Published in final edited form as:

Nature. 2010 May 20; 465(7296): 355–358. doi:10.1038/nature08970.

Modulation of *Shigella* virulence in response to available oxygen *in vivo*

Benoit Marteyn¹, Nicholas West^{1,†}, Douglas Browning², Jeffery Cole², Jonathan Shaw³, Fredrik Palm⁴, Joelle Mounier⁵, Marie-Christine Prévost⁶, Philippe Sansonetti^{5,7}, and Christoph Tang¹

¹Centre for Molecular Microbiology and Infection, Department of Microbiology, Flowers Building, Imperial College London, London, SW7 2AZ, UK

²School of Biosciences, The University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

³Division of Genomic Medicine, University of Sheffield Medical School, Beech Hill Road, Sheffield, S10 2RX, UK

⁴Department of Medical Cell Biology, Biomedical Center, Uppsala University, 751 23 Uppsala, Sweden

⁵Unité de Pathogénie Microbienne Moléculaire, and Unité INSERM786, Institut Pasteur, 28 rue du Dr Roux, F - 75724 Paris Cédex 15, France

⁶Plate forme de Microscopie électronique, Institut Pasteur, 25 Rue du Docteur Roux, F-75724 Paris cedex 15, France

⁷Collège de France, 11 Place Marcelin Berthelot, F-75231, Paris Cédex 05, France

Abstract

Bacteria co-ordinate expression of virulence determinants in response to localised microenvironments in their hosts. Here we show that *Shigella flexneri*, which causes dysentery, encounters varying oxygen concentrations in the gastrointestinal (GI) tract, which govern activity of its type three secretion system (T3SS); the T3SS is essential for cell invasion and virulence¹. In anaerobic environments (*e.g.* the GI tract lumen), *Shigella* expresses extended T3SS needles while reducing Ipa (Invasion plasmid antigen) effector secretion. This is mediated by FNR, a regulator of anaerobic metabolism that represses transcription of *spa32* and *spa33*, virulence genes that the switch in secretion through the T3SS. We demonstrate there is a zone of relative oxygenation adjacent to the GI tract mucosa, caused by diffusing from the capillary network at the tips of villi. This would reverse the anaerobic block of Ipa secretion, allowing T3SS activation at its precise site of action, enhancing invasion and virulence.

Shigella virulence depends on its ability to enter epithelial cells by delivering Ipa effectors *via* its T3SS into the host cell cytoplasm¹. Secretion through T3SSs is highly regulated. Initially, T3SS needle components are secreted until it reaches a pre-defined length²³. In inducing conditions, a switch then occurs allowing Ipa secretion through needles, mediating bacterial entry⁴.

For correspondence: Christoph M. Tang and Philippe Sansonetti, Tel: (44)-207-594-3072 and (33) 1-40-61-30-95, Fax: (44)-207-594-3076 and (33)-1-45-68-89-53, c.tang@imperial.ac.uk and psanson@pasteur.fr.

[†]Present address: Centenary Institute, Newtown, NSW 2042 Australia.

Author contributions

BM, NPW and JM performed experiments with *Shigella*. DFB and JAC provided technical support and advice for EMSAs and analysis of the fusions. JAS analysed the LPS, M-CP carried out EM, and FP performed the oxygen measurements. CMT and PS provided advice and overall direction, and wrote the paper.

Using the rabbit ligated gastrointestinal (GI) loop model ⁵⁶, we identified a colonisation-defective *S. flexneri* M90T mutant with a transposon in *fnr*, which encodes a regulator of anaerobic metabolism (supplementary Fig. 1). FNR is only active as a dimer containing an [4Fe-4S] cluster. The cluster dissociates in the presence of oxygen (O₂), destabilising the dimer, with loss of FNR activity ⁷. M90TΔ*fnr* was substantially attenuated for colonisation compared with the wild-type strain, M90T (competitive index [C.I.], 0.05), and this was restored by complementation (C.I. of M90TΔ*fnr*pBM2, 0.6). Furthermore, we challenged intestinal loops with strains individually. Infection with M90T led to abscesses, and shortening and destruction of villi (Fig. 1a). These alterations were significantly reduced following infection with M90TΔ*fnr* ($p < 0.01$, Fig. 1b and supplementary Table 1) and absent after challenge with a T3SS⁻ mutant, M90TΔ*mxiD* (Fig. 1c). Restoration of the invasive phenotype following complementation (M90TΔ*fnr*pBM2) confirmed the requirement of FNR *in vivo* (Fig. 1d).

To define the contribution of O₂ to *Shigella* virulence, epithelial cells were propagated in an aerobic environment, and either left there or transferred to an anaerobic cabinet for 30 min. and then challenged with bacteria grown in aerobic or anaerobic conditions, respectively. Bacterial entry into cells in an anaerobic cabinet was significantly higher than in an aerobic cabinet ($p < 0.05$, Fig. 1e). This was not caused by cell death or mediated by the host transcription factor HIF-1α⁸⁹ (supplementary Fig. 2). Instead, the enhanced cell entry of *Shigella* in the absence of O₂ was dependent on FNR (Fig. 1e).

