The role of the *Shigella flexneri* yihE gene in LPS synthesis and virulence

Bryn Edwards-Jones,¹ Paul R. Langford,² J. Simon Kroll² and Jun Yu¹

¹Centre for Molecular Microbiology and Infection, Department of Biological Sciences, Imperial College London, South Kensington Campus, London SW7 2AZ, UK
²Molecular Infectious Diseases Group, Department of Paediatrics, Faculty of Medicine, Imperial College London, St Mary’s Campus, London W2 1PG, UK

Previously, the authors have shown that inactivation of *Shigella flexneri* yihE, a gene of unknown function upstream of *dsbA*, which encodes a periplasmic disulphide catalyst, results in a global change of gene expression. Among the severely down-regulated genes are *galETK*, suggesting that the yihE mutant, Sh54, may inefficiently produce the UDP-glucose and UDP-galactose required for LPS synthesis. This paper demonstrates that LPS synthesis in Sh54 is impaired. As a result, Sh54 is unable to polymerize host cell actin, due to aberrant localization of IcsA, or to cause keratoconjunctivitis in guinea pigs. Furthermore, Sh54 is more sensitive to some antimicrobial agents, and exhibits epithelial cytotoxicity characteristic of neither wild-type nor *dsbA* mutants. Supplying *galETK* in trans restores LPS synthesis and corrects all the defects. Hence, it is clear that the *Shigella yihE* gene is important not only in regulating global gene expression, as shown previously, but also in virulence through LPS synthesis via regulating the expression of the *galETK* operon.

INTRODUCTION

*Shigella flexneri* is a facultative intracellular pathogen causing acute inflammatory enteritis in man. Following invasion of epithelial cells, *S. flexneri* escapes from phagocytic vacuoles into the cytoplasm through the action of Ipa proteins (Sansonetti, 2001). Bacteria then spread intra- and intercellularly by recruiting the cellular actin polymerization machinery, via N-WASP, through the unipolar surface protein IcsA (Cossart, 2000).

Lipopolysaccharide (LPS) is a major component of the Gram-negative bacterial envelope. It consists of lipid A, as well as an inner and an outer core oligosaccharide to which a repeating O antigen region is attached. Loss of LPS has been shown in many organisms, including *S. flexneri* (Okamura et al., 1983; Rajakumar et al., 1994), to affect virulence as well as other phenotypes such as colony morphology, biofilm production and outer membrane permeability (Genevaux et al., 1999; Nesper et al., 2001).

A *galU* mutant of *S. flexneri*, the LPS of which consists of only the lipid A and inner core region, was found to invade epithelial cells well, but had lost its ability to disseminate through a epithelial cell monolayer owing to aberrant localization of IcsA (Sandlin et al., 1995). Instead of being directed to the old pole of the bacterium, IcsA appeared to be evenly distributed around the surface of the *galU* mutant.

As would be expected, this mutant was unable to form plaques in L2 cell monolayers and failed to provoke keratoconjunctivitis in guinea pigs.

YihE is a 38.1 kDa cytoplasmic protein of unknown function that is present in many enteric bacteria. yihE lies upstream from *dsbA* and is co-transcribed with it. Although *dsbA* is expressed independently from a σ^70_ promoter, the transcript that originates from the CpxRA-regulated promoter upstream of yihE continues through both genes. Mutation of yihE in *Escherichia coli* reduces the amount of DsbA present in the periplasm, but the folding of proteins that require DsbA, such as PhoA, is unaffected (Belin & Boquet, 1994). The CpxRA two-component signal transduction system senses and responds to envelope stress caused by accumulation of misfolded proteins by up-regulating the expression of *dsbA*, *ppiA* and *degP* (Otto & Silhavy, 2002). Monitoring the biogenesis of P-pili is one such example (Hung et al., 2001). In addition, Cpx is responsible for surface sensing and adhesion in *E. coli* (Otto & Silhavy, 2002).

