ΔrfaC* strain was grown in presence of different concentrations of DNase I or in presence of pre-heated DNase I or without DNase I for 48 hours under static conditions at 37°C. Statistical analysis was performed using student t-test. *, P 0.01, against the biofilm formation by *ΔrfaC* strain without DNase I treatment. (**F**) biofilm formed by *ΔrfaC* strain under high-magnification (10000X) SEM. Arrows indicate the fibrous connections among bacteria.


A
autoaggregation
OD600
0.8
0.6
0.4
0.2
0
WT
Δwzy
ΔwaaL
ΔrfaC
B
biofilm
OD595
0.6
0.5
0.4
0.3
0.2
0.1
0
C
WT
Δwzy
ΔwaaL
ΔrfaC
D
X-Y
3D
SEM
WT
ΔrfaC
E
0.5
0.4
0.3
0.2
0.1
0
WT
0
10
50
100
100 (pre-heated)
ΔrfaC+Dnase I (μg/ml)
F

3

Invasion and adhesion ability of *Shigella flexneri* LPS mutants in comparison with the parental Sf301 strain

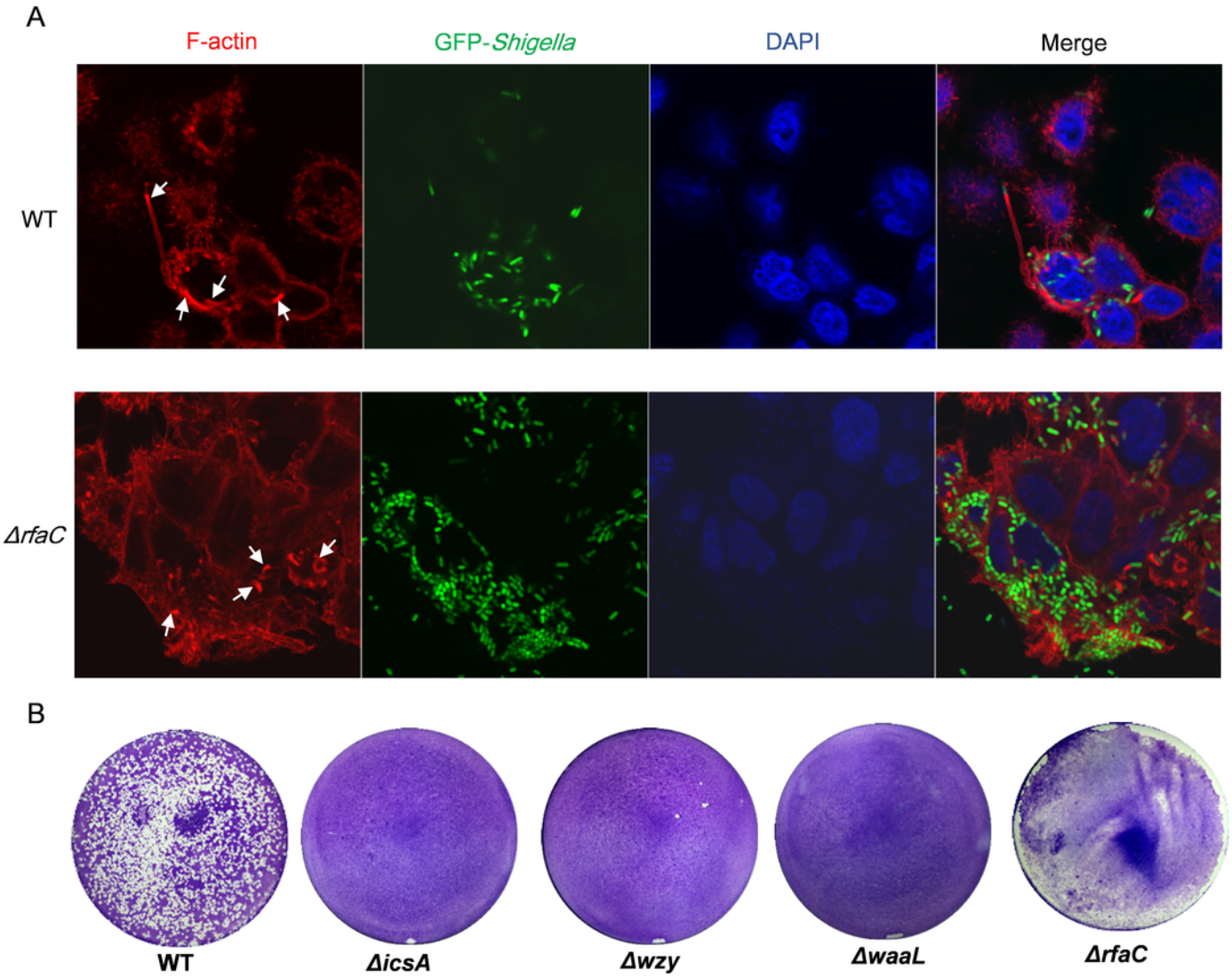
(**A**)Invasion and intercellular proliferation of Sf301 and its LPS mutants. (**B-C**) Initial adhesion of Sf301 and its LPS mutants at 30℃ and 37℃ to HeLa cells. Briefly, Bacteria were centrifuged onto HeLa cells (moi 10:1) to synchronize the infection. For adhesion assay, after washing, the cells were lysed with distilled water and the CFU was enumerated after plating. For invasion and proliferation assays, invasion was allowed to happen for 40min after centrifuge, followed by washing and 1-hour (invasion) or 4-hour (proliferation) incubation in gentamycin-containing (25μg/ml) medium before the cells were lysed with distilled water. Adhesion was defined as the total number of HeLa cell-associated bacteria and is shown as the percentage of input. Invasion and proliferation was defined as the total number of intracellular bacteria in HeLa cells. The mean ± SD of results from three independent experiments are shown. Statistical analysis was performed using student t-test. *, P 0.01. For fluorescence microscopy in **C**, GFP-expressing plasmid was electroporated into the *Shigella* strains, and adhesion assay was performed as described above, after washing, the cells were fixed in 3% paraformaldehyde/PBS and mounted with Anti-Fade solution (Invitrogen) containing DAPI onto glass slides. *Shigella* strains are in green and nuclei are blue. (**D**) SEM analysis of enhanced adhesion of *ΔrfaC S.flexneri* strain to HeLa cells. Bars in both images represent 30μm. (**E**) Levels of LDH in HeLa cell culture supernatant, 3h post-infection with *S. flexneri*. The mean ± SD of results from three independent experiments are shown. Statistical analysis was performed using student t-test. *, P 0.01.