To understand the basis of the increased cell entry, we investigated T3SS structure and function of *Shigella* grown with or without O₂. With O₂, M90T and M90TΔ*fnr* secreted the effectors IpaB, IpaC and IpaD following exposure to the inducer of secretion Congo red ⁴ (CR). CR-induced secretion of Ipas by M90T was reduced in anaerobic conditions and was accompanied by increased intra-bacterial Ipa levels ($p < 0.01$, Fig. 2a and supplementary Fig. 3). The reduced effector secretion in the absence of O₂ was not detected with M90TΔ*fnr* (Fig. 2a), while complementation of the *fnr* mutant restored low level Ipa secretion in anaerobiosis as observed with M90T. Additionally, SEM demonstrated that the number of visible needle tips was significantly higher on bacteria after growth in anaerobic compared with aerobic conditions (supplementary Fig. 4). This was not associated with any detectable change in the lipopolysaccharide profile, which affects the presentation of T3SS needles ⁵ (supplementary Fig. 5). Instead, TEM revealed a 25% increase in the average needle length during growth in anaerobic compared with aerobic conditions (62 vs. 48 nm respectively, $p < 0.001$, Fig. 2b), with less rigorous control of needle length in the absence of O₂ (S.D. of needle length 28 and 11 nm in anaerobic and aerobic environments, respectively). In contrast, needles produced by M90TΔ*fnr* were similar regardless of the presence of O₂ (average length 53 and 58 nm with and without O₂, respectively, Fig. 2c). Therefore in the absence of O₂, FNR mediates reduced Ipa secretion and elongation of T3SS needles.

As FNR is a transcription regulator, we next examined mRNA levels of *mxi/spa* pathogenicity island genes and their regulators by qRT-PCR ¹⁰ (Fig. 3a). There was no significant alteration in mRNA levels of genes encoding effectors, T3SS components, or regulators in the presence or absence of O₂. However there was a reduction in *spa32* and *spa33* mRNA levels (4.7- and 5.5-fold reduction respectively, $p < 0.01$) during anaerobic growth that was FNR dependent (Fig. 3a). Spa32 is required for control of needle length and the selection of substrates for secretion ²³, while dysregulation of Spa33 expression blocks Ipa secretion ¹¹. The activity of *lacZ* reporter fusions confirmed that the reduction of *spa32* and *spa33* mRNA levels is associated with decreased transcription in an FNR-dependent fashion (supplementary Fig. 6). The O₂ regulation of *spa32* and *spa33* fusions was abolished by modifying the two predicted FNR binding sites ¹² in the promoters of *spa32* and *spa33*

(−156 and −67, and −205 and −116 upstream of the initiation codons, respectively, supplementary Fig. 6), while FNR binds sequences upstream of *spa32* and *spa33* (Fig. 3b). Binding upstream of *spa33* was abolished by modification of the FNR binding sites (supplementary Fig. 7). These data demonstrate that FNR represses the *Shigella* virulence genes *spa32* and *spa33* in anaerobic conditions.

To establish whether FNR-mediated repression of *spa32* and *spa33* contributes to increased *Shigella* entry into cells in an anaerobic cabinet, we constructed M90T containing *spa32* and *spa33* under the control of either their native promoters (M90Tp32/33) or promoters with disrupted FNR boxes (M90Tp#32/#33). The presence in M90T of *spa32* and *spa33* alleles that are not repressed by FNR led to IpaB secretion during anaerobic growth (Fig. 3c), and loss of the increased entry of *Shigella* into cells in an anaerobic cabinet (invasion of strain with p#32/#33 vs. p32/33, $p < 0.01$, Fig. 3d); similar findings were obtained by inappropriate expression of *spa32* and *spa33* in anaerobic conditions with IPTG (supplementary Fig. 7).

To explain the enhanced entry of *Shigella* into epithelial cells following transfer into anaerobic cabinets, we hypothesized that bacteria encounter O_2 in the vicinity of host cells that promotes T3SS activation. We used microelectrodes to directly measure concentrations of O_2 above cells after they had been moved into an anaerobic cabinet¹³. There was sufficient O_2 (approximately 0.4%) 10 μm above cells to inactivate FNR¹⁴ and allow IpaB secretion by *S. flexneri* (Fig. 4a and b). While no O_2 was detected above cells following addition of the O_2 scavenger deoxy-haemoglobin, O_2 levels were unaffected by the addition of cytochrome *c*, which contains haem but does not bind O_2 ¹⁵. These data provide an explanation for the hyper-invasion of *Shigella* following transfer of cells to an anaerobic chamber, and indicate that remote from the cells, bacteria would assume a ‘primed state’ (with extended T3SS needles and impaired Ipa secretion), while residual O_2 in proximity to cells would allow T3SS activation and enhanced entry.