On DNA array analysis, a yihE mutant (Sh54) of *S. flexneri* serotype 5a, which has a 398 bp internal deletion in yihE, shows a global change in gene expression during exponential growth (Li et al., 2001). Many genes involved in energy metabolism and carbon degradation are down-regulated while some stress-inducible genes are up-regulated, although *dsbA* is unaffected. Amongst the severely depressed genes are *galETKM*, the galactose utilization operon, the expression

Abbreviation: LDH, lactate dehydrogenase.
of which is reduced by as much as 11-2-fold. Depression of the gal operon to this degree may be expected to reduce the cell’s ability not only to utilize galactose, but also to produce UDP-glucose and UDP-galactose. As both of these compounds are required for production of the outer core of LPS, we initiated the present study to investigate the consequences of the yihE mutation on LPS phenotype.

**METHODS**

**Strains, plasmid and bacterial growth conditions.** M90TS is a virulent strain of *Shigella flexneri* serotype 5a, which has been widely used in virulence studies (Sansonnetti et al., 1986). Derived from M90TS, Sh4 has an inactivated *dsbA* (dsbA::kan) (Yu, 1998) and Sh54 a 398 bp internal deletion in *yihE* (Li et al., 2001). The plasmid, pAA101, a kind gift from Professor Steve Busby at the University of Birmingham, UK, harbours a 4-8 kb *E. coli* genomic fragment containing *galetK* with a native promoter in pBR313.

*S. flexneri* strains were grown at 37°C overnight on tryptic soy agar (TSA) (Oxoid) containing 0-1% Congo red. Red colonies were inoculated into tryptic soy broth and grown to early exponential phase for cell infection. Ampicillin was added to a final concentration of 200 µg ml⁻¹ to select for the presence of plasmid pAA101.

**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 125 µl lysis buffer (0-1 M Tris/HCl pH 6-8, 2% SDS, 4% β-mercaptoethanol and 10%, v/v, glycerol) and boiled for 10 min. Proteinase K (30 µg, Sigma) was added and the mixture incubated for 1 h at 60°C. Samples were run on a Tris-Tricine gel (Schagger & von Jagow, 1987) and visualized by silver staining (Tsai & Frasch, 1982).

**Antimicrobial sensitivity and growth supplement tests.** Antimicrobial sensitivity tests were performed in a microtitre plate in which the antibacterial agent had been serially (1:2) diluted from 100 µg ml⁻¹ to 0-097 µg ml⁻¹ (1:2 dilution from 20% in the case of SDS). From a mid-exponential phase culture, 100 µl of bacterial suspension was added to an OD₆₀₀ of approximately 0-05. The plates were incubated overnight at 37°C, and bacterial growth scored by eye. Glucose and galactose requirements were tested by growth at 37°C overnight on M9 minimal agar with either glucose or galactose (0-4% in each case) as sole carbon source.

**Infection of HeLa cells, cell stain and microscopy.** HeLa cells were cultured in minimal essential medium (MEM) (Gibco-BRL), supplemented with 10% fetal bovine serum, at 37°C under 5% CO₂. Gentamicin protection assays were carried out as described by Sansonnetti et al. (1986). Infection was stopped at the appropriate time by extensive washing with PBS, and intracellular bacteria were recovered by lysing cells with 0-5% sodium deoxycholate and plating out serial dilutions on TSA plates. For microscopy, cells were fixed with 3-7% paraformaldehyde for 20 min and then stained with Giemsa, or Bodipy FL phallacidin and a mouse anti-IcsA antiserum followed by Texas red-conjugated goat anti-mouse antibody (Molecular Probes). Observations were made with a BH2-RFCA fluorescence microscope (Olympus Optical) and an LSM-510 confocal laser scanning microscope (Zeiss).

**Plaque formation assay.** This was carried out according to Oaks et al. (1985) using confluent CaCo-2 cell monolayers.

**Lactate dehydrogenase (LDH) activity assay.** The LDH assay was performed using the Cytotox 96 non-radioactive cytotoxicity assay system (Promega), 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 TherMo Max Plate Reader (Molecular Devices) was used.

**Sereny test.** The Sereny test for guinea pig keratoconjunctivitis was used to test the virulence of the *S. flexneri* strains in vivo.

