Supporting Online Material

Materials and Methods

Strains, plasmids and bacterial growth media

The bacterial strains and plasmids used in this study are described in Table S4. *S. flexneri* was grown in trypticase soy (TCS) broth, Luria Bertani (LB) broth, or on TCS agar plates supplemented with 0.01% (wt/vol) Congo Red (Sigma). The mutant library was constructed by independently mating pUTmini-Tn*5*Km2 plasmids carrying pre-selected signature tags (S1) into *Shigella flexneri* M90T (serotype 5a).

DNA manipulations

Plasmid DNA was extracted using the Flexi Prep method (Amersham) in accordance with manufacturer's instructions. For Southern hybridisation, DNA was immobilised onto nylon membranes by alkaline transfer, and probes radiolabelled with [α-^{32}P]dATP using the random primers method (New England Biolabs). Signature tags were initially amplified from chromosomal DNA (100 ng) or plasmid DNA (10 ng) in the presence of non-radioactive dNTPs (200 μmol each). PCR products were purified (PCR purification kit, Qiagen), and 5 μl used as target in second round PCRs that included [$^{32-}$P]-dCTP to radiolabel the tags. Complementation was performed by the conjugal transfer of pBBR1MCS-4 (S2) harbouring the complete gene of interest amplified using a proof-reading polymerase (all oligonucloetide primers used in this study are shown in Table S5). The nucleotide sequence of plasmids was determined using a Perkin Elmer ABI Prism 377 sequencer.

Deletion mutants were constructed by modification of the one-step method (S3). The target sequences were amplified from M90T with approximately 850-1000 bp of flanking DNA. The product was then ligated into the vector pACYC184 and the resulting plasmid transformed into *E. coli* DH5α harbouring pKD46. The kanamycin resistance gene of pKD4 was amplified by PCR using primers with an additional 50 bp of *Shigella* target sequence. The PCR product was electroporated into *E. coli* where it recombined within the corresponding sequence in the pACYC184 derivative, replacing the *Shigella* operon with the kanamycin resistance gene. Amplification of this sequence resulted in the Km^r gene flanked by 850 bp *Shigella* sequence for efficient recombination after introduction into M90T containing pKD46. The kanamycin resistance marker was subsequently removed as described (S3). Mutants were analysed by PCR to confirm the deletion.

Rabbit ligated ileal loop model

New Zealand White rabbits weighing 2.5–3 kg (Charles River Breeding Laboratories, Wilmington, MA) were used for experimental infections. From each animal, up to 12 intestinal ligated loops, each 5 cm in length, were prepared as described previously (S4). Bacteria were injected in 0.5 ml of saline into each loop. For STM or for evaluation of the competitive index (C.I.) of a mutant, each loop received a total dose of 10^5 CFU, and colonisation-defective mutants were identified by their failure to be recovered 16 h later. Animals were sacrificed at this time, the lumenal fluid aspirated, and *S. flexneri* recovered. C.I. was calculated as the proportion of mutant to wild-type bacteria recovered from animals, divided by the proportion of mutant to wild-type in the inoculum (S5), and results are expressed as the mean of at least 4 loops from two independent animals for each strain tested. For evaluation of virulence, 10^9 CFU of bacteria was injected into each loop, and left for 8 h before animals were sacrificed.

Ileal loops were dissected, a segment was opened longitudinally, and fixed in 4 % buffered formalin. Sections (5 μm) were obtained, and following haematoxylin-eosin-safranin (HES) and Giemsa staining, histopathological observation and analysis was performed. For each mutant and control strains, at least four samples originating from four different rabbits were examined.

Lipopolysaccharide purification and electrophoresis

Lipopolysaccharide (LPS) was extracted from *S. flexneri* as described previously (S6), with the exception that LB broth was used as growth media. LPS profiles were determined by Tricine-SDS Polyacrylamide gel electrophoresis (TSDS-PAGE) and was visualised by silver staining (S7).

Slide agglutination assays

Hydrophobicity assays were performed as described previously by mixing bacteria grown to mid log (5 $x10^9$ CFU/ml in PBS) with increasing concentrations of ammonium sulfate from 0.0625M to 2M in duplicate (S8). Serotype specific sera (Serotec) were used to examine strains for changes in LPS.

