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Novel role of the nitrite transporter NirC in *Salmonella* pathogenesis: SPI2-dependent suppression of inducible nitric oxide synthase in activated macrophages

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Activation of macrophages by interferon gamma (IFN-γ) and the subsequent production of nitric oxide (NO) are critical for the host defence against *Salmonella enterica* serovar Typhimurium infection. We report here the inhibition of IFN-γ-induced NO production in RAW264.7 macrophages infected with wild-type *Salmonella*. This phenomenon was shown to be dependent on the *nirC* gene, which encodes a potential nitrite transporter. We observed a higher NO output from IFN-γ-treated macrophages infected with a *nirC* mutant of *Salmonella*. The *nirC* mutant also showed significantly decreased intracellular proliferation in a NO-dependent manner in activated RAW264.7 macrophages and in liver, spleen and secondary lymph nodes of mice, which was restored by complementing the gene *in trans*. Under acidified nitrite stress, a twofold more pronounced NO-mediated repression of SPI2 was observed in the *nirC* knockout strain compared to the wild-type. This enhanced SPI2 repression in the *nirC* knockout led to a higher level of STAT-1 phosphorylation and inducible nitric oxide synthase (iNOS) expression than seen with the wild-type strain. In iNOS knockout mice, the organ load of the *nirC* knockout strain was similar to that of the wild-type strain, indicating that the mutant is exclusively sensitive to the host nitrosative stress. Taken together, these results reveal that intracellular *Salmonella* evade killing in activated macrophages by downregulating IFN-γ-induced NO production, and they highlight the critical role of *nirC* as a virulence gene.

INTRODUCTION

Salmonella enterica serovar Typhimurium is a broad-host-range pathogen that causes self-limiting gastroenteritis in immunocompetent humans. Mouse infection by *Salmonella* Typhimurium constitutes a model for the systemic syndrome associated with nontyphoidal salmonellosis in immunodeficient people. In mice, *Salmonella* Typhimurium proliferates in the phagocytic cells (Miller *et al.*, 1995). Macrophages express inducible nitric oxide synthase (iNOS), which then catalyses the generation of nitric oxide (NO) upon stimulation with interferon gamma (IFN-γ) and various other agents including *Salmonella* (Brett *et al.*, 2008; Chakravortty & Hensel, 2003; Cherayil & Antos, 2001; MacMicking *et al.*, 1997; Stuehr, 1997; Xie & Nathan, 1994). High NO production suppresses the growth of many intracellular pathogens such as *Leishmania major* (Evans *et al.*, 1993), *Toxoplasma gondii* (Suzuki *et al.*, 1988), *Mycobacterium bovis* (Hanano & Kaufmann, 1995) and *Salmonella* (Mastroeni *et al.*, 2000; Vazquez-Torres *et al.*, 2000). The antimicrobial property of NO is due to its ability to bring about widespread damage to DNA, proteins and lipids. In order to survive and thrive in the hostile environment of the host, *Salmonella* employs various strategies to avoid and persist in the presence of antimicrobial radicals such as NO (Lahiri *et al.*, 2008a) and the subsequently produced reactive nitrogen species (RNS). For example, *Salmonella* can prevent co-localization of the *Salmonella*-containing vacuole with iNOS in a SPI2 (*Salmonella* pathogenicity island 2)-dependent manner, protecting itself from the NO stress (Chakravortty *et al.*, 2002).

To counteract host nitrosative stress, *Salmonella* has evolved many RNS-metabolizing enzymes, such as the flavohaemoglobin HmpA (Bang *et al.*, 2006) for aerobic detoxification of NO, and the flavorubredoxin NorV and

Abbreviations: BMDM, bone-marrow-derived macrophages; FBS, fetal bovine serum; IFN-γ, interferon gamma; iNOS, inducible nitric oxide synthase; JAK-STAT, Janus kinase/signal transducer and activator of transcription; L-NIL, L-N^6-iminoethyllysine; MLN, mesenteric lymph nodes; NED, *N*-(naphthyl)ethylenediamine dihydrochloride; RNS, reactive nitrogen species; ROI, reactive oxygen intermediates; SOCS-3, suppressor of cytokine signalling-3; SPI2, *Salmonella* pathogenicity island 2.

Three supplementary tables and three supplementary figures are available with the online version of this paper.

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the periplasmic cytochrome *c* NrfA nitrite reductase for anaerobic detoxification of NO (Mills *et al.*, 2008). In addition, *Salmonella* has the *nir* operon, which is predicted to encode an NADH-dependent nitrite reductase (NirBD), which is potentially able to reduce nitrite to ammonia. The third gene of the *nir* operon, *nirC*, codes for a potential nitrite transporter, which is predicted to be a polytopic membrane protein having six transmembrane helices. Its homologue in *Escherichia coli* is induced during anaerobic growth in the presence of high concentrations of nitrite and is required for nitrite uptake (Clegg *et al.*, 2002). Although several studies have focused on the roles of other NO stress-combating genes in *Salmonella*, the role of NirC in this context has remained unknown.

IFN-γ, a very important secretory cytokine, plays a complex and central role in the resistance of mammalian hosts to pathogens. Over 200 genes are known to be regulated by IFN-γ via the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway. IFN-γ transcriptionally co-induces iNOS and two additional enzymes involved in substrate (L-arginine) and cofactor (tetrahydrobiopterin) synthesis for NO generation (Boehm *et al.*, 1997). On the other hand, SpiC, one of the crucial secreted virulence factors encoded by SPI2, inhibits IFN-γ signalling by inducing the host factor SOCS-3 (suppressor of cytokine signalling-3) (Uchiya *et al.*, 1999; Uchiya & Nikai, 2005). Thus, the level of SpiC in activated macrophages dictates the outcome of *Salmonella* infection by regulating IFN-γ-induced iNOS expression.