**RESULTS**

**LPS synthesis is impaired in Sh54**

As shown in Fig. 1, both M90TS and Sh4 (dsbA::kan) (Yu, 1998) exhibit the expected LPS pattern: a strong band at the bottom of the gel corresponding to the core with no O antigen attached, and a ladder pattern extending up the gel corresponding to an increasing number of attached O antigen repeats. However, Sh54 lacks a ladder of O antigen repeats and the core subunit is considerably smaller than that of M90TS. These results indicate that *yihE*, rather than *dsbA*, plays a role in LPS synthesis, most likely through depression of gal genes.

**galetK restores LPS synthesis in trans**

To demonstrate that depression of the *galetK* operon was the cause of the defective LPS synthesis, we transformed Sh54 with pAA101, a plasmid harbouring a 4-8 kb *E. coli* genomic fragment containing *galetK* with a native promoter in pBR313 (Steve Busby, University of Birmingham, personal communication). The presence of pAA101 was sufficient to reconstitute the galactose pathway, restoring...
the ability of Sh54 to grow on galactose as sole carbon source (Table 1). pAA101 also restored the Sh54 truncated LPS core to full length, enabling attachment of O antigen (Fig. 1, lane 4).

Outer membrane permeability of Sh54

It has been documented that altered LPS structure can affect outer membrane permeability (Nesper et al., 2001; Vaara, 1992), so we tested whether this was the case in Sh54. Sh54 did indeed show increased sensitivity to SDS, vancomycin, penicillin G and gentamicin (Table 1). The resistance to SDS, vancomycin and gentamicin was restored in Sh54/pAA101, suggesting that the cause of sensitivity in Sh54 was altered LPS structure. Sh54/pAA101 had high-level resistance to penicillin G because of the TEM1 β-lactamase expressed from the pBR313 plasmid backbone.

Intracellular motility of Sh54

galU and other LPS mutants all have defective intra- and intercellular motility due to aberrant localization of IcsA (Sandlin et al., 1995). We therefore examined the motility of Sh54 by staining S. flexneri-infected HeLa cells with Bodipy FL phallacidin. As shown in Fig. 2(a), M90TS was able to form characteristic actin comet tails in the host cells, indicating its ability to polymerize host actin to achieve intra- and intercellular spread. In contrast, the characteristic actin tails were not detected in Sh54-infected HeLa cells (Fig. 2b). Furthermore, using an anti-IcsA antibody, IcsA was detected on the entire bacterial surface of Sh54 within the host (Fig. 2b), indicating that Sh54 had similar aberrant localization of IcsA as the galU mutant reported previously (Sandlin et al., 1995). The same fluorescent labelling procedure, however, was not sensitive enough to detect IcsA on the surface of M90TS within HeLa cells (Fig. 2a).

Sh54 is cytotoxic to HeLa cells

In a gentamicin protection assay, Sh54 retained the typical invasive capacity of wild-type S. flexneri – 90 min post-infection, HeLa cells were densely populated with bacteria, comparable to M90TS-infected cells (Fig. 3a). Consistent with its invasive capacity, Sh54 secreted Ipa proteins to the

Table 1. Growth characteristics and chemical sensitivities of the S. flexneri strains used in this study

<table>
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<th>M90TS</th>
<th>Sh54</th>
<th>Sh54/pAA101</th>
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<td><strong>Growth on solid media</strong></td>
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<tr>
<td>MacConkey</td>
<td>++++</td>
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<td>Glucose*</td>
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<td>Galactose*</td>
<td>+/++</td>
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<td><strong>Chemical sensitivities (mg l&lt;sup&gt;-1&lt;/sup&gt;)</strong></td>
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<tr>
<td>Chloramphenicol</td>
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<td>0·10</td>
<td>0·10</td>
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<td>Gentamicin</td>
<td>25</td>
<td>12·5</td>
<td>25</td>
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<tr>
<td>Penicillin G</td>
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<td>0·39</td>
<td>&gt;100</td>
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<tr>
<td>SDS</td>
<td>5%</td>
<td>0·13%</td>
<td>5%</td>
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<td>Tetracycline</td>
<td>0·1</td>
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<td>Vancomycin</td>
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*Sole carbon source in M9 minimal medium. NG, No growth. †Minimal inhibitory concentration determination (mg l<sup>-1</sup>, unless otherwise stated).