To establish the relevance of these findings to *Shigella* invasion *in vivo*, we constructed two bacterial reporters for the presence of oxygen during host: pathogen interactions as introduction of a microelectrode into the GI lumen might disrupt O_2 homeostasis. Formation of the GFP fluorophore requires covalent bonding with O_2 ^{16,17}, and luciferase activity is dependent on O_2 and ATP¹⁸. M90T Δ *mxhD* pFPV25.1 expresses GFP with O_2 -dependent fluorescence^{16,17} while Lux-based luminescence of *E. coli* pXen5 provides a distinct reporter for the presence of O_2 (supplementary Fig. 8 and 9); *E. coli* was used as the host because this construct is not functional in *S. flexneri*. The luminescence of anaerobically grown *E. coli* pXen5 was measured in intestinal loops with an intact vascular supply or in loops in which the vessels had been occluded by clamping prior to challenge. Bacteria emitted significant luminescence in intestinal loops with an intact vascular supply within 15 min, while barely any signal was detected from loops that had had their afferent vessels occluded ($p < 0.001$, Fig. 4c and supplementary Fig. 10). To determine the location of intra-luminal O_2 within the GI tract, intestinal loops were infected with M90T Δ *mxhD* pFVP25.1, and tissue recovered 18 h later. Even though bacteria were present throughout the gut lumen (detected with an anti-LPS pAb), only *Shigella* located within approximately 70 μm of the epithelial surface (particularly at the villous tips) emitted significant fluorescence (Fig. 4d). This zone of relative oxygenation is provided by the vascular supply to the GI tract as the GFP signal is abolished on clamping the afferent vasculature (supplementary Fig. 11).

The status of the *Shigella* T3SS is precisely modulated by ambient O_2 tensions which vary at specific sites in the GI tract. In the anaerobic lumen of the intestine, T3SS needles will be extended but not competent for secretion. In contrast, the relatively aerobic zone adjacent to the mucosal surface would allow activation of the T3SS on bacteria at this site (Fig. 4e).

This zone of oxygenation depends on the vascular supply probably by diffusion of O₂ from the capillary network at villous tips¹⁹. Expression of elongated needles would optimise the presentation of the T3SS translocon for engaging host cells²⁰. Furthermore precise regulation of secretion of effectors would allow bacteria to deliver maximal amounts of Ipa at the exact time and place *in vivo* needed to trigger efficient manipulation of the cytoskeleton and cell entry²¹.

The presence of available O₂ in localised regions in the intestine may be under-appreciated and could have important biological consequences. This 'aerobic zone' might dictate the outcome of interactions with the extensive intestinal microbiota, acting as an innate immune barrier to protect the mucosal surface from anaerobic bacteria, while being recognised as a signal to promote invasion by pathogens such as *Shigella*, *Salmonella* and *Listeria monocytogenes*²². FNR and possibly other regulators (such as the ArcBA system) may mediate other effects aside from bacterial invasion, especially in the context of the inflammatory response to infection. Similar mechanisms of modulating virulence in response to O₂ may also have evolved in other intestinal pathogens, as FNR boxes are present upstream of genes required for T3SS function in *Yersinia* spp., and serovars of *Salmonella enteritidis* (supplementary Table 2).

Materials section

Bacterial strains, growth conditions and LPS analysis

All strains and plasmids in this study are described in Supplementary Table 2. *E. coli* was grown in Luria Bertani broth while *Shigella* was propagated in Trypticase soy media (TCS, Oxoid) or on TCS plates with Congo Red (0.01%, Sigma). Experiments under different oxygen (O₂) concentrations were performed in an anaerobic cabinet (Whitley DG250), or in a cabinet in which O₂ tensions can be controlled (Plaslabs). Antibiotics were added at the following concentrations: chloramphenicol, 50 µg/ml; ampicillin, 100 µg/ml; kanamycin 50 µg/ml. For LPS analysis, whole cell lysates of bacteria grown in liquid culture to an O.D. A₆₀₀ of <0.2 were prepared in LPS loading buffer, treated with proteinase K, then subjected to Tricine SDS-PAGE analysis²³, and visualised by silver staining (Pierce).

DNA manipulations

The regulatory regions of *spa32* and *spa33* (from 300 bp upstream of the start codon to 15 bp downstream) were amplified by PCR with NG900/901 and NG902/903, respectively, and ligated into the *Sma*I and *Bam*HI sites of pRS415²⁴ (supplementary Fig. 6). All oligonucleotides are shown in supplementary Table 4). For transcriptional fusions, the regulatory regions of *spa32*, *spa33* and P6 (located upstream *spa47*²⁵ and supplementary Fig. 6) were amplified with primers SG87/88, SG81/89 and SG94/26, and ligated in pQF50.1 which contains a promoterless *lacZ* as a reporter (kind gift from C. Parsot²⁶). Putative FNR binding sites upstream of *spa32* and *spa33* were modified by a two step-PCR procedure²⁷. *spa32* FNRbox1 (TTGAAGAAATTCAA, inverted repeats underlined) and *spa32* FNRbox2 (TTGATTAAAAGAAA) were altered to TCGAGGAAATACAG and CTCATTAAACGGAA using primers SG25/26 and SG27/28, respectively (for details see supplementary Fig. 6). *spa33* FNRbox1 (TTGATAGCAGTCAA) and *spa33* FNRbox2 (TTGACGCTAACGAA) were altered to TCGATAGCACAGCA and TCCACGCTAAGGAA with primers SG29/30 and SG31/32, respectively. To generate p32/33, the open reading frames and promoters of *spa32* and *spa33* were amplified with SG13 and SG14, and ligated into pBBR1MCS-4. The FNR boxes in this plasmid were modified as above to generate p#32/#33. For IPTG inducible over-expression, *spa32* and *spa33* were amplified with NG1390/1391 and NG1369/1370, and ligated into the *Nde*I/

*Bam*HI sites of pET21b and the *Nde*I/*Xho*I sites of pET28b (Invitrogen), respectively. Constructs were verified by DNA sequencing.

