**Shigella**-mediated oxygen depletion is essential for intestinal mucosa colonization

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Pathogenic enterobacteria face various oxygen (O₂) levels during intestinal colonization from the O₂-deprived lumen to oxygenated tissues. Using *Shigella flexneri* as a model, we have previously demonstrated that epithelium invasion is promoted by O₂ in a type III secretion system-dependent manner. However, subsequent pathogen adaptation to tissue oxygenation modulation remained unknown. Assessing single-cell distribution, together with tissue oxygenation, we demonstrate here that the colonic mucosa O₂ is actively depleted by *S. flexneri* aerobic respiration—and not host neutrophils—during infection, leading to the formation of hypoxic foci of infection. This process is promoted by type III secretion system inactivation in infected tissues, favouring colonizers over explorers. We identify the molecular mechanisms supporting infectious hypoxia induction, and demonstrate here how enteropathogens optimize their colonization capacity in relation to their ability to manipulate tissue oxygenation during infection.

Pathogenic enterobacteria virulence and the survival and function of first-line immune cells (particularly neutrophils) are highly modulated by oxygen (O₂)¹—⁴. Most virulent enterobacteria (*Shigella* species, *Listeria* species, *Salmonella* species, pathogenic *Escherichia coli* and *Yersinia pestis*) are well adapted to these changing microenvironments⁵, suggesting that adaptability to various O₂ levels represents a selective advantage crucial for their infectious capacity. The colonization process involves three key steps: degradation of the mucus layer; invasion of the epithelium, mediated by the type III secretion system (T3SS); and formation of primary foci of infection. We previously demonstrated that *Shigella flexneri* T3SS activation requires O₂, which diffuses on the epithelial surface⁶. However, tissue oxygenation modulation during the dissemination of enteropathogens and its impact on colonization and immune response efficiency remain largely unknown, although hypoxia has previously been reported in a 2,4,6-trinitrobenzene sulfonic acid-induced colitis model of inflammation⁷.

In this work, we use *S. flexneri* as a model to show that O₂ is depleted during tissue colonization by enteropathogens, leading to the formation of hypoxic foci of infection. We designed a quantitative image analysis method allowing the assessment of tissue oxygenation at the single-cell level for both bacteria and neutrophils. We demonstrate that *S. flexneri* aerobic respiration is essential for O₂ depletion within the colonic mucosa—not neutrophils, as previously demonstrated in a non-infectious model of inflammation⁸. We show that formation of hypoxic foci of infection is the primary *S. flexneri* colonization strategy, which is promoted by the repression of T3SS activity. Our results demonstrate that the interaction between *S. flexneri* and immune cells occurs mainly in the absence of O₂. We anticipate that our results will have a significant impact on new vaccine and antibiotic development strategies, as well as reappraisal of immune response and host–pathogen interactions under low-O₂ conditions.

We recently observed that hypoxia was induced within the colonic mucosa on *S. flexneri* infection⁹ using a hypoxia reporter, EF5 (ref. ⁹). Here, measuring relative hypoxia levels from the epithelial surface to the submucosa (Supplementary Table 1), we report results that confirm this preliminary observation (Fig. 1a) and show that, during infection, compared with basal conditions, the degree of hypoxia increased significantly to a depth of 130 μm (Fig. 1b; < 0.05). Under basal conditions, the ‘physiological hypoxic status’ of the epithelium was confirmed, as previously reported in the mouse¹⁰ (Fig. 1b, uninfected tissues).