In the study reported here, we have shown that the NO response and iNOS induction in IFN-γ-activated macrophages (both pre- and post-treatment) is downregulated following infection with wild-type *Salmonella* in a *nirC*-dependent manner. We further demonstrate that the *nirC* mutant shows a higher level of SPI2 repression under nitrosative stress and elicits an increased phosphorylation of STAT-1 in IFN-γ-activated macrophages. This leads to increased iNOS induction and thereby controls the NO response of the host macrophages. This study is believed to be the first to show that NirC is a major nitrite transporter in *Salmonella* and is required for its virulence in mice. It further demonstrates the intricate relationship between NirC and SPI2 and its importance in determining the fate of *Salmonella* pathogenesis.

METHODS

Bacterial strains, plasmids and growth conditions. The strains and plasmids used for this study are listed in Supplementary Table S1, available with the online version of this paper. The wild-type (WT) *S. enterica* serovar Typhimurium strain 12023 used was a kind gift of Professor Michael Hensel, Max von Pettenkofer-Institute for Hygiene and Medizinische Mikrobiologie, Germany. The bacterial cultures were grown at 37 °C in Luria broth (LB) in the presence of nalidixic acid, carbenicillin or kanamycin at 50 μg ml $^{-1}$. Heat-killed bacteria were prepared by heating the cultures at 80 °C for 20 min.

Preparation of bone-marrow-derived macrophages (BMDM). As described by Pan *et al.* (2006), femurs were collected aseptically from mice, and after removal of muscle, the ends of the bones were cut off and the marrow was flushed out. The cells were centrifuged at 1000 r.p.m. for 10 min and resuspended in Dulbecco's modified Eagle medium (DMEM; Gibco) [with 4.5 g D-glucose and 110 mg sodium pyruvate per litre and with the addition of 30 % L929 cell-conditioned medium (see below), 10 % heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U penicillin ml^{-1}, 100 μg streptomycin ml^{-1}], and 30 ml aliquots were added to 150 mm-style non-tissue-culture-treated dishes. The plate was placed in a tissue culture incubator in 10 % CO_2 at 37 °C, and after 4 days 10 ml additional medium was added. When the cells reached confluence, the medium was removed, and the cells were washed once and detached by a 15 min exposure to ice-cold PBS. The detached cells were suspended by pipetting, centrifuged at 1000 r.p.m. for 5 min, resuspended in DMEM-L929 conditioned medium, and plated in tissue culture plates. The cells were observed to be >90 % CD11b positive by fluorescence-activated cell sorting analysis. The BMDM were plated at 1×10^6 cells ml^{-1} in 24-well tissue culture plates 6 h prior to use in experiments.

The L929 cell line was a kind gift of Professor M. S. Shaila, Department of Microbiology, Indian Institute of Science, Bangalore, India. L929 conditioned medium was produced by plating L929 cells in 75 cm^2 tissue culture flasks at an initial density of 1×10^6 cells ml^{-1} in DMEM (high glucose) supplemented with 10 % non-heated FBS, 2 mM L-glutamine, and penicillin/streptomycin as described above. After 5 to 7 days in culture, when the adherent cells were fully confluent, the culture supernatant was centrifuged at 2000 r.p.m. for 10 min, aliquoted and stored at −20 °C.

Cell culture and bacterial infection. The murine macrophage-like cell line RAW264.7 was a kind gift of Professor Anjali Karande, Department of Biochemistry, Indian Institute of Science, Bangalore, India. RAW264.7 cells were maintained in a 37 °C incubator with 5 % CO_2 in DMEM (Sigma) supplemented with 10 % heat-inactivated FBS (Sigma).

Bacterial infection of macrophages was performed as described previously (Chakravortty *et al.*, 2002; Lahiri *et al.*, 2008b). Briefly, the strains were grown to stationary phase in LB with the respective antibiotic. The OD_{600} of the cultures was adjusted with LB to 0.3 and the bacteria were washed once with PBS. Appropriate dilutions of the bacterial cultures were made in cell culture medium and added to the macrophage cells growing in tissue culture plates at different multiplicities of infection (m.o.i.) as stated in the figure legends. Bacteria were centrifuged onto the cells at 500 ***g*** for 5 min. After infection for 25 min, cells were washed three times with PBS and incubated for 1 h in the cell culture medium containing 25 μg gentamicin ml^{-1} (Sigma). The medium was replaced with medium containing 10 μg gentamicin ml $^{-1}$ for the rest of the experiment.

For the enumeration of the intracellular bacteria, macrophages were washed three times with PBS, lysed with 0.1 % Triton X-100 for 10 min at room temperature, and serial dilutions were plated onto LB agar with the respective antibiotics. In some experiments, chloramphenicol (15 μg ml^{-1}), recombinant murine IFN-γ (Peprotech, 100 U ml $^{-1}$), aminoguanidine (Sigma, 100 μM) or L-NIL (L-N^6-iminoethyllysine; Calbiochem, 10 μM) was added to the cells along with 10 μg gentamicin ml^{-1}.

Construction of the *nirC* mutant *Salmonella* strain. The *nirC* (STM 3476) mutation was engineered in *Salmonella* Typhimurium following the one-step deletion strategy as described by Datsenko & Wanner (2000). Briefly, transformants carrying a red helper plasmid (pKD46) were grown in LB with ampicillin and 10 mM L-arabinose at 30 °C to an OD_{600} of 0.35–0.4 and then made electrocompetent by

washing three times with ice-cold 10 % (v/v) glycerol and MilliQ water. PCR product containing the kanamycin-resistance gene (from plasmid pKD4) flanked by sequences upstream and downstream of *nirC* was obtained with the sets of primers described in Supplementary Table S2. This DNA was then electroporated into *Salmonella* Typhimurium carrying pKD46. The *nirC* mutant was selected by kanamycin resistance and confirmed by PCR using the confirmatory primers. In the knockout strain, a 1.5 kb band was amplified whereas in the WT strain with confirmatory primers a gene-specific band of 800 bp was observed.