A
Intracellular bacteria (% input)
80
60
40
20
0
WT
△wzy
△waaL
△rfaC
*
*
invasion
proliferation
B
Adhering bacteria (% input)
25
20
15
10
5
0
WT
△wzy
△waaL
△rfaC
*
*
adhesion
adhesion 30℃
C
WT
Δwzy
ΔwaaL
ΔrfaC
30℃
37℃
D
WT
ΔrfaC
E
Cell toxicity (%)
12
10
8
6
4
2
0
WT
Δwzy
ΔwaaL
ΔrfaC

4

Actin-based motility of *Shigella flexneri* LPS mutants in comparison with the parental Sf301 strain

(**A**) Fluorescence microscopy analysis of F-actin in HeLa infected by Sf301 and its *ΔrfaC* mutant strain. Invasion assay was performed as previously described. F-actin is stained by TRITC-phalloidin (red), *Shigella* bacteria are green and nuclei are blue (DAPI). White arrows in the upper panel indicate the long protrusion with actin comets assembled by Sf301. White arrows in the lower panel indicate the short actin-tail formed by the *ΔrfaC* mutant bacteria. (**B**) Plaque formation of Sf301 and its LPS mutants on HeLa cell monolayer. Plaque assays were performed as described in Methods and Materials.

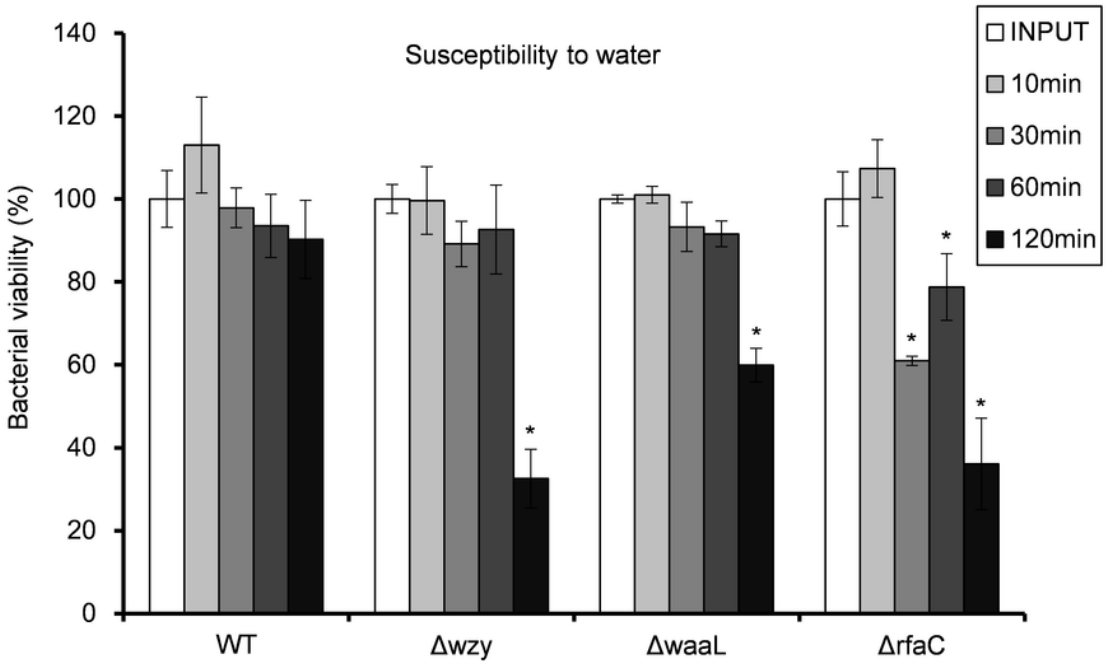

A
F-actin
GFP-Shigella
DAPI
Merge
WT
ΔrfaC
B
WT
ΔicsA
Δwzy
ΔwaaL
ΔrfaC

5