Bactericidal assays of S. flexneri

Bacteria were grown in liquid culture to mid-log phase (O.D. A_{600} of 0.6), enumerated, spun at 12,000 *xg* for 1 minute, then re-suspended in physiological saline. Equal volumes of the bacterial suspension and serial dilutions of the test solutions were combined in wells of a microtitre dish. HBD-1 and HNP-1 (Sigma) assays were performed in 0.5% tryptone. Following one to two h incubation at 37°C, the number of surviving bacteria was determined by plating, and results expressed as

the percentage of the original inoculum. All assays were performed at least in triplicate.

Tissue culture

Invasion assays were performed with HeLa cells seeded onto glass coverslips in 12-well tissue culture plates (Life technologies) and grown until semi-confluent. *S. flexneri* strains were diluted from overnight liquid cultures and grown at 37°C until they had reached an optical density at A_{600} of 0.4 (S9). Cells were infected with an MOI of 100. After 10 min (for initial adhesion) and two hours (for invasion), cells were washed three times, fixed then stained for bacteria before and after permeabilization; external and internalised bacteria were then enumerated by microscopy.

Transmission electron microscopy

In order to reveal bacterial surface sugars with the highest possible resolution, two electron microscopy procedures were used. (a) For ruthenium red, bacteria were harvested by centrifugation (8000 *xg*, 5 min.) and washed in PBS (S10). Pellets were fixed in 3 % (w/v) glutaraldehyde, 0.075 % (w/v) ruthenium red in cacodylate buffer 0.1 M for one hour in the dark at room temperature. Samples were then washed three times in cacodylate buffer with ruthenium red, post-fixed for two hours in the dark in 1 % (w/v) osmium tetroxide, 0.075 % (w/v) ruthenium red, then washed twice in cacodylate buffer and water. Samples were then dehydrated through graded ethanol series of 10, 30, 50, and 95 % for 15 min each, then washed twice for 15 min each in pure ethanol, then twice in propylene oxide. Samples were finally re-suspended in 1/1 propylene oxide/spurr resin overnight and transferred to pure spurr resin that

polymerized at 60°C for 48 hours. Blocks were thin sectioned, post-stained with uranyl acetate and Reynold's lead citrate. Observations were performed on a Jeol JEM 1010 electron microscope at 80 kV. (b) Alternatively, samples were cryofixed by slam-freezing with a Reichert MM80 on a gold metal mirror at –196°C with liquid nitrogen (S11). Substitutions were performed for 16 hours at –90°C in pure anhydrous acetone containing 2.5% osmium tetroxide followed by temperature rises of 5°C:hour to 4°C and final incubation was carried out in spurr at –20°C. After infiltration and polymerisation, samples were thin-cut and examined. For negative staining, cultured bacteria in their exponential phase were absorbed on copper grids freshly glow discharged and negatively stained with uranyl-acetate 2% following two brief and gentle washings with distilled water.

Scanning and immunogold electron microscopy

For scanning electron microscopy (SEM) analysis, cells were applied to a glass coverslip, and fixed with 0.1% glutaraldehyde/4% paraformaldehyde (vol/vol) in 0.1M Sorensen buffer (pH 7.2) at 4°C for 30 min. The fixed bacteria were incubated in PBS supplemented with 0.25% NH_4Cl for 20 min then washed extensively with PBS. Samples were incubated in Protein Block (Aurion) for 30 min. Following incubation at 25°C for 1h with the primary antibody (anti-IpaB mAb H16 at a 1:150 dilution), the samples were washed and incubated for an additional 1h with the secondary antibody conjugated to 10 nm colloidal gold. Preparations were washed with PBS and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight at 4°C, then washed three times for 5 min (each time) in 0.2 M cacodylate buffer (pH 7.2), post-fixed for 1 h in 1% (wt/vol) osmium tetroxide in 0.2 M cacodylate buffer (pH 7.2), and finally rinsed with distilled water. Bacteria were

dehydrated through a graded series of ethanol (25, 50, 75 and 95% for 5 min each concentration). Samples were then dehydrated for 10 min in 100% ethanol followed by critical point drying with CO_2.