Fig. 2. Fluorescence labelling and confocal microscopy of S. flexneri-infected HeLa cells. Bodipy FL phallacidin was used to stain actin filaments and an anti-IcsA antiserum was used to stain surface expressed IcsA followed by Texas red-conjugated goat anti-mouse antibody. (a) M90TS-infected HeLa cells. Arrows indicate comet tails of polymerized actin filaments formed by M90TS. The anti-IcsA antibody failed to detect the unipolarly expressed IcsA protein under the conditions used. (b) Sh54-infected HeLa cells. IcsA was detected on the entire surface of the bacteria so that all bacteria were fully fluorescently stained (arrows). No actin tails were observed. Bars, 100 μm.
same level as the wild-type (data not shown). However, viable counts for Sh54 at 90 min were about 100 times lower than for M90TS (Fig. 3c). This cannot be explained simply by the sensitivity of Sh54 to gentamicin (Table 1), because a time-course analysis indicated that intracellular viable counts of Sh54 were very similar to those of M90TS at 30 and 60 min post-infection (data not shown). We therefore suspected that intracellular Sh54 might cause loss of integrity of the host cell membrane, allowing penetration by gentamicin from the culture medium.

To investigate this possibility, we assayed the level of LDH in the cell culture medium 90 min post-infection. LDH is a stable cytoplasmic enzyme that is only released on loss of membrane integrity. The LDH level in the supernatant of Sh54-infected HeLa cells was more than twice that of the M90TS-infected cell supernatant (Fig. 3d) (Student’s t-test, \( P < 0.001 \)). Transformation of Sh54 with pAA101 resulted in significant complementation. Viable counts recovered for Sh54/pAA101 were about tenfold higher than for Sh54, although still nearly tenfold lower than for M90TS (Fig. 3c). The level of LDH in the culture supernatant of Sh54/pAA101-infected cells was slightly higher than with M90TS, but significantly lower than with Sh54 (\( P = 0.037 \)). Incubation of HeLa cell monolayers with culture supernatants from M90TS and Sh54 resulted in the release of similar low levels of LDH (data not shown), indicating that the unidentified cytotoxic factor(s) produced during Sh54 invasion was not released during growth in vitro.

**Sh54 is attenuated in virulence**

Sh54 formed no plaques on CaCo-2 cell monolayers, but complementation with pAA101 restored the formation of wild-type plaques (Fig. 4). The aberrant intracellular motility of Sh54 (Fig. 2) offered one explanation for the plaqueless phenotype, but not in a straightforward fashion, because Sh54 apparently possessed epithelial cytotoxic activity that led to self-destruction (Fig. 3). The Sereny test (guinea pig keratoconjunctivitis) results indicated that Sh54 did not cause any manifestation of conjunctivitis during a period of 2 weeks’ observation. M90TS, on the other hand, provoked a typical keratoconjunctivitis 72 h post-infection.

**Fig. 3.** (a) HeLa cell invasion by *S. flexneri* M90TS; (b) HeLa cell invasion by *S. flexneri* Sh54. Photos were taken 90 min post-infection. Bars, 250 \( \mu \text{m} \). (c) Intracellular viable counts of *S. flexneri* recovered from HeLa cells 90 min post-infection; (d) levels of LDH in HeLa cell culture supernatant, 90 min post-infection with *S. flexneri*. Error bars represent ±2SE.

**Fig. 4.** Plaque formation by *S. flexneri* strains. Complementation of Sh54 with pAA101 restored the formation of wild-type plaques. Experiments were performed using confluent CaCo-2 cell monolayers in 60 mm petri dishes, as described by Oaks et al. (1985).
DISCUSSION