Antibodies and fluorescent dyes

Murine α -*Shigella* LPS pAbs²⁸ were diluted 1:500 for immunofluorescence microscopy. FITC-conjugated goat anti-rabbit and Phalloidin-Rhodamine red X (RRX)-conjugated donkey anti-mouse antibodies (Jackson Immunoresearch Antibodies) were used at a final dilution of 1:500. Dapi (Invitrogen) was used at 1 μ g/mL. Rabbit α -FNR pAbs (kind gift from Professor Jeff Green, University of Sheffield, UK), anti-RecA (MBL), α -IpaB, α -IpaC and α -IpaD²⁹ were used at a 1:10,000 final dilution. HRP-conjugated α -His mAb (Qiagen) was used at a final dilution of 1:2,000.

Cell culture

HeLa epithelial cells were grown to semi-confluency in aerobic conditions in 24-well tissue culture plates. Before challenge, cells were either maintained under aerobic conditions or transferred to an anaerobic cabinet (Whitley DG250) for 30 min. Cells were challenged at an MOI of 100 with bacteria grown in liquid culture under aerobic or anaerobic conditions. For aerobic growth, bacteria were grown in air overnight in 5 ml LB with shaking in a 37 °C incubator. Cultures were then diluted 1 in 100 into 5 ml fresh media in a 20 ml universal and then grown with vigorous shaking until the O.D. A_{600} reached 0.2. For anaerobic growth, bacteria were propagated in exactly the same way except cultures were performed in an anaerobic cabinet in media which had been pre-incubated in the cabinet for at least 16 hrs. Bacteria were spun onto cells by centrifugation at 2,000 $\times g$ for 10 min. For adhesion assays, cells were immediately washed three times in PBS, then fixed in 3% paraformaldehyde (PFA) for 15 min.

To measure bacterial entry, cells were challenged as above, incubated for 30 min at 37°C, after which gentamicin (50 μ g/ml) was added for 1 hr to kill extracellular bacteria. Cells were washed with PBS, lysed with 500 μ L 1% saponin in PBS, and bacteria recovered by plating. Results are expressed as the number of CFU/ml of cell lysate, and are the average of at least three independent experiments performed in triplicate. Statistical significance was calculated using the Student's T-test. When required, IPTG (1 mM final concentration) was added to the cells in tissue culture wells at the time of bacterial challenge. Cells were exposed to mimosine (Sigma, final concentration 800 μ M), which inhibits degradation of HIF α for 1 h before challenge with bacteria³⁰. The constitutively HIF α –expressing epithelial cell lines Rcc10 VHL^{-/-} and corresponding control VHL^{+/+} (kindly provided by P. Maxwell⁸³¹) were cultured and infected as above.

For immunofluorescence microscopy, cells were grown on 12 mm glass coverslips, challenged as above, washed, then fixed in 3% paraformaldehyde (PFA) for 15 min, and washed again. Coverslips were incubated for 30 min with primary antibodies diluted in 10% horse serum/0.1% saponin in PBS, washed with PBS containing 0.1% saponin, then incubated with the secondary conjugated antibodies for 30 min. Samples were washed three times in 0.1% saponin in PBS, then in PBS, and finally in water, mounted in Prolong (Molecular Probes), and examined by laser-scanning confocal microscopy (Zeiss Axiovert 100M or LSM510).

Cell viability was determined by staining with 0.01% propidium iodide (PI, Sigma) in PBS for 10 min³². PI only penetrates into and stains the DNA of non-viable cells. As a positive control, cells were killed by incubation in 3% PFA for 15 min. Fluorescence was measured using a FACS flow cytometer (BD systems) recording at least 10,000 events. Data were

analysed with CellQuest Pro software (BD Biosciences), and expressed as percentage survival.

To evaluate the response of bacterial O₂ reporters, M90TΔ*mxlD* pFPV25.1 or *E. coli* pXen were grown anaerobically in a controlled atmosphere chamber (Plaslabs) in TCS media at 37 °C until the O.D. A₆₀₀ reached 0.2. The cabinet was then adjusted to 5%, 10%, 15% or 20% O₂, with the ambient oxygen tension monitored with an oxygen meter. At each step, bacteria and PFA were incubated for 15 min. To measure fluorescence an aliquot of the culture was mixed with an equal volume of equilibrated PFA. For luminescence, bacteria were transferred into an air tight tube within the chamber. Fluorescence of bacteria was assessed by confocal microscopy, and the results are the average of at least 150 bacteria from three different fields. Luminescence was measured with an IVIS camera (Xenogen). Experiments were performed on three different occasions.

The *luxABCDE* operon present on pXen5 (Xenogen) was introduced into *E. coli* by transformation. Luminescence was measured using an IVIS camera (Xenogen), and images quantified using the Living Image 3.1 software (Xenogen) and expressed as a Total flux (p/s), as recommended by the manufacturer.