Construction of the *nirC*-complemented strain and *spiC*-over-expressing strain. DNA extracted from WT *Salmonella* Typhimurium was used as a template to amplify the *nirC* gene or the *spiC* gene using primers listed in Supplementary Table S2. The amplified product was purified and the inserts along with vector pQE60 were digested with *Bam*HI and *Hin*dIII. The vector and insert were mixed at 1 : 3 molar concentrations and ligated at 16 °C for 16 h. The vector-containing inserts were then transformed into *E. coli* competent cells and plated on LB-carbenicillin plates after 1 h incubation in LB medium. The colonies were screened for plasmids having the appropriate insert by restriction digestion, and the purified plasmid containing *nirC* was then transformed into Δ*nirC* electro-competent cells. The *spiC*-containing plasmid was transformed in both the WT and Δ*nirC* background.

Determination of the nitrite concentration. Nitrite (NO_2^-) accumulation in the supernatants of cultured macrophages, used as an indicator of NO production, was measured by the Griess reaction with sodium nitrite as a standard. A 50 μl sample of the supernatant was incubated for 10 min with 50 μl of a solution containing *N*-(naphthyl)ethylenediamine dihydrochloride (NED) (0.01 %) and sulfanilamide (0.1 %) in 5 % phosphoric acid. The absorbance was then measured at 540 nm.

Nitrite uptake assay. Nitrite uptake was determined by measuring nitrite disappearance from the uptake mixture as described by Wu & Stewart (1998). Assay mixtures contained cell suspension (1×10^8 bacteria), 40 mM glucose, and 80 mM MOPS/NaOH buffer (pH 8.0) in a final volume of 5.0 ml. Assays were initiated by adding $NaNO_2$ at a final concentration of 75 μM. The assay tube (5 ml) was incubated at 37 °C with shaking. At defined time intervals, a 100 μl sample of the culture was mixed with 50 μl 1 % (w/v) sulphanilamide in 1 M HCl and 50 μl 0.01 % (w/v) naphthylethylene diamine dihydrochloride. The presence of nitrite was indicated by the appearance of a dark pink colour that developed immediately. The concentration of nitrite in the supernatant was determined by measuring the absorbance at 540 nm using sodium nitrite as standard. Uptake rates (micromoles of nitrite produced per minute per 10^8 bacteria) were determined until 16 h of growth.

Construction of the *nirC* and *spiC* reporter plasmids. The pHG86 plasmid, which carries a promoterless *lacZ* gene downstream from a unique MCS site, was used for cloning of the promoter constructs. DNA extracted from WT bacteria was used as a template to amplify the *nirC* promoter (500 bp) or *spiC* promoter (500 bp) using primers listed in Supplementary Table S2. The amplified products were purified using the Eppendorf 'Gel Cleanup' kit and the insert along with vector pHG86 was digested with *Eco*RI and *Bam*HI. The vector and insert were mixed at 1 : 3 molar concentrations and ligated at 16 °C for 16 h. The vector-containing insert was then transformed into *E. coli* DH5α competent cells, which were plated on LB-ampicillin plates after 1 h incubation in SOC medium. The colonies were screened for plasmids with the appropriate insert, and isolated plasmids were then transformed into either the WT or the Δ*nirC* strain.

Determination of the effects of acidified nitrite on *nirC* promoter activity. WT *Salmonella* Typhimurium harbouring either the *nirC*: : *lacZ* promoter construct or the empty vector were grown overnight in LB and were further subcultured at 37 °C in a shaker incubator until they reached an OD_{600} of 0.4. The NO donor $NaNO_2$ (Sigma, freshly prepared in distilled water) and H_2O_2 (Sigma) were used to determine the effects of RNS and ROI (reactive oxygen intermediates) stress on *nirC* transcription. The NO donor was added to LB at pH 5.0 along with 100 μl samples of exponential-phase *Salmonella* culture adjusted to the same OD_{600} (0.2). After 1, 3 and 9 h of exposure, equal OD_{600} (0.3) aliquots of different samples were examined for β-galactosidase activity and serial dilution was performed for enumeration of bacteria. Five hundred microlitres of OD-adjusted culture of each sample was centrifuged at 10 000 ***g***. The pellet was resuspended in Z-buffer (Na_2HPO_4, 60 mM; NaH_2PO_4, 40 mM; KCl, 10 mM; $MgSO_4.7H_2O$, 1 mM; β-ME, 50 mM; pH 7.0). Then 20 μl chloroform and 10 μl 0.1 % SDS were added to the resuspended pellet along with 200 μl *o*-nitrophenyl β-D-galactopyranoside (ONPG, Sigma) (4 mg ml^{-1}). After 1 h incubation in the dark at 28 °C, 500 μl 1 M $NaCO_3$ was used to stop the reaction. Finally, the absorbance was measured at 420 nm. β-Galactosidase activity (Miller units) was expressed relative to the respective empty vector controls.