Taking into account the potential heterogeneity of the bacteria population¹¹, we developed a quantitative imaging strategy at the single-bacteria level, as previously reported¹¹, to localize individual bacteria and assess hypoxia levels (Fig. 1c and Supplementary Fig. 1a–c). First, we revealed that most bacteria (99.71%; Supplementary Fig. 1a) were located within foci of infection formed in the colonic extracellular matrix (clustered bacteria, defined as having at least six neighbouring bacteria within 16 μm) and not dispersed within the mucosa (dispersed bacteria, defined as having a closest neighbour beyond 30 μm; Fig. 1c and Supplementary Fig. 1a). We demonstrated that *S. flexneri* intracellular bacteria and assess hypoxia levels (Fig. 1d–f; *P* < 10⁻¹⁰). This result implies that *S. flexneri* adaptation to low O₂ levels is crucial for tissue colonization. We demonstrated that a *S. flexneri* Δftr mutant strain (where FNR is a fumarate and nitrate reductase regulator that mediates the adaptation of *S. flexneri* to anaerobiosis¹²) did not propagate within foci of infection and was
Fig. 1 | Hypoxia is specifically induced by Shigella within foci of infection. a, Hypoxia was detected in guinea pig colonic mucosa 8 h after infection with the S. flexneri Sa pGFP strain (green) using the EF5 reporter. EF5 was immunodetected with anti-EF5-Cy3 (red). Neutrophils were labelled with the Myelotracker-Dylight405 marker (blue). Scale bar, 50 µm. b, Hypoxia profiles through the colonic mucosa. The EF5 level is reported as the mean over all aligned profiles (thick lines) ± s.d. (shaded areas) against tissue depth (n = 128, 61 and 48 profiles for the M90T, uninfected (UI) and no-EF5 conditions, respectively). The left-hand grey bracket above the curves represents the range of depths for which the EF5 levels were significantly different between the M90T and uninfected conditions (two-sided Student’s t-test, P < 0.05). c, Individual bacteria were detected by quantitative image analysis (red dots; see Supplementary Fig. 1a), and two populations of S. flexneri were defined as either: (1) clustered (if at least six bacteria were within 16 µm diameter, forming a focus of infection); or (2) dispersed (if its closest neighbour was located beyond 30 µm). Scale bar, 20 µm. d, Detection of clustered and dispersed bacteria populations within the colonic mucosa 8 h after infection. Scale bar, 50 µm. e, f, Hypoxia levels (imaged in e and quantified in f) were compared at single dispersed and clustered bacteria levels by quantitative image analysis. Scale bars in e, 20 µm. Hypoxia levels were significantly higher around clustered bacteria compared with dispersed bacteria (one-way ANOVA with Tukey’s test, P < 0.05). g, Individual neutrophils (orange dots), stained with Myelotracker-Dylight405 (ref. 12), were localized in infected tissues not stained with EF5. ***P < 0.001. h, Individual neutrophils (orange dots), stained with Myelotracker-Alexa405 (ref. 12), were localized in tissues by quantitative image analysis (red dots; see Supplementary Fig. 1a), and two populations of S. flexneri were defined as either: (1) clustered (if at least six bacteria were within 16 µm diameter, forming a focus of infection); or (2) dispersed (if its closest neighbour was located beyond 30 µm). Scale bar, 20 µm. Hypoxia levels were significantly higher around clustered bacteria compared with dispersed bacteria (one-way ANOVA with Tukey’s test, P < 0.05). i, Hypoxia (EF5) levels detected around neutrophils (averaged over the five closest) were higher around clustered bacteria compared with dispersed bacteria (distance from n = 61,119 and 186 bacteria to the closest neutrophil for the clustered and dispersed bacteria, respectively; ***P < 10−10). In the box plots of panels f, h and i, the central mark indicates the median, and the bottom and top edges of each box indicate the 25th and 75th percentiles, respectively. The whiskers extend to the most extreme data points that were not considered to be outliers, and the outliers were not plotted. Outliers were considered as data points further away than 1.5x the range from the first to third quartile, respectively, above or below the third and first quartile.

avirulent (Supplementary Fig. 2a), as previously reported1. Also, the neutrophil population was reduced—a sign of limited inflammation (Supplementary Fig. 2a). In a rabbit ileal loop model, we confirmed that FNR was specifically required for S. flexneri propagation in an inflammatory environment, but not mandatory for its fitness within the intestinal lumen (Supplementary Fig. 2b).

Neutrophils represent the most abundant immune cell population recruited on Shigella infection, and their activation has previously
been identified as being responsible for hypoxia induction in a 2,4,6-trinitrobenzene sulfonic acid-induced colitis model of inflammation. Neutrophils were specifically labelled with Myelotrackr (also known as MUB40—a neutrophil lactoferrin marker), allowing single-cell analyses (Fig. 1g). We observed that neutrophils were in closest proximity to clustered bacteria compared with dispersed bacteria (Fig. 1h), and that the level of hypoxia quantified at a single-neutrophil level was significantly higher when located in the vicinity of clustered bacteria (Fig. 1i). Hypoxia levels measured at the single-bacteria level around clustered and dispersed S. flexneri remained similar in conventional and neutropenic animals (Fig. 2a,b), suggesting that neutrophil contribution is not an essential factor for hypoxia induction. As expected, a significantly reduced number of neutrophils were detected in the infected colonic mucosa of neutropenic animals; the S. flexneri population was only mildly reduced (Supplementary Fig. 2c). Neutrophil hypoxia levels were compared with the closest bacteria hypoxia profiles; for more than 95% of neutrophils, hypoxia levels were not found to be significantly higher than the hypoxia generated by bacteria in their vicinity (Supplementary Fig. 2d).

Since the contribution of neutrophils seemed limited, O2 consumption mediated by S. flexneri was investigated as a potential cause of local O2 depletion observed within foci of infection. The S. flexneri population density in foci was estimated from confocal images to be $3.9 \times 10^{10} \pm 2.7 \times 10^{9} \text{cells ml}^{-1}$ for clustered bacteria, compared with $1.3 \times 10^{10} \pm 1.1 \times 10^{9} \text{cells ml}^{-1}$ for bacteria dispersed in the tissue (Fig. 2c,d). The impact of bacterial density on S. flexneri O2 consumption kinetics was then assessed in vitro using a dedicated device allowing the inoculation of bacteria in a sealed anoxic chamber containing culture medium stabilized at 40 mmHg O2, reflecting estimated physiological O2 levels (Fig. 2e). The O2 consumption rate was correlated with the S. flexneri population cell density (Fig. 2f). At a cell density measured within foci of infection (between $1 \times 10^{9}$ and $5 \times 10^{9} \text{cells ml}^{-1}$), complete O2 depletion by S. flexneri occurred rapidly ($t_{\text{anoxia}} = 2.3 \pm 1.1 \text{min}$; Fig. 2f). Similar results were obtained with other pathogenic enterobacteria, including E. coli, Salmonella typhimurium, Listeria monocytogenes and Y. pestis, although not with Lactobacillus casei—an aerotolerant anaerobe (Supplementary Fig. 3). When a similar experiment was conducted at a density corresponding to the dispersed population, complete O2 depletion was not achieved over the measurement period (Fig. 2f). The co-incubation of neutrophils with S. flexneri at a pathophysiological cell density ($8.1 \times 10^{9} \pm 5.4 \times 10^{9} \text{cells ml}^{-1}$; Fig. 2d) had no impact on the timing of complete O2 depletion compared with S. flexneri alone (clustered: $t_{\text{anoxia}} = 2.4 \pm 1.0 \text{min}$; $P > 0.05$; Fig. 2g). A mild increase in O2 consumption was observed when co-incubating neutrophils with S. flexneri at a cell density corresponding to the dispersed population or with heat-killed bacteria ($t = 10 \text{min}$; $P < 0.01$ and $P < 0.05$, respectively), although complete O2 depletion was not achieved over the measurement period (Fig. 2g). We demonstrate here that O2 depletion during infection seems to be driven by S. flexneri, while neutrophils contribute comparatively much less.