in vitro susceptibility to water (A) and *in vivo* colonization ability (B) of *Shigella flexneri* LPS mutants in comparison with the parental Sf301 strain.

(**A**)Bacteria from mid- exponential phase were collected and resuspended into distilled water at the density of 10^7CFU/ml, bacterial viability was evaluated as output CFU/input CFU X 100% at indicated time intervals. The mean ± SD of results from three independent experiments are shown. Statistical analysis was performed using student t-test. *, P 0.01, against bacterial survival level of sf301 wild type. (**B**) To assess the colonization ability of the *Shigella* strains, 10^8 CFU were inoculated into the rectum of guinea pigs, and bacterial load in the feces was determined by the number of colonies on MacConkey Agar as described by Methods and Materials. The mean ± SD of results from three individual animals are shown.

A


Susceptibility to water
Bacterial viability (%)
140
120
100
80
60
40
20
0
INPUT
10min
30min
60min
120min
*
WT
Δwzy
ΔwaaL
ΔrfaC

B

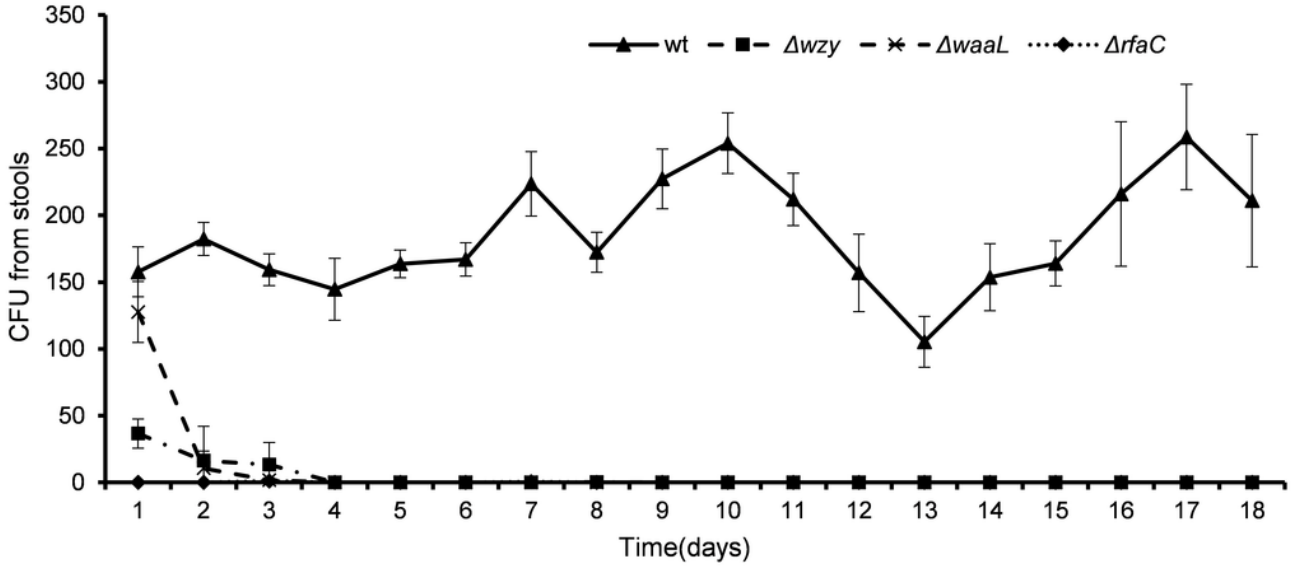

wt
Δwzy
ΔwaaL
ΔrfaC
CFU from stools
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
Time(days)