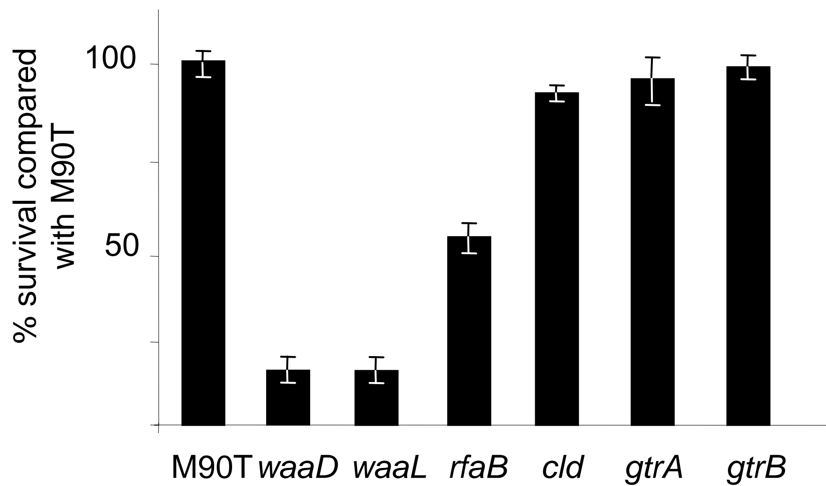
Dried specimens were sputter coated twice with carbon, with a BALTEC MED010 evaporator and were examined and photographed with a JEOL JSM 6700F field emission scanning electron microscope operating at 5 or 7 Kv. Images were acquired simultaneously from the upper SE detector and the YAG BSE detector.

Molecular Modeling.

Models of two O-specific polysaccharides corresponding to *S. flexneri* 5a and unglucosylated serotypes were obtained as follows. A 3D structure of tetra- (non-glucosylated) or penta- (serotype 5a) saccharide built at SWEET server (S12) was taken as a starting point for the construction. It was then manually corrected for glycosidic ϕ,ψ dihedral angles obtained from NMR data (S13, S14) for unglucosylated and branched glucosylated oligosaccharide, using Accelrys Inc. InsightII software. For each serotype a structural model containing its respective 15 repeating units was built interactively and submitted to energy minimization under dihedral angle constraints using Accelrys Discover program with AMBER-Homans force field.

Figure S1

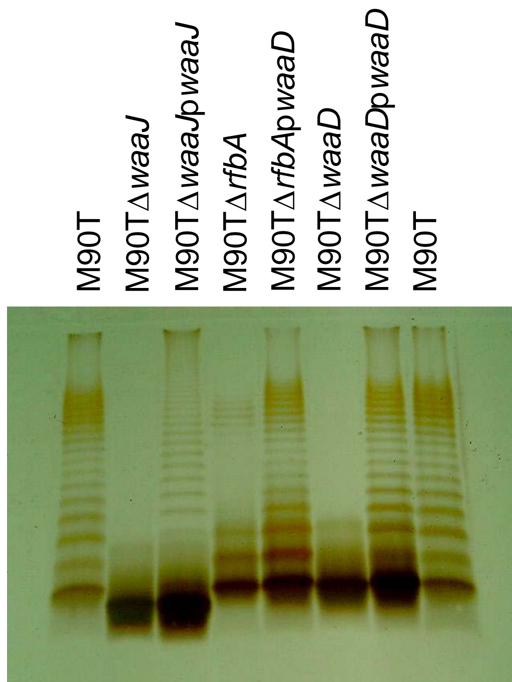
Sensitivity of LPS mutants to HNP-1, 2.5 μg/ml



Sensitivity of strains to HNP-1 (2.5 μg/ml). Bacteria were incubated with HNP-1 for 1 hr and the number of surviving bacteria determined by plating to solid media. Assays were performed in triplicate, and the results are expressed as the percent survival compared with the wild-type isolate, M90T. The error bars show the standard deviation.

Figure S2

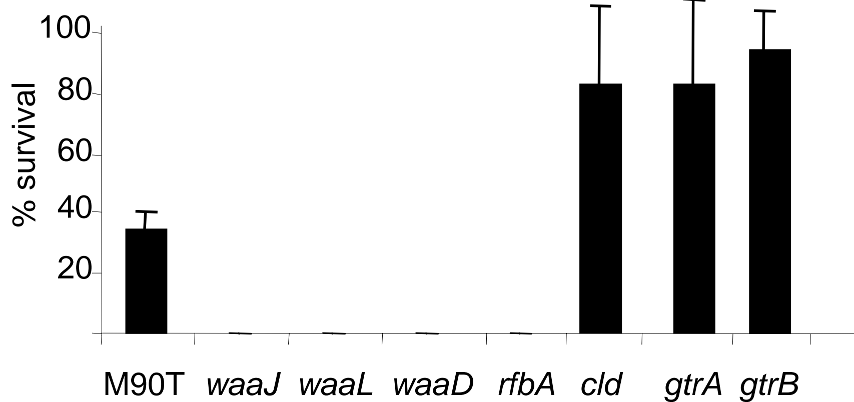
LPS profile of mutants and complemented strains



TSDS-PAGE analysis of mutants affected in LPS biosynthesis and the complemented strains (indicated above each lane).