We reasoned that S. flexneri aerobic respiration is responsible for the O2 depletion observed in vivo. At the end of the aerobic respiratory chain, O2 is reduced to H2O by terminal cytochrome oxidases6: S. flexneri expresses two cytochrome oxidases named bd-I (CydAB) and bd-II (AppCB), similarly to E. coli (S. flexneri cyoAB genes are truncated) (Fig. 3a). Therefore, we engineered S. flexneri ΔcydAB and ΔappCB mutants and noted that their cellular morphologies were indiscernible from the wild type (Supplementary Fig. 4a).

We showed that the bd-I complex (CydAB), but not the bd-II complex (AppCB), was essential for S. flexneri’s ability to consume O2 (Fig. 3b). Consistently, cytochromes b and d expressed by S. flexneri belong mainly to the bd-I complex (Supplementary Fig. 4b), and S. flexneri ΔcydAB displayed a growth defect in the presence of O2 compared with the wild-type strain, confirming that aerobic respiration is defective in this mutant (Supplementary Fig. 4c).

The S. flexneri ΔcydAB (bd-I) mutant was not virulent in vivo, O2 depletion was not observed, and foci of infection were not formed in the colonic mucosa (Fig. 3c,d), although it remained invasive and was phagocytosed by neutrophils as per the wild-type strain in vitro (Supplementary Fig. 4d). Consistent with previous results (Fig. 2f,g), the co-incubation of neutrophils (with or without diphenyleiodonium chloride (DPI)—a neutrophil NADPH oxidase inhibitor) with S. flexneri ΔappCB (bd-II) had no impact on the timing of anoxia induction (Supplementary Fig. 4e). When similar experiments were conducted with S. flexneri ΔcydAB, or on activation with N-Formyl-Met-Leu-Phe (FmLF), a mild increase in O2 consumption was observed and abolished by DPI (Supplementary Fig. 4e; $P < 0.05$), although anoxia was not reached in both conditions over the measurement period.

We confirmed that glucose transporter 1 (GLUT1)—a hypoxia-induced glucose transporter—was overexpressed in non-infected epithelial cells (physiological hypoxia), but also within S. flexneri foci of infection (Fig. 3f and Supplementary Fig. 5a), although not on S. flexneri ΔcydAB mutant infection (Supplementary Fig. 5a). To confirm that S. flexneri ΔcydAB avirulence was caused by its defect in O2 consumption, we co-infected guinea pigs with S. flexneri pGFP wild-type and ΔcydAB pDsRed mutant strains. We confirmed that hypoxia was induced within foci of infection (GLUT1 stabilization; Fig. 3f), mediated by the S. flexneri wild-type strain, and we demonstrated that, in this context, the S. flexneri ΔcydAB mutant was able to colonize the colonic mucosa together with the wild-type strain (Fig. 3f and Supplementary Fig. 5b). The relative amounts of S. flexneri wild-type and ΔcydAB mutant strains in the lumen (comparing plasmid-cured BS176 and BS176ΔcydAB mutant strains; Fig. 3f and Supplementary Fig. 5b) or within the colonic mucosa (comparing M90T and M90TΔcydAB mutant strains; Fig. 3f and Supplementary Fig. 5b) were similar (competitive index values of ~1), as observed in vitro under anoxic conditions (Fig. 3g). We therefore confirmed our hypothesis that S. flexneri aerobic respiration was the primary cause of hypoxia induction in vivo, explaining its essential role for tissue colonization6. The ability of S. flexneri to grow in the absence of O2 supports the expansion of foci, whose formation appears to be the preferential strategy for tissue colonization (composed of 99.71% total bacteria; Supplementary Fig. 1a).

We hypothesized that the repression of S. flexneri’s dissemination capacity may promote foci of infection extension by favouring colonizing over exploring bacteria. S. flexneri’s ability to invade host cells relies on the T3SS, whose activity is significantly reduced in hypoxic conditions4. To assess the level of T3SS activity of individual bacterium within colonic tissues, we measured the fluorescence signal produced by a S. flexneri strain harbouring the pTSA plasmid, which has a fast-maturating eGFP under the control of a T3SS activity-dependent promoter6.