Western blot analysis. RAW264.7 cells were seeded in 6-well plates (5×10^5 cells per well). At 12 h after infection or treatment, cells were scraped from the bottom of the wells, lysed in SDS-PAGE sample buffer and boiled for 5 min at 95 °C. Aliquots containing equal amount of protein (100 μg) were loaded onto 10 % gel and transferred onto the nitrocellulose membrane using a mini-gel transfer apparatus (Bio-Rad). The membranes were treated with 5 % skimmed milk solution for 1 h to block non-specific binding and then incubated for 2 h with rabbit polyclonal antibody against mouse iNOS (Sigma-Aldrich; 1 : 10 000), rabbit polyclonal antibody against mouse P-STAT-1 (Cell Signalling; 1 : 1000) or rabbit polyclonal antibody against mouse β-actin (Sigma; 1 : 1000). The blots were further treated with goat anti-rabbit IgG–horseradish peroxidase conjugate (AP-Biotech, 1 : 2000) for 2 h. The immune complexes on the blots were detected with enhanced chemiluminescence substrate (Perkin Elmer) and exposed to Eastman Kodak XAR X-ray film.

Determination of the effects of NO on SPI2 expression. SPI2 expression was induced by culturing *Salmonella* Typhimurium in low-osmolarity (8 μM $MgCl_2$) N salts mediume (Deiwick *et al.*, 1999). Briefly, strains harbouring the *spiC*: : *lacZ* promoter construct were grown overnight in high-magnesium N salts medium (5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 38 mM glycerol, 0.1 % Casamino acids supplemented with 10 mM $MgCl_2$ and 100 mM Tris/HCl), pH 7.6. The bacteria were subcultured in this medium and grown at 37 °C in a shaker incubator until they reached an OD_{600} of 0.5. SPI2 expression was induced by switching the bacteria to 8 μM $MgCl_2$ N salts medium, pH 6.9. The NO donor $NaNO_2$ (Sigma, freshly prepared in distilled water) was used to determine the effect of RNS on SPI2 transcription. The NO donor was added to fresh 8 μM $MgCl_2$ N salts medium, pH 5.0, along with 100 μl of OD-adjusted *Salmonella* Typhimurium cultures that had been grown for 1 h in 8 μM $MgCl_2$ N salts medium. The expression of the *spiC*: : *lacZ* promoter construct was quantified spectrophotometrically as β-galactosidase activity using the substrate ONPG (Sigma) as described above. β-Galactosidase activity was expressed as fold decrease in Miller units from the control values where nitrite was not added.

Synthesis of SPI2 cDNA from bacterial cultures. Total RNA was isolated from the bacterial cultures grown in 8 μM $MgCl_2$ N salts medium after exposure to nitrite stress at pH 5.0 as described before. The samples were resuspended in TRIzol reagent (Sigma) and the

total RNA was isolated according to the manufacturer's protocol. Complementary cDNA was synthesized at 42 °C for 30 min using MMLV reverse transcriptase (Promega) and was used as template for standard PCR with gene-specific forward and reverse primers as indicated in Supplementary Table S2.

Localization of nitrotyrosine residues by fluorescence microscopy. The formation of peroxynitrite molecules in the IFN-γ-activated macrophages was determined by standard immunocytochemical methods. Cells infected with WT *Salmonella* Typhimurium or the *nirC* mutant were washed free of medium and the cells were fixed for 10 min in paraformaldehyde (3.5 %) at room temperature and washed in PBS. Fixed cells were then incubated for 1 h with rabbit anti-mouse nitrotyrosine IgG (Upstate Biotechnology) diluted 1 : 250 and mouse anti-LPS antibody (1 : 500, Hytest) in PBS containing 2 % BSA, 2 % goat serum and 0.2 % saponin. The cells were then washed three times in PBS and incubated in identical conditions with goat anti-rabbit IgG conjugated to Cy5 (Jackson Laboratory, 1 : 100) and goat anti-mouse IgG conjugated to Cy2 (Jackson Laboratory, 1 : 100) for 1 h. Samples were viewed with a confocal laser-scanning microscope equipped with an argon laser (Zeiss).

Determination of the *nirC* and the *spiC* promoter activity in the activated RAW264.7 cell line. RAW264.7 cells were seeded into 24-well plates (2×10^5 cells; three wells per time point) and were infected with WT *Salmonella* Typhimurium carrying either the *nirC* or the *spiC* promoter and with the *nirC* mutant carrying the *spiC* : : *lacZ* promoter construct. Cells were also infected with WT *Salmonella* carrying pHG86 empty vector as a control. At selected time points post-infection, as described by Szeto *et al.* (2009), cells were washed twice with PBS and harvested using a total of 1 ml ice-cold lysis buffer per well (1 % Triton X-100, 0.1 % SDS, 1 mM PMSF, 5 mM NaF, 5 mM NaV, 10 μg aprotinin ml^{-1}, 1 μg leupeptin ml^{-1}, 10 μg pepstatin ml^{-1}). A 200 μl sample of cell lysate from each time point was used to enumerate intracellular bacteria by dilution plating. The remaining 800 μl of cell lysate was centrifuged at 10 000 ***g*** for 3 min, and the pellet was resuspended in 50 μl PBS and mixed with 13 μl chloroform. The expression of the *spiC* : : *lacZ* promoter construct in different bacterial pellets was quantified spectrophotometrically as β-galactosidase activity using the substrate ONPG (Sigma). β-Galactosidase activity was expressed as fold increase compared to control infection.

Mouse experiments. Six- to eight-week-old $iNOS^{-/-}$ mice (B6.129 P2-Nos2 <tm1Lau>/J, Jackson Laboratory) and congenic B6 mice and BALB/c mice were maintained under specific-pathogen-free conditions in the Central Animal Facility, Indian Institute of Science, Bangalore, India. All the procedures with animals were carried out as approved by the animal ethics committee of the Institute. Bacterial strains were grown with shaking overnight at 37 °C, centrifuged, washed, resuspended to an appropriate concentration in sterile PBS and administered to mice at the indicated doses. For the organ infiltration experiment, 5 days after infection, spleens were taken aseptically. The organs were weighed and homogenized in 1 ml PBS. The homogenate was centrifuged and plated at different dilutions to determine the number of bacteria.