Electron Microscopy

For TEM, bacteria were grown on solid medium overnight and a single colony was re-suspended in distilled water. Bacteria were negatively stained with 2% uranyl acetate on glow discharged copper grids. The samples were observed in a Jeol 1200EXII or a JEM 1010 microscope (Jeol Company, Tokyo Japan) at 80-kV with an Eloise Keenview camera. Images were recorded with Analysis Pro Software version 3.1 (Eloise SARL, Roissy, France). The length of at least 200 needles was measured on three separate occasions, and the results presented using a non-linear regression model (Loess, locally weighted scatterplot smoothing, GraphPad, Prism software).

For SEM, bacteria were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h on coverslips coated with poly L-lysine, washed three times in 0.2 M cacodylate buffer (pH 7.2), then post-fixed for 1 h in 1% (wt/vol) osmium tetroxide in 0.2 M cacodylate buffer (pH 7.2). Samples were rinsed with distilled water, dehydrated through a graded series of ethanol then critical point dried with CO₂. Dried specimens were sputtered with 10 nm gold palladium with a GATAN Ion Beam Coater and examined with a JEOL JSM 6700F field emission scanning electron microscope operating at 5 kV. Images were acquired with the upper SE detector and the lower secondary detector.

Western blot analysis

Bacteria were grown in 500 ml TCS liquid media in the presence or absence of O₂ until the O.D. A₆₀₀ reached 0.2; IPTG was added to a final concentration of 1 mM as required. Bacteria were harvested by centrifugation for 15 min at 3,000 *x g*, washed, then re-suspended in 10 ml PBS. Cells were spun again for 5 min at 3000 *x g*, and re-suspended in 200 μl of PBS. Secretion was induced by adding Congo Red (CR, final concentration, 0.05%) under aerobic or anaerobic conditions, and incubated for 20 min at 37 °C. Bacteria (representing the cellular fraction) were collected by centrifugation at 3000 *x g*, and the supernatants (representing the secreted fraction) retained. Total protein concentrations were measured by the method of Bradford (Biorad). Proteins were separated by 10% SDS-PAGE and either visualised by Coomassie blue staining or transferred to nitrocellulose membranes using semi-dry transfer, and incubated with the primary antibodies diluted in PBS/5% milk/0.01% Tween20 (Sigma) for 1 h. Membranes were washed in PBS three times, then incubated with secondary antibodies for 1 hr before washing. Antibody binding was detected

with chemiluminescence (ECL kit, GE Healthcare). Quantification of expression levels was performed by densitometry of scanned blots using the ImageJ software and performed at least on two independent occasions. Statistical significance was calculated by the Student's T-test.

EMSA assays

Gel retardation assays were carried out as described previously using purified FNR^{D154A}, an allele that forms dimers under aerobic conditions³³. In brief, purified 300 bp *spa32* and *spa33* promoter fragments (supplementary Fig. 6), and the modified alleles were end-labelled with [γ -³²P]-ATP with 10 U/ μ l T4 polynucleotide kinase (New England BioLabs). Approximately 0.5 ng of each labelled fragment was incubated with varying amounts of FNR^{D154A} in 10 mM potassium phosphate (pH 7.5), 100 mM potassium glutamate, 1 mM EDTA, 50 μ M DTT, 5% glycerol and 25 μ g ml⁻¹ herring sperm DNA. After incubation at 37°C for 20 min, samples were separated on 6% polyacrylamide gels containing 2% glycerol. Gels were analysed using a Bio-Rad Molecular Imager FX and Quantity One software (Bio-Rad).

Quantitative real-time reverse transcriptase (RT) PCR

Bacteria were grown in liquid LB media until an OD₆₀₀ 0.2. The cultures were immediately transferred to a new tube containing a 1 in 5 volume of 5% phenol pH 4.3 (Sigma) in ethanol. Samples were kept on ice for 30 min, then bacteria harvested by centrifugation at 10,000 $\times g$ for 10 min. The pellets were re-suspended in TE buffer containing 50 mg/ml lysozyme. RNA was isolated with the RNeasy method (Qiagen), and quantified by measuring ratio of the O.D. A₂₆₀/A₂₈₀ of samples. DNA was digested by treatment with DNase I (Sigma), then cDNA was synthesized with reverse transcriptase (Quantitect, Qiagen). *polA*, *ipaB*, *ipaC*, *ipaD*, *mxiH*, *spa47*, *spa13*, *spa32*, *spa33*, *spa24*, *virF*, and *virB* were amplified using the SensiMix DNA kit (Quantance) with primers described in supplementary Table 4 with a Rotor-gene RT-PCR machine (Corbett Research). Results are the average of duplicate experiments each performed on three independent occasions. Data were analysed with Rotor Gene 3000 (Corbett Research) software.

Analysis of promoter fusion activity

Bacteria were grown to an OD₆₀₀ 0.2 in 10 ml of TCS media and harvested by centrifugation at 5,000 $\times g$ for 10 min. Pellets were re-suspended in 500 μ l of PBS, sonicated and further centrifuged at 13,000 $\times g$ for 20 min and the supernatants collected. β -galactosidase (β gal) activity was measured at 37°C by assaying degradation of ortho-nitrophenyl- β -galactoside (ONPG)³⁴. The protein concentration of lysates was determined by the method of Bradford, and the results are the mean values of three measurements performed on two independent samples, and are expressed in Miller Units/mg of total cellular protein.