The positive correlation between O2 availability and T3SS activity in the presence of the secretion inducer Congo red was confirmed in vitro (fast-maturating eGFP fluorescent signal and IgG secretion; Fig. 4a,b; $P < 0.001$). We also previously confirmed O2-dependent type III secretion of effectors in vitro by western blot1. The activity of S. flexneri T3SS was also assessed in vivo within foci of infection (Fig. 4c). Performing a single-bacteria analysis, we demonstrated that S. flexneri T3SS was mostly inactive in infected tissues (87.55% total bacteria; Fig. 4d,e; $n = 265$ bacteria). These results confirm that hypoxic conditions encountered in foci of infection block T3SS activation, hence limiting S. flexneri dissemination to adjacent host cells and promoting tissue colonization through foci expansion.

Here, we show that intestinal mucosa colonization by S. flexneri occurs through the formation and expansion of hypoxic foci of infection. In summary, while O2 is essential for S. flexneri T3SS-dependent epithelium invasion1, its depletion is conversely mandatory for...
subsequent tissue colonization. The ability of *S. flexneri* to survive this low-oxygenated environment, as well as the associated blockade of the T3SS, contributes to the expansion of foci of infection.

Our results reveal that the adaptation of invasive pathogens to various O$_2$ levels is essential for tissue colonization. In fact, to our knowledge, pathogenic microorganisms that are not facultative anaerobes are not invasive. We demonstrate that O$_2$ depletion by pathogenic enterobacteria may be considered a community behaviour, querying previous concepts such as the O$_2$ availability hypothesis. Our results reveal that the adaptation of invasive pathogens to varying O$_2$ levels is essential for tissue colonization. In fact, to our knowledge, pathogenic microorganisms that are not facultative anaerobes are not invasive. We demonstrate that O$_2$ depletion by pathogenic enterobacteria may be considered a community behaviour, querying previous concepts such as the O$_2$ availability hypothesis.

pathophysiological environments. In particular, improved methods will be required to study the population heterogeneity within foci with regard to O$_2$ levels. For example, >40% of individual bacteria were neither clustered nor dispersed in neutropenic animals (Supplementary Table 1), highlighting the importance of foci formation in response to neutrophil recruitment and antimicrobial activity.

O$_2$ depletion by pathogenic enterobacteria is expected to modulate the survival and function of immune cells, particularly neutrophils. Important O$_2$-dependent antimicrobial functions,
Fig. 3 | Aerobic respiration is required for hypoxia induction and efficient colonic mucosa colonization by Shigella in vivo. a. Genetic organization of cytochrome oxidases (bo₃, bd-I and bd-II) encoding genes in E. coli K12 and S. flexneri 5a. The cyoA and cyoB genes are not functional in S. flexneri due to genetic insertions. b. S. flexneri 5a ΔcydAB (bd-I) was unable to consume O₂ in vitro in a medium of RPMI 1640 + 10 mM HEPES stabilized at pH₀ = 40 mmHg, and had a growth defect in the presence of O₂ (n = 3 independent experiments; P < 10⁻⁴, two-way ANOVA; see Supplementary Table 5). O₂ tensions are expressed as means ± s.d., WT, wild type. c. S. flexneri 5a ΔcydAB (bd-I) pGFP (green) was avirulent in vivo compared with the WT and the ΔappCB (bd-II) mutant strain 8 h after infection. Hypoxia and neutrophils were detected with anti-EF5-Cy3 (red) and Myelotracker-Dylight405 (ref. 12) (blue), respectively. White asterisks indicate foci of infection. Scale bars, 50 µm. The experiment was repeated independently three times with similar results. d. Tissues infected with the ΔacydAB strain were not inflamed compared with the WT strain, as indicated by the presence of solid stools in the colon lumen (means ± s.d.; P > 0.8; see Supplementary Table 6). e. Numbers of foci of infection were significantly reduced in tissues infected by the ΔacydAB strain compared with the WT strain (means ± s.d.; ***P < 10⁻⁴; see Supplementary Table 7). f. GLUT1 was detected with a monoclonal antibody (magenta) within S. flexneri 5a pGFP (green) hypoxic foci of infection 8 h after infection. S. flexneri 5a ΔacydAB pDsRed (red) was able to colonize the colonic mucosa on co-infection with the WT strain (S. flexneri 5a pGFP). Plasmid-cured BS176 and BS176ΔacydAB remained in the luminal compartment. Neutrophils were labelled with Myelotracker-Dylight405 (ref. 12) (blue). Scale bars, 70 µm. See Supplementary Fig. 5 for extended imaging. This experiment was repeated independently three times with similar results. g. M90T/V90TΔacydAB and BS176/BS176ΔacydAB competitive indices were determined in co-cultures in vitro (–O₂ conditions) and on guinea pig co-infection in vivo (lumen and colonic mucosa). Competitive index = 1 means no growth difference (means ± s.d.; n = 5 biologically independent animal samples).

such as reactive oxygen species production, are likely to be altered. The contribution of alternative electron transfer machineries for O₂ depletion and enteropathogen virulence, such as the recently described L. monocytogenes extracellular electron transport⁴¹, requires further investigation. The respective contributions of enteropathogens, commensal flora and host cells (that is, neutrophil
activation, epithelial cell death, and so on) in O2 depletion may be further investigated in other acute or chronic models of inflammation. In conclusion, this ‘battle to breathe’ is anticipated to be crucial for the outcome of infection, and will have to be taken into account in the development of truly effective antibiotics or vaccines targeting pathogens in low-O2 environments for efficient clearance or protection.