The competitive index assays (Beuzon & Holden, 2001) were performed by oral infection of mice with mixtures of the WT and Δ*nirC* or the WT and complemented Δ*nirC* strain in a 1 : 1 ratio in an inoculum of 0.5×10^6 bacteria. After 5 days of infection, homogenized samples of liver, spleen and MLN of infected mice were plated on different antibiotic plates and the competitive index of the Δ*nirC* strain was calculated in two independent experiments. As a control experiment, the competitive indices of a different WT strain were calculated in the same way.

Serum nitrite assays. Mice sera were collected from WT- or Δ*nirC*-infected mice. Each group consisted of three mice. The serum was deproteinized to reduce turbidity by precipitation using methanol/diethyl ether. Experiments were performed at room temperature. A nitrite standard solution was serially diluted (200–1.6 μM) in duplicate in a 96-well, flat-bottomed polystyrene microtitre plate (Polylab). The diluting medium was used as standard blank. After loading the plate with samples (100 μl), addition of VCl_3 (100 μl) to each well was rapidly followed by addition of the Griess reagents, sulfanimide (100 μl) and NED (100 μl). The absorbance at 540 nm was measured in a plate reader after incubation for 30 min in the dark (Miranda *et al.*, 2001).

Statistical analysis and software. Each assay was repeated at least three times. *In vitro* data were analysed by paired *t* test (two sample, equal variance) and *P*-values below 0.05 were considered significant. FACS data were plotted and analysed using WinMDI 2.9 software. Results of mouse challenge studies were evaluated by using Mann–Whitney *U* tests from the GraphPad Prism 4.0 software. Differences between experimental groups were considered significant for $P<0.05$. Immunoblots and gels were quantified using Multi Gauge V2.3 software.

RESULTS

NirC in *Salmonella* Typhimurium is a conserved membrane-spanning nitrite transporter

In silico analysis predicted that the NirC protein in *Salmonella* has six membrane-spanning domains as known in *E. coli*. Bioinformatics analysis against non-redundant protein databases revealed that several bacterial proteins share significant homology with NirC. All of them are known nitrite or formate transporters from different bacteria. More precise comparison of NirC in all *Salmonella* species also revealed its conserved nature (Supplementary Fig. S1).

Infection of IFN-γ-activated RAW264.7 macrophages with WT *Salmonella* Typhimurium suppresses IFN-γ-induced NO synthesis in a *nirC*-dependent manner

Macrophages play a critical role in the clearance of pathogens from the host (Lindgren *et al.*, 1996). In IFN-γ-activated RAW264.7 macrophages we sought to determine the effect of *Salmonella* Typhimurium infection on the nitrite response. Treatment of RAW264.7 macrophages with IFN-γ led to an increased production of nitrite (a measure of NO synthesis) in the culture supernatant. However, when the IFN-γ-treated cells had been infected with the WT *Salmonella* at an m.o.i. of 150, 100, 10 or 1 for 12 h, a striking reduction in the nitrite production was observed in the culture supernatants (Fig. 1a). This difference was seen even when the cells were pretreated with IFN-γ for 12 h before performing the infection (Fig. 1b). The phenomenon was abolished at very low m.o.i., suggesting the fact it might be dependent on a bacterial protein. No decrease in cell number or viability was observed in any of the experimental conditions as