Figure S3

Sensitivity of strains to human complement



Sensitivity of strains to 10% human complement. Bacteria were incubated with normal human serum (pooled from six donors) for 1 hr and the number of surviving bacteria determined by plating to solid media. Assays were performed in triplicate and the results are expressed as the percent survival compared with the number of the bacteria in the inoculum. The error bars show the standard deviation.

Table S1 Competitive index of complemented mutants

Strain	CI
M90TΔ*waaJ*p*waaJ*	0.7
M90TΔ*waaL*p*waaL*	0.65
M90TΔ*rfbA*p*rfbA*	0.88

Table S2 Slide agglutination confirms serotype switching of strains

Strain	Agglutination with antisera against		
	1a	2a	5a
M90T	-	-	+
2457T	-	+	-
13737	+	-	-
M90TΔ*gtrA*	-	-	-
M90TΔ*gtrB*	-	-	-
M90TΔ*gtrV*	-	-	-
M90TΔ*gtr*	-	-	-
M902a	-	+	-
M90TΔ*gtr* p1a	+	-	-
M90TΔ*gtr* p2a	-	+	-
M90TΔ*gtr* p5a	-	-	+

+ indicates agglutination, - indicates lack of agglutination.

Table S3 Slide agglutination assay

Strain	Concentration of ammonium sulphate		
	0.5M	1M	2M
M90T	n.d.	n.d.	5 min
M90TΔ*gtrA*	n.d.	n.d.	5 min
M90TΔ*gtrB*	n.d.	n.d.	5 min
M90TΔ*waaL*	7 min	1 min	1 min

Results show the mean time to aggregation visible to the naked eye
n.d., indicates agglutination was not detectable after 30 min observation.

Table S4 **Initial adherence of strains**

Strain	No. of adherent bacteria/cell
M90T	0.68 (0.08)
M90TΔ*gtr*	0.93 (0.07)

The results represent the number of external bacteria per epithelial cell, with standard deviations shown in brackets. The results are from three separate experiments and were derived from scoring at least 300 cells.

Table S5 **Bacterial strains and plasmids**

Strain/plasmid	Description	Source or reference
S. flexneri		
M90T	*Shigella flexneri* serotype 5a, wild–type	Infect. Immun. 1982 **35:** 852
BS176	Virulence plasmid cured derivative of M90T	Microb. Pathog. 1987 **1:** 53
M90T Nal^R	Nalidixic acid resistant M90T	
2457T	*Shigella flexneri* serotype 2a, wild–type	NCTC
13737	*Shigella flexneri* serotype 1a	NCTC
M90TΔ*waaJ*	M90T containing Tn*5* in *waaJ*	This study
M90TΔ*waaL*	M90T containing Tn*5* in *waaL*	This study
M90TΔ*waaD*	M90T containing Tn*5* in *waaD*	This study
M90TΔ*rfbA*	M90T containing Tn*5* in *rfbA*	This study
M90TΔ*waaJ*p*waaJ*	Complemented mutant	This study
M90TΔ*waaD*p*waaD*	Complemented mutant	This study
M90TΔ*rfbA*p*rfbA*	Complemented mutant	This study
M90TΔ*gtrA*	M90T containing Tn*5* in *gtrA*	This study
M90TΔ*gtrB*	M90T containing Tn*5* in *gtrB*	This study
M90TΔ*gtrV*	Deletion mutant of *gtrV* in M90T	This study
M90TΔ*cld*	M90T containing Tn*5* in *cld*	This study
M90TΔ*gtr*	M90TΔ*gtrABV*	This study
M902a	M90TΔ*gtr* transformed with *gtrABII*$^+$ (from 2457T)	This study
M90TΔ*gtr* p1a	M90TΔ*gtr* containing pNW78	This study
M90TΔ*gtr* p2a	M90TΔ*gtr* containing pNW77	This study
M90TΔ*gtr* p5a	M90TΔ*gtr* containing pNW86	This study
E. coli		
E. coli DH5α	*F' endA1 supE44 thi-1 hsdR17*(rK$^-$mK$^+$) *recA1 gyrA relA1* Δ*(lacIZYA-argF)U169 deoR* (ϕ *80 dLac*δ*(lacZ)M15)*	Gibco-BRL
E. coli S17-1 λ *pir*	*recA thi pro hsdR*$^-$ *M*$^+$ RP4::2-Tc::Mu::Km Tn*7* lysogenized with λ *pir* phage	J. Bacteriol. 1998 **170:** 2575