**Methods**

**Bacterial strains and growth conditions.** *Shigella flexneri* 5a (Shigella) strains (wild-type M90T, plasmid-cured BS176, BS176ΔcydAB, M90TΔfnr mutant and BS176Δfnr mutant) and *Staphylococcus aureus* were propagated in Trypticase soy media (TCS; Oxoid) or on TCS plates, supplemented with Congo red (0.01%; Sigma–Aldrich) for the *Shigella* strains. *E. coli*, *S. typhimurium* and *L. monocytogenes* were cultured in Luria Bertani medium. *L. casei* was grown in De Man, Rogosa and Sharpe broth (Sigma–Aldrich). *S. flexneri* M90T was used as a wild-type *Shigella* strain. Targeted deletion of appCB and cydAB genes was performed using lambda red recombination using the primers appCB1 (5'-ATGTTGGAATGTCATTGATTATCCTATGGAGCTGAGCAGG-3') and appCB2 (5'-TTATCGCGAGCTCGAGGAGTTTCTGGTGCTGCTTGCACCTC-3') with plasmid pKD4 to generate a polymerase chain reaction product containing a chloramphenicol resistance cassette flanked on each end with 50 base pairs corresponding to the *S. flexneri* appCB genes. Primers cydAB1 (5'-TTAGTCAGTTGAGCTCGAGGAGTTTCTGGTGCTGCTTGCACCTC-3') and cydAB2 (5'-ATGTTGGAATGTCATTGATTATCCTATGGAGCTGAGG-3') were used with plasmid pKD4 to generate a polymerase chain reaction product containing a kanamycin resistance cassette flanked on each end with 50 base pairs corresponding to the *S. flexneri* cydAB genes. Knockout generation was performed under anoxic conditions to limit the potential toxicity from inactivating the respiratory pathway. Isolated mutants were passaged on Trypticase soy agar plates containing 0.01% Congo red, to confirm virulence plasmid maintenance.

*S. flexneri* 5a fluorescent strains (wild-type M90T, M90TΔcydAB, plasmid-cured BS176, BS176ΔcydAB, M90TΔfnr mutant and BS176Δfnr mutant) were generated by transforming the pFPV25.1 (AmpR) or pDsRed (AmpR) plasmids, as indicated. Antibiotics were added as necessary at the following concentrations: chloramphenicol, 50 µg ml⁻¹; ampicillin, 100 µg ml⁻¹; and kanamycin 50 µg ml⁻¹. Experiments under different O2 concentrations were performed in an anaerobic cabinet (DG250; Don Whitley) or in a cabinet in which O2 tensions could be controlled (HyperOxystation H35; Don Whitley).

To assess the level of T3SS activity, *S. flexneri* 5a was transformed with pT3AR, allowing the constitutive expression of cyan fluorescent protein (CFP; cyan) and inducible expression of the fast-maturing eGFP (dependent of the T3SS activity).

**Cytochrome quantification.** The cellular contents of cytochrome b and d haemes were calculated as described by Dejean et al., taking into account the respective molar extinction coefficient values (18.0 and 27.9 kM⁻¹ cm⁻¹, respectively), and the reduced minus oxidized spectra recorded using a dual-beam spectrophotometer (DW2000; Aminco). Briefly, *S. flexneri* wild-type, and AcdyAB and ΔappCB mutant strains were grown until an optical density measured at a wavelength of 600 nm (OD₆₀₀) = 2 was reached. For each measurement, 100 OD units were centrifuged for 10 min at 5,000 r.p.m., and bacterial pellets were resuspended in distilled water up to 2 ml (1 ml for the oxidized condition and 1 ml for the reduced condition (dithionite addition)). The cytochrome maximum and minimum absorbance values were measured at 560 and 575, and 630 and 655 nm for cytochromes b and d, respectively, and expressed in mol OD⁻¹.

**Cell culture.** Blood collection and neutrophil purification. All participants gave written, informed consent in accordance with the Declaration of Helsinki principles. Peripheral human blood was collected from healthy patients at the Investigation Clinique et Acces aux Ressources Biologiques service of the Institut Pasteur (Authorization Direction Clinique number: 2008-68). Human blood was
collected from the antecubital vein into tubes containing sodium citrate (3.8% final) as anticoagulant molecules. Guinea pig blood samples were collected from anaesthetized animals by cardiac puncture.

Human or guinea pig neutrophils were purified as described previously. Briefly, whole-blood samples were centrifuged at 450g for 15 min. Platelet-rich plasma was collected and centrifuged at 2,500g for 20 min to form platelet-poor plasma. Blood cells were resuspended in NaCl 0.9% and dextran sulfate (0.72%). After 30 min sedimentation, the neutrophil-containing upper layer was centrifuged at 240g for 10 min. The resuspended pellet was separated, and a two-layer Percoll (GE Healthcare) gradient (51:42%) by centrifugation at 240g for 20 min.