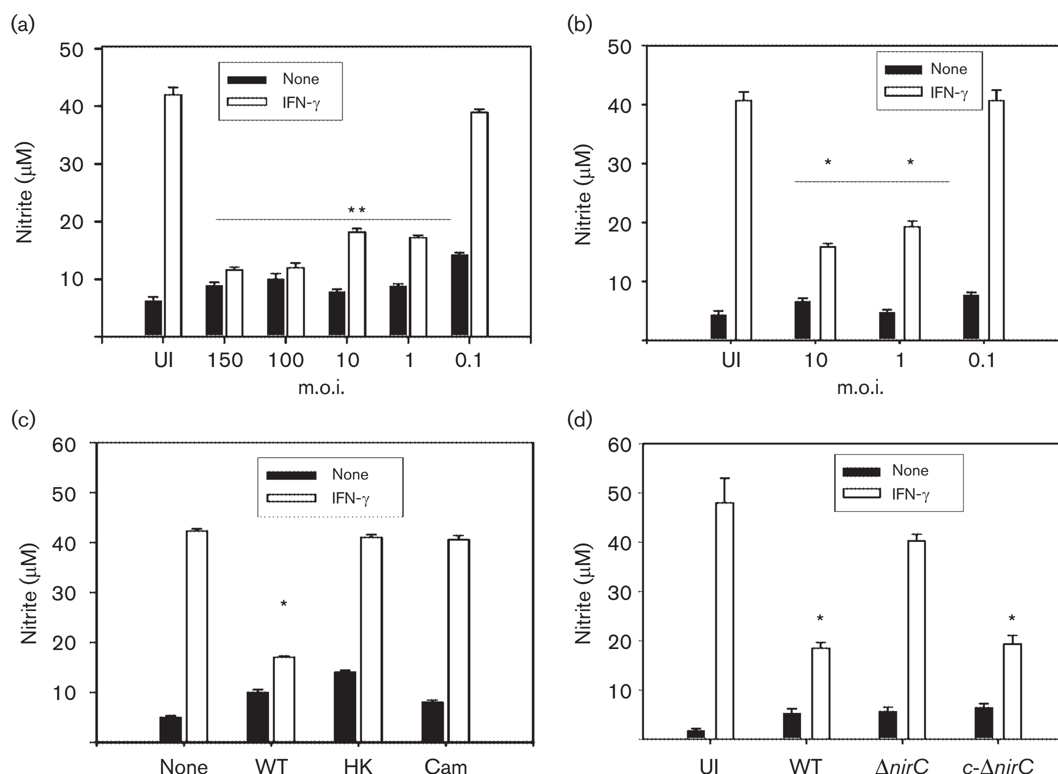

Fig. 1. (a–c) Nitrite production is suppressed in IFN-γ-activated RAW264.7 macrophages by WT *Salmonella* Typhimurium. (a) RAW264.7 cells were infected for 12 h with WT *Salmonella* at various m.o.i., with or without simultaneous IFN-γ treatment. (b). RAW264.7 cells were pretreated with IFN-γ for 12 h and then infected for 12 h with WT *Salmonella* at various m.o.i. (c) RAW264.7 cells were infected at an m.o.i. of 10, in the presence or absence of IFN-γ, with heat-killed WT *Salmonella* (HK), and with live WT bacteria with or without chloramphenicol (Cam; 15 μg ml^{-1}) treatment. Production of nitrite was determined in culture supernatants by Griess reaction. Values are expressed as mean ± SD of one of three independent experiments performed in triplicate. (d) NO production is suppressed in IFN-γ-activated RAW264.7 macrophages by WT *Salmonella* in a Δ*nirC*-dependent manner. RAW264.7 cells were infected at an m.o.i. of 10 with WT *Salmonella*, *nirC* mutant and the *nirC*-complemented strain (*c*-Δ*nirC*). Production of nitrite was determined in culture supernatants by Griess reaction. Statistical significance was defined as follows: *, $P<0.05$; **, $P<0.01$ (Student's *t* test). UI, uninfected. The concentration of IFN-γ was 100 IU ml^{-1} in each case.

indicated by MTT (tetrazolium) assay (Supplementary Fig. S2). We carried out all subsequent experiments with an m.o.i. of 10 and IFN-γ was added at 25 min after infection to mimic the *in vivo* infection system, wherein cytokines are produced after bacterial infection. Heat-killed *Salmonella* did not show the response seen with viable bacteria. We also observed that WT-induced downregulation of nitrite was abrogated by chloramphenicol treatment to inhibit prokaryotic protein synthesis (Fig. 1c).

We hypothesized that the nitrite transporter NirC might modulate the host NO response in favour of the pathogen. Therefore, we analysed the nitrite production by the *nirC* mutant. As shown in Fig. 1(d), the *nirC* mutant showed a heightened NO response upon IFN-γ treatment when compared to the WT bacteria. The *nirC*-complemented strain displayed inhibition of IFN-γ-induced NO production similar to the WT bacteria. Hence, Δ*nirC* *Salmonella* has lost its ability to downregulate the IFN-γ-induced nitrite production.

Regulation of the *nirC* promoter

An assay system to mimic the RNS stress is acidified nitrite (Kim *et al.*, 2003). Therefore, the influence of nitrite stress on the expression of the *nirC* promoter was examined using acidified $NaNO_2$. The β-galactosidase activity of the *nirC*::*lacZ* promoter construct was measured. As shown in Fig. 2(a) the promoter activity was increased up to twofold after 3 h stress with 500 μM $NaNO_2$ exposure (acidic and normal pH). Anaerobic incubation also led to a similar increase in the promoter activity. The promoterless construct (empty vector) showed negligible β-galactosidase activity under all conditions tested. Hence, for the empty vector the β-galactosidase activity is shown for only one

representative condition. When oxidative stress was conferred by adding 500 μM H_2O_2, no change was observed in the transcription of the *nirC* promoter construct, suggesting high specificity of this transporter for nitrite (Fig. 2a). In infected RAW264.7 cells, IFN-γ induction led to a threefold increase in the *nirC* promoter activity (Fig. 2b).

nirC-deficient *Salmonella* Typhimurium is unable to transport nitrite

It has been reported that in *E. coli*, deletion of the *nirC* gene decreased nitrite uptake (Clegg *et al.*, 2002). Hence, the rate of nitrite uptake by the *nirC* mutant and WT bacteria cultured in minimal medium was assessed. Strikingly, when nitrite consumption from an initial concentration of 75 μM was measured, the WT bacteria rapidly consumed nitrite whereas in the Δ*nirC Salmonella* the consumption was significantly less, demonstrating that NirC is responsible for nitrite uptake in *Salmonella* under this aerobic experimental condition. The rate of consumption of nitrite in the complemented strain was comparable to that of the WT (Fig. 2c).

nirC-deficient *Salmonella* is attenuated in activated macrophages

The growth of the WT and Δ*nirC* strains was comparable in both LB and pH 7 minimal medium (data not shown). The role of the *nirC* gene in intracellular survival in RAW264.7 macrophages was then investigated. As shown in Fig. 3(a), there was an almost 12-fold increase in the WT from 2 to 16 h in the absence of IFN-γ treatment, whereas with IFN-γ treatment it was 3.5-fold. The Δ*nirC* strain under similar conditions was attenuated in the macrophages: it showed a 6-fold increase from 2 to 16 h in the absence of IFN-γ, and no replication in the presence of IFN-γ. In the case of infection with the complemented strain the growth was similar to that of the WT. Similar results were observed in BMDM: the *nirC* strain was significantly attenuated in the presence of IFN-γ (Fig. 3b). This attenuation was abrogated when the survival assay was performed in the presence of the iNOS inhibitor L-NIL. In order to better understand whether inability to use nitrite could lead to this attenuation, we further checked the survival of a Δ*nirBD* strain (a kind gift of Professor Michael Hensel), in which nitrite transport is normal. The data presented in Supplementary Fig. S3 indicate that the reductase knockout strain grew just as well as the WT strain. Furthermore, the NO response as determined by Griess assay was also similar to that of the WT strain (data not shown). Hence, it can be inferred that the inability to transport nitrite in the Δ*nirC* strain is responsible for the observed attenuation. Thus, the transport of nitrite is the essential step, not the reduction per se.