Table 1(on next page)

Strains and plasmids in this study

Strains	Relevant genetype, phenotype and description	reference
Shigella flexneri		
Sf301	*Shigella flexneri* 2a strain	(Jin et al. 2002)
301*ΔicsA*	Sf301 *ΔicsA* (+51 to +3255): KRC	This work
301*Δwzy*	Sf301 *Δwzy* (+26 to +1075): KRC	This work
301*ΔwaaL*	Sf301 *ΔwaaL* (+6 to +1155): KRC	This work
301*ΔrfaC*	Sf301 *ΔrfaC* (+33 to +832): KRC	This work
Plasmids		
pKD4	Template plasmid for λ Red recombination system	(Datsenko & Wanner 2000)
pKD46	λ Red recombinase expression plasmid	(Datsenko & Wanner 2000)
pCP20	λ Red FLP-recombinase expression plasmid	(Datsenko & Wanner 2000)

1

Table 2(on next page)

oligonucleotides used in this study

Name	Sequence	target
icsARedF	ATGAATCAAATTCACAAATTTTTTTGTAATATGACCCAATGTTCACAGGGGTGTAGGCT GGAGCTGCTTC	KRC targeting *icsA*
icsARedR	AAGGTATATTTCACACCCAAAATACCTTGGGTGTCTCTGTAACTGTTATTATGGGAATT AGCCATGGTCC	KRC targeting *icsA*
icsAF	GACCCAATGTTCACAGGG	*icsA*
icsAR	TGGGTGTCTCTGTAACTG	*icsA*
wzyRedF	ATAACTTCCCTATTTTTAACATCCTTTATTTTGCTCCAGAAGTGAGGTTAGTGTAGGCTG GAGCTGCTTC	KRC targeting *wzy*
wzyRedR	ATAACATTTTTATGTATTGAACTGATTATTGGTGGTGGTGGAAGATTACTATGGGAATT AGCCATGGTCC	KRC targeting *wzy*
wzyF	TTGCTCCAGAAGTGAGG	*wzy*
wzyR	GTGGTGGTGGAAGATTAC	*wzy*
waaLRedF	CTCAACATTATTTTTCTCTCTCGAGAAAAAAAAACTGGATAGCGTACTGGAGTGTAGGCT GGAGCTGCTTC	KRC targeting *waaL*
waaLRedR	TTGTTTTTCATCGCTAATAATAAGCCGGCGTAAACGCCTAATAAATTTGGATGGGAATT AGCCATGGTCC	KRC targeting *waaL*
waaLF	ACTGGATAGCGTACTGG	*waaL*
waaLR	TAAGCCGGCGTAAACGC	*waaL*

rfaCRedF	CACTGATGCCCAGCAGGCAATCCCAGGGATTAAGTTTGACTGGGTGGTGGGTGTAGGC TGGAGCTGCTTC	KRC targeting *rfaC*
rfaCRedR	AAGAGACATACTTGTAGAACGACACTCTACTTGATTCTTCCCATACCCACATGGGAATT AGCCATGGTCC	KRC targeting *rfaC*
rfaCF	GGGATTAAGTTTGACTGG	rfaC
rfaCR	GTAGAACGACACTCTAC	rfaC

1

Table 3(on next page)

Bacterial viability of the strains after two-hour serum killing

Treatment	Heated inactivated serum (%)	Pooled human serum (%)
WT	90.6±13.5	63.4±6.4
Δwzy	85.5±7.6	0
ΔwaaL	111.1±11.4	0
ΔrfaC	89.9±10.5	0.0023±0.0004

1

2

Table 4(on next page)

Scores of Sereny test

	WT			*Δwzy*			*ΔwaaL*			*ΔrfaC*		
	#1	#2	#3	#1	#2	#3	#1	#2	#3	#1	#2	#3
day1	2	2	2	0	0	0	0	0	0	0	0	0
day2	3	3	3	0	0	0	0	0	0	0	0	0
day3	3	3	3	0	0	0	0	0	0	0	0	0
day4	3	3	3	0	0	0	0	0	0	0	0	0
day5	3	3	3	0	0	0	0	0	0	0	0	0
day6	3	3	3	0	0	0	0	0	0	0	0	0
day7	3	3	3	0	0	0	0	0	0	0	0	0

1