Neutrophils were collected, and remaining red blood cells were removed using CD233a (glycophorin) microbeads (negative selection; Miltenyi Biotec).

For functional assays, human or guinea pig purified neutrophils were cultured in RPMI 1640 (Life Technologies) supplemented with 10 mM HEPES (Life Technologies).

Epithelial cell culture. The Hep2 cell line was purchased from the American Type Culture Collection and tested for Mycoplasma contamination. Hep2 epithelial cells were cultured in DMEM medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific). For functional assays, Hep2 cells were incubated in DMEM medium supplemented with 10 mM HEPES, without foetal calf serum.

Antibodies and fluorescent dyes. Hypoxia detection in guinea pigs was achieved by using the fluorochrome EF5 (molecular weight 800 Da) and a mouse monoclonal antibody against the 19 kDa EF5 component (ab40084; Abcam), used at a 1:40 dilution, and a mouse anti-EF5-Cy3 conjugated with a Cy3 fluorophore (ready-to-use solution; University of Pennsylvania). 4′,6-Diamidino-2-phenylindole (Invitrogen) was used at 1 mg/mL.

Neutrophils were cultured in both the Myelotracker-Dylight405 markers, binding to lactoferrin stored in specific and tertiary granules. GLUT1 was detected with a mouse monoclonal antibody (ab80084; Abcam), used at a 1:40 dilution, and a secondary antibody conjugated with an Alexa 647 fluorophore (Thermo Fisher Scientific).

Bacterial cell infection in vitro. For cell entry assays, Hep2 epithelial cells were seeded (5 × 10⁵ cells well⁻¹) and grown overnight in 24-well tissue culture plates under the indicated conditions (0, 5 and 21% O₂). Neutrophils were directly seeded (5 × 10⁵ cells well⁻¹) in 24-well tissue culture plates. Cells were challenged at a multiplicity of infection of 100 (Hep2) or 20 (neutrophils), with bacteria grown until the mid-log phase was reached in liquid culture under the same conditions. Bacteria were spun briefly onto cells by centrifugation at 2,000g for 10 min.

To measure bacterial entry, cells were challenged as above and incubated for 30 min at 37 °C, after which gentamicin (50 µg mL⁻¹) was added for 30 min to kill extracellular bacteria. Cells were then washed three times with phosphate buffered saline (PBS) and lysed with 1 mL 1% saponin in PBS, and bacteria were recovered by plating to solid media. The results are expressed as the number of colony-forming units (c.f.u. mL⁻¹) 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.

In vitro O₂ quantification. Gas-proof glass tubes were first left in an anaerobic air chamber overnight. The tubes were then filled with RPMI 1640 culture medium supplemented with 10 mM HEPES pre-stabilized at 37 °C and 100 µM HgCl₂. Bacteria and neutrophils were prepared under similar conditions in adequate culture media and initiated immediately after bacteria or cell inoculation. The results were repeated in two independent experiments on homogenized luminal and colonic mucosa bacteria content (M90T versus M90TΔfnrΔcydAB mutant, and BS176 versus BS176ΔcydAB mutant).

Colonic tissue processing for quantitative analysis. Immunostaining. For immunofluorescence samples used for quantitative analyses, 30-µm-thick transversal colon sections were stained using a modified protocol from Arena and colleagues. Briefly, tissues on slides were fixed with 4% PFA for 10 min at room temperature. They were then washed with 1x PBS and stained using an EF5-Cy5 antibody solution (supplied by the University of Pennsylvania) supplemented with 0.1% Triton, 1% bovine serum albumin and Myelotracker-Dylight405 (1 µg/mL⁻¹) overnight at 4 °C. The following day, the slides were washed with 1x PBS and mounted with ProLong Gold (Invitrogen).

Image analysis. Hypoxia profiles through colonic mucosa. The luminal propria was delineated using Fiji. Intensity profiles were generated from 10-µm-thick lines on maximum-intensity projections of the 3D images in the EF5 channel averaged with a Gaussian filter with σ = 10 µm (Fig. 1a). The profiles ran into the tissue, orthogonally to the luminal propria, and their locations were selected to traverse an infectious focus for infected tissues and randomly arranged for non-infected tissues.

The hypoxia curves of Fig. 1b were generated by measuring several profiles that run through the lamina propria from the apical surface to the basal surface (Fig. 1a). Within one condition (infected or non-infected), all profiles were aligned with respect to the first local maximum (crossing the lamina propria surface). After alignment, we had the EF5 signal distribution (one value per profile), which is expressed as a function of the tissue depth. We then computed the mean EF5 signals (thick lines in Fig. 1b) and their standard deviation (shaded areas in Fig. 1b), and reported them as a function of the tissue depth.

We then performed a Student’s t-test at each position along the tissue depth, comparing the distribution of EF5 signal at a given depth between profiles through tissues of infected (red) and non-infected (blue) animals, for all depths. We then reported the range of depths for which this test revealed significant differences as a grey bracket over the plot. Figure 1b displays one of three repetitions. The infected and uninfected curves were made, respectively, by averaging 128 and 61 profiles. Sample sizes were adjusted to reach statistical differences between compared conditions. A blind analysis was used for image acquisition.