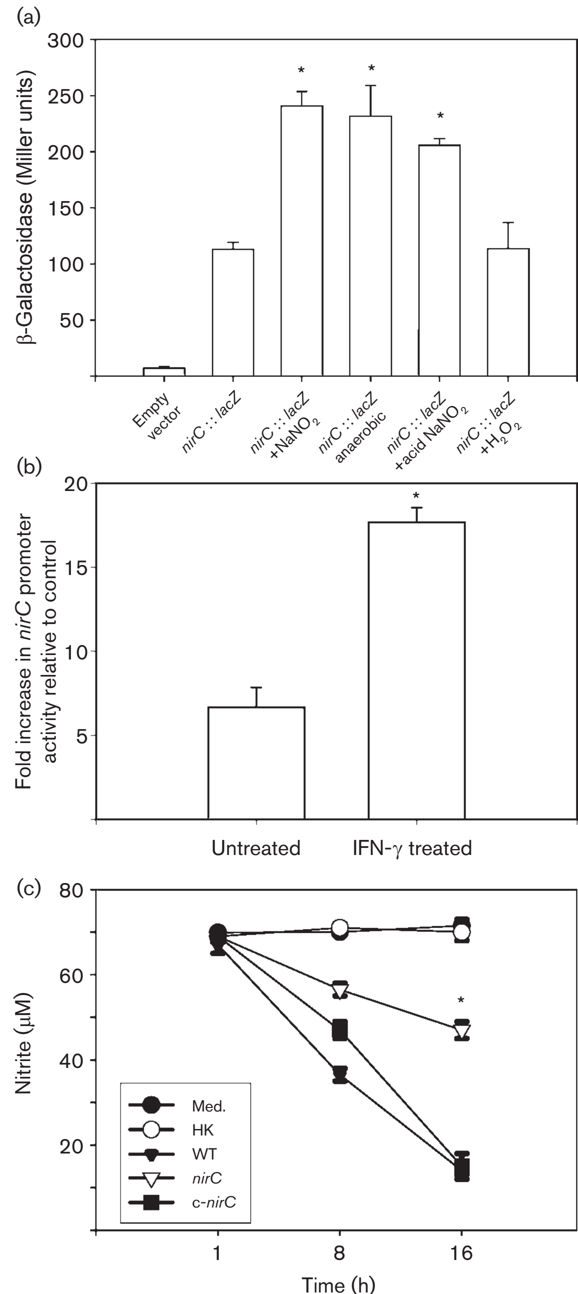

Fig. 2. $NaNO_2$ stress and anaerobic growth upregulate the expression of the *nirC* gene. (a) *nirC* transcription was quantified by measuring β-galactosidase activity of the strain expressing a *nirC* : : *lacZ* promoter construct. The bacteria were grown in low-osmolarity M salts medium with or without the presence of the nitrite donor $NaNO_2$, or H_2O_2, or under anaerobic conditions; promoter activity was measured in Miller units after 3 h of stress. (b) RAW264.7 cells were infected with the WT strain expressing a *nirC* : : *lacZ* promoter construct, with or without IFN-γ treatment. At 12 h post-infection cell lysates were collected and β-galactosidase activity was determined. (c) WT, *nirC* mutant and complemented Δ*nirC* strains were cultured in defined glucose medium buffered with MOPS (pH 8.0). Nitrite uptake was measured as described in Methods. Statistical significance was defined as follows: *, $P<0.05$ (Student's *t* test).

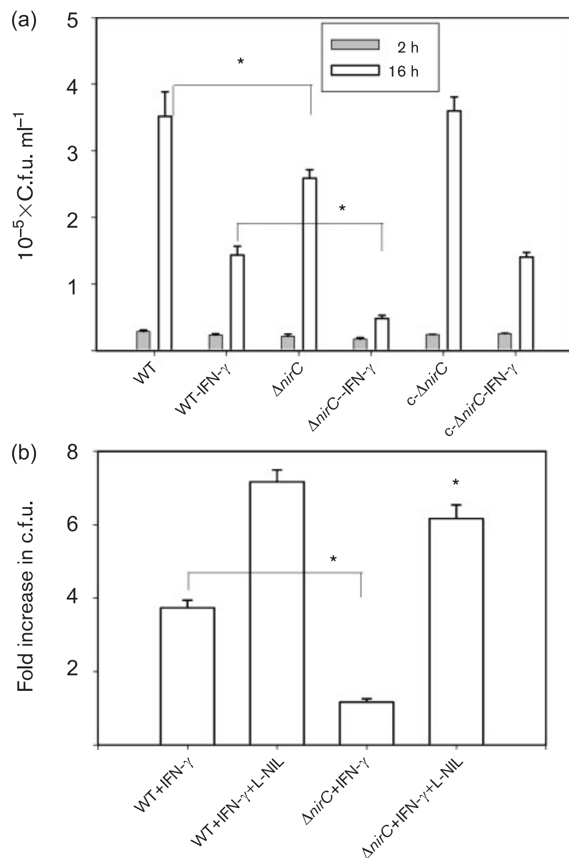

Fig. 3. Intracellular survival assays. (a) RAW264.7 macrophages both with and without IFN-γ stimulation were infected with WT or Δ*nirC* bacteria at an m.o.i. of 10. Infected macrophages were lysed at 2 and 16 h post-infection and the bacterial loads were determined in triplicate. (b) Fold increase in c.f.u. in BMDM between 2 h and 16 h after infection (data from three independent experiments). The WT- or Δ*nirC*-infected cells (m.o.i. 10) were treated with IFN-γ only or with IFN-γ plus L-NIL. Statistical significance was defined as follows: *, $P<0.05$ (Student's *t* test).