Rabbit ligated ileal loop model

For evaluation of the virulence and measurement of competitive indices (C.I.), experiments were performed on four or more independent occasions in at least two loops in four animals. Bacteria were grown in liquid media for three hours at 37°C, and a total 10⁷ CFU of bacteria consisting of equal numbers of two strains in 500 μ l of physiological water were injected into loops. Bacteria were recovered from the loops by plating homogenates to solid media with (to recover mutant strains) or without antibiotics (to recover the mutant and wild-type strains). The competitive index was calculated as the ratio of mutant to wild-type bacteria recovered from animals divided by the ratio in the inoculum; the results are the average of at least four samples originating from four different rabbits.

For histopathological analysis, ileal loops were fixed in 4 % buffered formalin, embedded in paraffin, 5 μm sections obtained, and stained with haematoxylin-eosin-safranin or Giemsa. The extent of damage to villi was quantified by measuring their volume-to-length ratio and the presence of indentations, ruptures of the mucosal surface, and abscesses. Results are the average of measurements from at least 25 villi from two independent samples for each strain³⁵. For immunohistochemical staining, samples were washed in PBS, incubated at 4°C PBS containing 12% sucrose for 90 min, then in PBS with 18% sucrose overnight, and frozen in OCT (Sakura) on dry ice. 7 μm sections were obtained using a cryostat CM-3050 (Leica). The immunostaining was performed as for immunofluorescence of infected cells, and the antibodies were used at the same concentrations. Stained samples were examined by laser-scanning confocal microscopy (Zeiss Axiovert 100M or LSM510), as described above.

The vascular supply to intestinal loops was interrupted by exposing the afferent mesenteric vessels which were occluded with a clamp. To detect luciferase and GFP activity in the loops, bacteria were grown under anaerobic conditions for 4 hrs in LB media then 10^7 CFU injected into the lumen of individual ligated loops. The total luminescence of each injected loop was measured with an IVIS camera system (Xenogen) and expressed as a Total flux (p/s), as recommended by the manufacturer. To detect GFP fluorescence, the corresponding loops were sectioned and fixed in 3 % PFA and processed as above. Stained samples were observed by laser-scanning confocal microscopy (Zeiss Axiovert 100M). The results are the average of duplicate experiments each performed on three independent occasions.

Measurement of oxygen

HeLa cells were grown at 37 °C in room air supplemented with 5% CO₂. Plates with the cells were transferred into an anaerobic cabinet, and media discarded and replaced with media that had been equilibrated in the cabinet for over 18 hrs. An oxygen microelectrode sensor (Oxy-4, Unisense, Aarhus, Denmark) connected to a PA-2000 (Unisense) was advanced into the media to 10 μm from the top of the cells. The oxygen sensor was calibrated at 37 °C as described previously³⁶. Measurements were performed in triplicate on three occasions at 30 and 60 mins after replacement of the media. For experiments in the presence of deoxy-haemoglobin (Hb, Sigma) and cytochrome *c* (cyt, Sigma), saturated solutions were prepared in deionised water, and added to cells at a final concentration of 6 μM to media kept under aerobic or anaerobic conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Fondation pour la Recherche Médicale, the Royal Society, an ERC Advanced Grant (HOMEOPATH), and the European Union (QLRT-1999-00938). Work in CMT's laboratory is supported by the Wellcome Trust and The Medical Research Council, and PJS is a Howard Hughes Medical Institute scholar. Imaging was performed at the PFID station (Institut Pasteur). We are grateful to Professor Jeff Green (University of Sheffield) for the α -FNR pAbs and advice. Rachel Exley, David Holden and Katrina Ray provided helpful suggestions and reviewed the manuscript; we thank Marie-Anne Nicola for technical help.

References

1. Phalipon A, Sansonetti PJ. Shigella's ways of manipulating the host intestinal innate and adaptive immune system: a tool box for survival? *Immunol Cell Biol.* 2007; 85:119–29. [PubMed: 17213832]