Hypoxia measurements at the single-cell level. Automatic segmentation of individual S. flexneri and neutrophils was carried out using TrackMate, yielding their x, y, and z coordinates and their local hypoxia levels, measured from the EF5 channel averaged with a 3D Gaussian filter with σ = 2 µm. Together, bacterial and
neutrophil positions and the manual annotations were processed using MATLAB (MathWorks) for further analysis (Figs. 2 and 3).

We inspected infection foci manually and estimated their radius to be approximately 16 μm. Clustered bacteria were therefore defined as bacteria that have at least six other bacteria in a neighbourhood of 16 μm in radius from their position (Supplementary Fig. 1a). Isolated bacteria were defined as those having no other bacteria in a neighbourhood of 30 μm in radius from their position. Bacteria that did not fall into these two categories were not included in the analysis (Supplementary Table 1).

Mean hypoxia spatial profile generated by bacteria. The hypoxia profile around single bacteria was calculated by averaging the EF5 signal (filtered with σ = 2 μm) of all pixels at a fixed distance from the closest bacterium (Supplementary Fig. 1b). Pixel distance to the closest bacterium was calculated thanks to the image distance transform, taking into account the non-isotropy of the pixel size (0.2 μm pixel⁻¹ in x and y, and 1 μm pixel⁻¹ in z). Locations outside the tissue were not included in the profile. Pixels were then pooled in 1-μm bins from 0–50 μm. All of the EF5 intensity of pixels in a single bin contributed to the mean and standard deviation at the bin distance, and built the profiles of Supplementary Fig. 2d. Single bins incorporated between 600 and 3 × 10⁶ measurements for a single image. This procedure was repeated on 16 images, treating clustered and dispersed bacteria separately. These hypoxia profiles averaged EF5 levels over all pixels at a specified distance, thereby cancelling contributions from other potential sparse hypoxia sources such as neutrophils.

To investigate whether neutrophils contribute a significant addition to this hypoxia, we sought the closest bacterium for each neutrophil and categorized them as dispersed or clustered. We then reported on the hypoxia profile (the EF5 level) of the neutrophil at the measured distance to the closest bacterium, depending on whether the closest bacterium was dispersed or clustered. A one-sided t-test was used to determine whether or not the neutrophil hypoxia could be explained by the bacteria hypoxia profile at this distance (dots) or if it was significantly larger (crosses; significance level: 5%). Less than 5% of neutrophils had significantly higher hypoxia levels than were generated by bacteria over 16 repetitions of this analysis.

Bacteria and neutrophil density. Whole tissue densities (Supplementary Fig. 2a,c,d) were calculated by counting the number of neutrophils or bacteria, regardless of their clustering status, in the total tissue volume imaged for each experimental condition. The clustered Shigella density (Fig. 2d) was calculated by counting the number of neighbors in a sphere of 16 μm around the 61,119 clustered bacteria detected in the conventional animals (the MOMT + EF5 condition). The dispersed Shigella density was calculated by counting the number of dispersed bacteria in the volume of the imaged tissue averaged over the 37 tissue sections of the same condition. The neutrophil density was calculated by counting the number of neutrophils in the volume of the imaged tissue averaged over a subset of 16 sections of the same condition.

T3SS activity reporter signal analysis. We expressed the TSAR T3SS activity reporter in the S. flexneri wild-type strain; EF5 injected guinea pigs were infected with this strain as described above. The reporter emits in the cyan channel (CFP) for all bacteria and in the green channel for bacteria that are positive for T3S activity (fast-maturing cFP; Rep⁺). cFP levels corresponding to active T3SSs were determined in vitro by culturing S. flexneri 5a pTSAR in the presence or absence of O₂, with or without Congo red (0.01%). More than 100 individual bacteria were analysed for each condition in vitro. We then imaged the tissue sections, focusing on bacteria foci, and proceeded to a similar analysis. We quantified signals in 2,488 wild-type bacteria located in foci of infection.

Statistical analysis. All statistical analyses were performed using MATLAB software (MathWorks). Comparisons with two groups (Figs. 1b,h,i and 2b) were supported with unpaired Student’s t-tests. Comparisons with three groups or more were supported by one-way analysis of variance (ANOVA) tests (Figs. 1d,3a, and 4b and Supplementary Figs. 4b,e and two-way ANOVA tests (Figs. 2g and 3b and Supplementary Fig. 4c), followed by a post-hoc Tukey’s test.

When P-values for the significance of pairwise comparisons were calculated (either from a Student’s t-test or a post-hoc Tukey’s test after an ANOVA test) and displayed on figures, we used the following convention: not significant (NS; P ≥ 0.05); *0.01 ≤ P < 0.05; **0.001 ≤ P < 0.01; ***P < 0.001.

Box plots. When results are presented in the shape of a box plot (Figs. 1h,i and 2b), the central mark indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The whiskers extend to the most extreme data points not considered to be outliers, and the outliers are not plotted. Outliers are data points that are further away than 1.5x the range from the first to the third quartile, respectively, above or below the third and first quartile.