We have previously shown, by DNA array analysis, that inactivation of yihE and dsbA results in different patterns of gene expression during exponential growth of S. flexneri (Li et al., 2001). Significantly, the galETK operon, involved in galactose utilization, is severely depressed in the yihE mutant, Sh54, but not in the dsbA mutants, Sh4 (dsbA::kan) and Sh42 (dsbA33G). This suggests that Sh54 may not produce sufficient UDP-glucose and UDP-galactose for LPS synthesis. In this study, we have demonstrated that Sh54 has altered LPS structure (Fig. 1). Expression of galETK in trans restored the wild-type LPS structure, confirming that galETK depression is the cause of defective LPS production. The truncated LPS in Sh54 is likely to be identical to that of a previously reported galU mutant, which also lacked UDP-glucose and UDP-galactose at the inner core after the second heptose residue (Sandlin et al., 1995). However, unlike the ‘rough’ galU strain (Sandlin et al., 1995), Sh54 formed small, smooth colonies on solid media and grew slowly in broth (data not shown). These phenotypes are consistent with the fact that many genes involved in small molecule metabolism, in addition to galETK, including those in the tricarboxylic acid cycle, are down-regulated in Sh54 (Li et al., 2001).

With a truncated LPS, Sh54 is more sensitive than the wild-type to various antimicrobial agents (Table 1). It has been well documented that a ‘deep rough’ LPS structure can increase permeability to hydrophobic agents such as SDS and vancomycin, possibly by increased levels of phospholipids in the outer leaflet of the outer membrane (Nesper et al., 2001; Vaara, 1992). Changes in LPS structure leading to increased sensitivity to gentamicin, possibly through an increase in self-promoted uptake, have also been documented (Genevaux et al., 1999). This suggests that increased sensitivity to penicillin G may be due to subtle perturbations in the overall structure of the outer membrane. Transcriptome analysis revealed no significant difference between M90TS and Sh54 in the expression of the major porins (Li et al., 2001), and the SDS-PAGE outer membrane protein profiles of M90TS, Sh54 and Sh54/pAA101 were identical (data not shown).

It is now clear that yihE and dsbA play different roles in modulating Shigella virulence, though their expression is subjected to the same transcriptional regulation by cpxRA (Belin & Boquet, 1994). yihE mainly dictates actin-based cellular motility via its role in LPS synthesis, through regulation of the galETK expression that determines surface expression of IcsA (Fig. 2). DsbA, on the other hand, is vital for intracellular survival and growth (Yu, 1998), though its key role in the secretion of Ipa proteins into the protrusions also affects cell-to-cell spread, and this, in part, contributes to the cellular growth defect of the mutants (Yu et al., 2000).

Sh54 possesses an unusual epithelial cytotoxic phenotype, which is characteristic of neither wild-type nor dsbA mutants (Fig. 3). As the supernatant from Sh54 culture has no epithelial cytotoxicity, the undiscovered cytotoxic substance(s) is more likely to be produced after bacterial entry into the host cells. Despite having an epithelial cytotoxic phenotype in vitro, Sh54 is attenuated, as shown by the guinea pig keratoconjunctivitis model. In the light of the recent finding that LPS is required for epithelial adherence and activation of the ERK pathway leading to production of IL-8 (Köhler et al., 2002), the attenuation of Sh54 in vivo is likely to be multi-factorial.

How inactivation of yihE leads to galETK depression, and indeed a global change of gene expression, is an important question to answer for a better understanding not only of Shigella virulence but also of the bacterial stress response and bacterial physiology in general. It is known that the cpxRA two-component signal transduction system and $\sigma^{E}$ work together to govern the extracytoplasmic stress response caused by accumulation of misfolded proteins (Raivio & Silhavy, 1999). Many genes in the cpxRA regulon encode either protein-folding catalysts (such as DsbA, which catalyses disulphide bond formation, and PpiA, which catalyses peptidyl-prolyl cis/trans isomerization), or proteases (such as DegP), which respond directly to the presence of misfolded proteins in the periplasm. The YihE protein, on the other hand, has been predicted to be cytoplasmic (Belin & Boquet, 1994). So YihE probably plays a regulatory role in the bacterial envelope stress response, which is consistent with inactivation of yihE triggering a global change of gene expression (Li et al., 2001).

To our knowledge, this is the first study that relates YihE, via regulation of the gal operon, with a phenotype showing an impaired pattern of LPS synthesis. Further functional studies on YihE are required for a full explanation of the phenotype observed.

ACKNOWLEDGEMENTS

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