2. Magdalena J, et al. Spa32 regulates a switch in substrate specificity of the type III secretion of *Shigella flexneri* from needle components to Ipa proteins. *J Bacteriol.* 2002; 184:3433–41. [PubMed: 12057936]
3. Tamano K, Katayama E, Toyotome T, Sasakawa C. *Shigella* Spa32 is an essential secretory protein for functional type III secretion machinery and uniformity of its needle length. *J Bacteriol.* 2002; 184:1244–52. [PubMed: 11844752]
4. Bahrani FK, Sansonetti PJ, Parsot C. Secretion of Ipa proteins by *Shigella flexneri*: inducer molecules and kinetics of activation. *Infect Immun.* 1997; 65:4005–10. [PubMed: 9316999]
5. West NP, et al. Optimization of virulence functions through glucosylation of *Shigella* LPS. *Science.* 2005; 307:1313–7. [PubMed: 15731456]
6. Sansonetti PJ, et al. Alterations in the pathogenicity of *Escherichia coli* K-12 after transfer of plasmid and chromosomal genes from *Shigella flexneri*. *Infect Immun.* 1983; 39:1392–402. [PubMed: 6341237]
7. Kiley PJ, Beinert H. The role of Fe-S proteins in sensing and regulation in bacteria. *Curr Opin Microbiol.* 2003; 6:181–5. [PubMed: 12732309]
8. Maxwell PH, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature.* 1999; 399:271–5. [PubMed: 10353251]
9. Esteban MA, et al. Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. *Cancer Res.* 2006; 66:3567–75. [PubMed: 16585181]
10. Tobe T, et al. Temperature-regulated expression of invasion genes in *Shigella flexneri* is controlled through the transcriptional activation of the *virB* gene on the large plasmid. *Mol Microbiol.* 1991; 5:887–93. [PubMed: 1857209]
11. Schuch R, Maurelli AT. Spa33, a cell surface-associated subunit of the Mxi-Spa type III secretory pathway of *Shigella flexneri*, regulates Ipa protein traffic. *Infect Immun.* 2001; 69:2180–9. [PubMed: 11254573]
12. Hoitink CW, Woudt LP, Turenhout JC, van de Kamp M, Canters GW. Isolation and sequencing of the *Alcaligenes denitrificans* azurin-encoding gene: comparison with the genes encoding blue copper proteins from *Pseudomonas aeruginosa* and *Alcaligenes faecalis*. *Gene.* 1990; 90:15–20. [PubMed: 2116366]
13. Melican K, et al. Bacterial infection-mediated mucosal signalling induces local renal ischaemia as a defence against sepsis. *Cell Microbiol.* 2008; 10:1987–98. [PubMed: 18549455]
14. Becker S, Holighaus G, Gabrielczyk T, Uden G. O₂ as the regulatory signal for FNR-dependent gene regulation in *Escherichia coli*. *J Bacteriol.* 1996; 178:4515–21. [PubMed: 8755879]
15. Marti MA, et al. Dioxygen affinity in heme proteins investigated by computer simulation. *J Inorg Biochem.* 2006; 100:761–70. [PubMed: 16442625]
16. Hansen MC, Palmer RJ Jr, Udsen C, White DC, Molin S. Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. *Microbiology.* 2001; 147:1383–91. [PubMed: 11320140]
17. Takahashi E, et al. In vivo oxygen imaging using green fluorescent protein. *Am J Physiol Cell Physiol.* 2006; 291:C781–7. [PubMed: 16738007]
18. Ghisla S, Hastings JW, Favaudon V, Lhoste J-M. Structure of the oxygen adduct intermediate in the bacterial luciferase reaction: ¹³C nuclear magnetic resonance determination. *Proc Natl Acad Sci U S A.* 1978; 75:5860–3. [PubMed: 16592595]
19. Torres Filho IP, Leunig M, Yuan F, Intaglietta M, Jain RK. Non-invasive measurement of microvascular and interstitial oxygen profiles in a human tumor in SCID mice. *Proc Natl Acad Sci U S A.* 1994; 91:2081–5. [PubMed: 8134352]
20. Tamano K, et al. Supramolecular structure of the *Shigella* type III secretion machinery: the needle part is changeable in length and essential for delivery of effectors. *Embo J.* 2000; 19:3876–87. [PubMed: 10921870]
21. Jaumouille V, Francetic O, Sansonetti PJ, Tran Van Nhieu G. Cytoplasmic targeting of IpaC to the bacterial pole directs polar type III secretion in *Shigella*. *Embo J.* 2008; 27:447–57. [PubMed: 18188151]
22. Pentecost M, Otto G, Theriot JA, Amieva MR. *Listeria monocytogenes* invades the epithelial junctions at sites of cell extrusion. *PLoS Pathog.* 2006; 2:e3. [PubMed: 16446782]