Direct O₂ consumption quantification in vitro. Direct O₂ quantifications were performed in gas-proof glass punchable tubes (368430; BD Vacutainer Z) using an oximeter (OXY-Meter; Unisense) combined with a needle sensor for piercing (OX-NP). Data were recorded using SensorTrace Logger software (Unisense). Glass tubes were first opened in an anaerobic cabinet (MiniMACS DG250; Don Whitley) for 30 min to ensure the absence of exogenous O₂, were removed from the tube (anoxic atmosphere). Such conditioned tubes were subsequently filled with 2 ml of a culture medium (RPMI 1640 + 10 mM HEPES for neutrophils) and pre-stabilized at 37°C and pO₂ = 40 mM[Hg] (5% O₂) in a hypoxic chamber (in vivo 500; Don Whitley) (Fig. 2c). Before their inoculation, bacteria (S. flexneri 5a wild-type and mutant strains, E. coli, S. typhimurium, L. monocytogenes, S. aureus or L. casei) were grown under similar conditions until OD₅₇₀ = 0.3 was reached. Bacteria alone or with neutrophils were resuspended at the indicated concentrations in RPMI 1640 + 10 mM HEPES. When DPI (Sigma–Aldrich) was added at a final concentration of 25 μM, N-Formyl-Met-Leu-Phe (Sigma–Aldrich) at 1 μM. Direct O₂ quantification was performed over time, and initiated immediately after bacteria or cell inoculation. The results were averaged from at least three independent measurements.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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**Author contributions**

B.S.M., J.-Y.T. and E.T.A. designed the experiments, interpreted the data and wrote the paper. M.A. designed the *Shigella* mutants. G.N., L.I., A.A., M.F. and F.-X.C.-V. contributed to studying the *Shigella* mutants in vitro and in vivo. J.-Y.T. conducted quantitative analysis of the data. A.D., S.L.S. and P.J.S. contributed to data interpretation.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated
- Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

Software and code

Policy information about availability of computer code

Data collection
- Leica LAS X v3.3.0
- Yokagawa CellVoyager software v1.06.06

Data analysis
- Fiji, version for Java8 (http://fiji.sc/)
- TrackMate, version 3.x (https://imagej.net/TrackMate)
- MATLAB v2017a, The Mathworks.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | In each condition described in the study, we used a minimum of 4 animals; more animals could not be processed due to facility constraints and were sufficient for statistical analyses. |
| Data exclusions | No exclusion of data was done. |
| Replication | All conditions were tested in a single experiment (infection, tissue processing, labelling and imaging), which was repeated on three independent occasions; all replicates were carried out successfully. |
| Randomization | All image acquisitions and processing were done on randomized, blind samples, so as not to bias results based on expected outcomes of the various conditions. |
| Blinding | Please see above statement. |

Reporting for specific materials, systems and methods

Materials & experimental systems

N/A Involved in the study

☐ Unique biological materials
☐ Antibodies
☐ Eukaryotic cell lines
☐ Palaeontology
☐ Animals and other organisms
☐ Human research participants

Methods

N/A Involved in the study

☐ ChIP-seq
☐ Flow cytometry
☐ MRI-based neuroimaging

Antibodies

Antibodies used

EF5 hypoxia reporter (http://www.hypoxia-imaging.org/) - anti-EF5 antibody (ELK3-51) conjugated to Cy3 (University of Pennsylvania); DAPI (Invitrogen, ref 62248, 1 mg/ml, 1:1000 dilution); MUB40-Dylight405 marker (Anderson, M. C. et al. 2018, 1 mg/ml, 1:1000 dilution); GLUT-1 mouse monoclonal antibody (Abcam, ref ab40084, clone SPM498, 1:40 dilution) - goat anti-mouse secondary polyclonal antibody conjugated with Alexa647 (Thermofisher Scientific, ref A32733, 1:500 dilution); mouse anti-Shigella 5a LPS monoclonal antibody (produced in our laboratory by Armelle Phalipon, 1:1000 dilution) - goat anti-mouse IgG HRP-conjugated polyclonal antibody (Sigma Aldrich, ref A0168, 1 ml, 1:500 dilution)

Validation

Validation of anti-EF5 antibody conjugated to Cy3 was first guaranteed by the manufacturer. We validated it on non-infected guinea pig colonic samples, reporting the physiological hypoxia (positive staining of the colonic epithelium), as previously described (Karhausen, JCI, 2004). The primary mouse anti-LPS antibody has been validated in many manuscripts published by our laboratory over the last decade.
**Eukaryotic cell lines**

Policy information about cell lines

| Cell line source(s) | Hep2 cell line (Hela derivative); the cell line was purchased at ATCC (ATCC CCL-23). |
|---------------------|-------------------------------------------------------------------------------------|
| Authentication      | The authentication of the cell line was guaranted by the company.                  |
| Mycoplasma contamination | Tested negative for mycoplasma every week                                        |
| Commonly misidentified lines (See ICLAC register) |                                                                                   |

**Animals and other organisms**

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Young (3-week old), female Hartley guinea pigs (Charles River laboratories) were used in this study. |
|--------------------|--------------------------------------------------------------------------------------------------|
| Wild animals       | This study did not involve wild animals.                                                         |
| Field-collected samples | This study did not involve samples collected from the field.                                    |