Table S5 (cont) **Bacterial strains and plasmids**

Strain/plasmid	Description	Source or reference
DH *gtrV*	pNW81 in DH5α	This study
pCR2.1 Topo	TA cloning vector	Invitrogen
pUTMiniTn*5*Km2	Tn5 transposon on suicide vector	J. Bacteriol. 1990 **172:** 6568
pBBR1MCS-4	Complementation vector	Gene 1995 **166:** 175
pNW83	pBBR1MCS-4 containing *gtrABV*	This study
pNW77	pCR2.1 Topo containing *gtrABII*$^{=}$ (from 2457T)	This study
pNW78	pCR2.1 Topo containing *gtrABI*$^{=}$ (from 13737)	This study
pNW81	*gtrV* operon in pACYC184	This study
pNW86	pCR2.1 Topo containing *gtrABV*$^{=}$ (from M90T)	This study
p*waaD*	*waaD* in complementation vector	This study
p*waaJ*	*waaJ* in complementation vector	This study
p*rfbA*	*rfbA* in complementation vector	This study
pKD46	encoding red recombinase	PNAS 2000 **97:** 6640
pKD4	Amplifyication of kan^{R}	PNAS 2000 **97:** 6640
pACYC184	Cloning vector	Nucleic Acids Res. 1988 **16:** 355

Table S6 Oligonucleotides used in this study

Primer	Sequence (5′ to 3′)	Purpose
pr9	TCCAACCCTATGAATACTAC	complementation of *waaJ*
pr10	AAGGATCAATTATCAGAATCG	complementation of *waaJ*
pr11	TTGCTGGTGGTACAACAACC	complementation of *rfbA*
pr12	TAGCACATCAGGAATTTCAG	complementation of *rfbA*
pr15	TTGAACCAAATTTATTAGGCG	complementation of *waaD*
pr16	TGGCATGATTGGGCGAACTAC	complementation of *waaD*
pr21	CTACGGCTGTAAGGGAATG	complementation of *cld*
pr22	GCGGTGTGTTTTTTTACAGGACG	complementation of *cld*
pr27	AAGCTTAACCTACGACCTTCGCATTACG	Amplification of *gtr* operon
pr30	GGATCCTATTGATGCAAAACTTAGC	Amplification of *gtr* operon
pr31	TTAAAACATCATATGTAAAAAAACTATTTTTCT TAATTGGTGTATTTTTCATTGCAGTTATTTTGA TGTATGTGTAGGCTGGAGCTGCTTCG	amplification of kan^r
pr32	TTAAGGCTGTTTTTTTATTAATGTCATCGTCCA TCCTGCAGGGTTGAACTTGAAAGAATACGATT CTCCTCATATGAATATCCTCCTTAG	amplification of kan^r
pr33	AGACATAGCAAGTATCGACCAATTGAGACTTG GATGATAGACTTCATGCCTTTCAGAGCTCATTG ATTAAATAAGTGTAGGCTGGAGCTGCTTCG	amplification of kan^r
pr38	TTGGATCCTCTTGTCGTTCCTGTCTTC	deletion of the *gtr* operon
pr37	TTGGATCCAGTGCGGTCAGTTGAATGG	deletion of the *gtr* operon.
pr43	GGATCCTTACGACAACGCTCCGATGG	amplification of *gtr* operon of *S. flexneri* 1a
pr44	GGATCCAGGTTCCCGATGACATTCTCAAG	amplification of *gtr* operon of *S. flexneri* 1a
pr45	GCATGCTGTTACGACAACGCTCCGATG	amplification of *gtr* operon of *S. flexneri* 2a
pr46	GCATGCCAGAGGCAAAATACCTGGGGAC	amplification of *gtr* operon on *S. flexneri* 2a

References for Supplemental data

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