23. Exley RM, et al. Available carbon source influences the resistance of *Neisseria meningitidis* against complement. *J Exp Med*. 2005; 201:1637–45. [PubMed: 15897277]
24. Podkovyrov SM, Larson TJ. A new vector-host system for construction of lacZ transcriptional fusions where only low-level gene expression is desirable. *Gene*. 1995; 156:151–2. [PubMed: 7737510]
25. Sasakawa C, Komatsu K, Tobe T, Suzuki T, Yoshikawa M. Eight genes in region 5 that form an operon are essential for invasion of epithelial cells by *Shigella flexneri* 2a. *J Bacteriol*. 1993; 175:2334–46. [PubMed: 8385666]
26. Farinha MA, Kropinski AM. Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. *J Bacteriol*. 1990; 172:3496–9. [PubMed: 2111810]
27. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A*. 2000; 97:6640–5. [PubMed: 10829079]
28. Phalipon A, et al. Identification and characterization of B-cell epitopes of IpaC, an invasion-associated protein of *Shigella flexneri*. *Infect Immun*. 1992; 60:1919–26. [PubMed: 1373401]
29. Menard R, Sansonetti P, Parsot C, Vasselon T. Extracellular association and cytoplasmic partitioning of the IpaB and IpaC invasins of *S. flexneri*. *Cell*. 1994; 79:515–25. [PubMed: 7954817]
30. Warnecke C, et al. Activation of the hypoxia-inducible factor-pathway and stimulation of angiogenesis by application of prolyl hydroxylase inhibitors. *Faseb J*. 2003; 17:1186–8. [PubMed: 12709400]
31. Esteban MA, et al. Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. *Cancer Res*. 2006; 66:3567–75. [PubMed: 16585181]
32. Darzynkiewicz Z, et al. Features of apoptotic cells measured by flow cytometry. *Cytometry*. 1992; 13:795–808. [PubMed: 1333943]
33. Browning DF, Beatty CM, Wolfe AJ, Cole JA, Busby SJ. Independent regulation of the divergent *Escherichia coli* *nrfA* and *acsP1* promoters by a nucleoprotein assembly at a shared regulatory region. *Mol Microbiol*. 2002; 43:687–701. [PubMed: 11929525]
34. Miller J. *Experiments in Molecular Genetics*. 1972
35. D’Hauteville H, Khan S, Maskell DJ, Kussak A, Weintraub A, Mathison J, Ulevitch RJ, Wuscher N, Parsot C, Sansonetti PJ. Two *msbB* genes encoding maximal acylation of lipid A are required for invasive *Shigella flexneri* to mediate inflammatory rupture and destruction of the intestinal epithelium. *J Immunol*. 2002; 168:5240–5251. [PubMed: 11994481]
36. Liss P, Nygren A, Revsbech NP, Ulfendahl HR. Intrarenal oxygen tension measured by a modified Clark electrode at normal and low blood pressure and after injection of x-ray contrast media. *Pflugers Arch*. 1997; 434:705–11. [PubMed: 9306002]

Figure 1. Influence of anaerobiosis and FNR on *Shigella* invasion

(a) Histological analysis of the GI tract infected with *S. flexneri* strains as indicated. There was marked destruction of the mucosal surface with loss of villi and infiltration of the submucosa with inflammatory cells following challenge with wild-type strain, M90T. These changes are less pronounced after challenge with M90TΔfnr (b) or a strain lacking the T3SS (M90TΔmxuD, c). (d) Complementation of *fnr* (M90TΔfnr pBM2) restores the pathological changes. Sections were stained with Evans blue; bars, 50 μm. (e) Epithelial cell invasion by M90T, M90TΔfnr and M90TΔfnr pBM2 in an aerobic or anaerobic cabinet (n, 5 independent experiments). Error bars show the S.D. and asterisks indicate $p < 0.05$ (Student's T-test).

Figure 2. T3SS structure and function are modified by ambient O₂

(a) Secreted (Sec.) and cytoplasmic (Cell) Ipas detected by Western analysis. Secretion induced by Congo Red (CR) was diminished in anaerobic conditions, with effectors retained in the bacteria; this is FNR dependent. Strains and growth conditions are indicated.

Detection of T3SS needles (white triangles) on M90T (b) and M90T Δfnr (c) by TEM. The average needle length (>200 needles counted in n=3 experiments, $\pm$ S.D.) on strains is shown and results displayed applying a Loess model. Needles on M90T were significantly longer following growth in anaerobic than aerobic conditions ($p < 0.001$, Student's T-test), and the needle length was more varied (S.D. ± 28 vs. 11 nm).

Figure 3. Regulation of *Shigella* virulence genes by O₂ and FNR

(a) qRT-PCR analysis of relative mRNA levels in the presence (dashed line) and absence (black boxes) of O₂; the reduction of *spa32* and *spa33* mRNA levels under anaerobic conditions are FNR dependent (n=3 independent experiments). (b) Binding of FNR to regions upstream of *spa32* and *spa33* by EMSA. (c) IpaB secretion detected by Western blot analysis 30 min. after transferring anaerobically grown bacteria to different O₂ concentrations (indicated). Strains including M90T, and M90T containing *spa32* and *spa33* under the control of their native promoters (M90Tp32/33) or promoters with disrupted FNR boxes (M90Tp#32/#33). (d) Epithelial cells entry of bacteria in cabinets with (white bars) or without (black bars) O₂. Error bars, S.D.; ** indicates $p < 0.01$; n=3 independent experiments.

Figure 4. The presence of O₂ encountered by *Shigella* in vitro and in vivo

(a) HeLa cells were transferred to an anaerobic cabinet with or without deoxy-haemoglobin or cytochrome *c* (6 μM) in the media, and oxygen tensions measured 10 μm above cells at times afterwards (n=3 independent experiments, error bars, S.D.). (b) M90T grown anaerobically or aerobically was transferred to different O₂ concentrations with CR. Secreted (Sec.) and bacterial (Cell) IpaB was detected 30 min. later. (c) Luminescence of *E. coli* pXen5 after introduction into loops (arrowed) with unclamped or clamped vascular supply. (d) Loops were challenged with M90TΔ*mxiD* pFPV25.1, and tissue obtained 18 h later; host cell nuclei stained blue, and bacteria and actin stained red. GFP-positive bacteria

were detected in a zone within 70 μm of villi (arrowed). (e) *Shigella* is 'primed' within the anaerobic lumen of the GI tract; O_2 from capillaries creates a permissive environment for T3SS activation.