Increased formation of peroxynitrite is observed in IFN-γ-treated macrophages when infected with Δ*nirC* as compared to WT *Salmonella* Typhimurium

In order to understand the precise effect of the high NO in the Δ*nirC Salmonella*-infected macrophages in response to IFN-γ, we looked at the formation of peroxynitrite. The most potent bactericidal effect of NO appears to be via reaction with O_2^- leading to the formation of the highly toxic peroxynitrite ($OONO^-$), which can kill *Salmonella* (De Groote *et al.*, 1995). Peroxynitrite generated in the cells cannot be detected directly, but sites of damage due to the reaction of peroxynitrite can be assessed by the immunohistochemical staining of nitrotyrosine. The results presented in Fig. 4 indicate that in activated macrophages infected with WT *Salmonella*, the level of nitrotyrosine residues was low and showed little colocalization with the bacteria. But in the case of Δ*nirC* infection there was enhanced peroxynitrite formation and increased colocalization with the bacteria. When aminoguanidine was used to inhibit the formation of RNS, there was no staining of peroxynitrite, indicating the specificity of the antibody (Fig. 4). To check the amount of ROS produced, extracellular H_2O_2 production was compared in the macrophages. There was no significant difference in the H_2O_2 production (data not shown).

Downregulation of IFN-γ-induced NO synthesis due to *nirC*-dependent reduction in iNOS expression

To further examine the correlation between NO production and iNOS expression, we looked at the translational levels of iNOS in infected and uninfected cells with IFN-γ treatment. Expression of iNOS protein was quantified by Western blot analysis (Fig. 5a). IFN-γ-stimulated macrophages showed increased iNOS expression compared to the control cells, accompanied by a fivefold increase in nitrite level. Cells that were infected with WT *Salmonella* along with IFN-γ treatment showed a marked reduction in the iNOS protein levels, which were also reflected in the nitrite production in the culture supernatant as compared to IFN-γ-stimulated macrophages alone. In contrast, the expression of iNOS protein was not downregulated following infection with Δ*nirC Salmonella*. Upon IFN-γ treatment in the RAW264.7 cells the relative intensity of iNOS protein was 60 %. However, upon WT infection the IFN-γ-treated macrophages exhibited only 30 % relative intensity for iNOS, whereas in the case of macrophages infected with the Δ*nirC Salmonella* it was around 55 % (Fig. 5b). We obtained similar results in flow cytometric analysis of iNOS protein (data not shown). Taken together, these results clearly indicate that the reduction in IFN-γ-induced iNOS expression seen in RAW264.7 macrophages infected with WT *Salmonella* does not occur in a *nirC* mutant background.

The IFN-γ-induced high NO response in Δ*nirC Salmonella*-infected macrophages is due to *nirC*-dependent increase in STAT-1 phosphorylation

Subsequent studies focused on the signal transduction pathways that govern IFN-γ-induced NO synthesis. As phosphorylation of STAT-1 is a crucial downstream signalling event of the IFN-γ pathway (Boehm *et al.*, 1997), experiments were conducted to check the amount of P-STAT-1 in infected cell lysates. The Δ*nirC* strain allowed a higher level of STAT-1 phosphorylation in the activated macrophages than did the WT bacteria. The level of P-STAT-1 at 8 h post-infection in the WT-infected and IFN-γ-activated macrophages was more than twofold lower than in those infected with the *nirC* mutant (Fig. 5c). These results indicate that the high NO output in the Δ*nirC*-infected macrophages arises due to the increased phosphorylation of STAT-1.

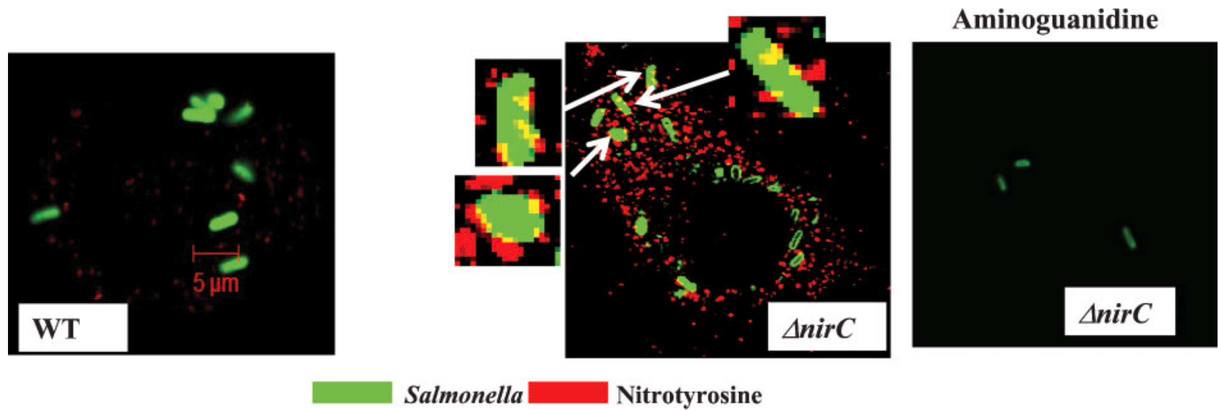

Fig. 4. (a) *nirC* mutant *Salmonella* Typhimurium shows enhanced peroxynitrite formation. The sites of peroxynitrite formation were detected by immunostaining with an anti-nitrotyrosine antibody and a Cy5-conjugated secondary antibody. Samples were analysed by confocal laser-scanning microscopy; representative images for the localization of the bacterial cells (green) and nitrotyrosine residues (red) are shown. A control experiment with aminoguanidine is shown on the far right.

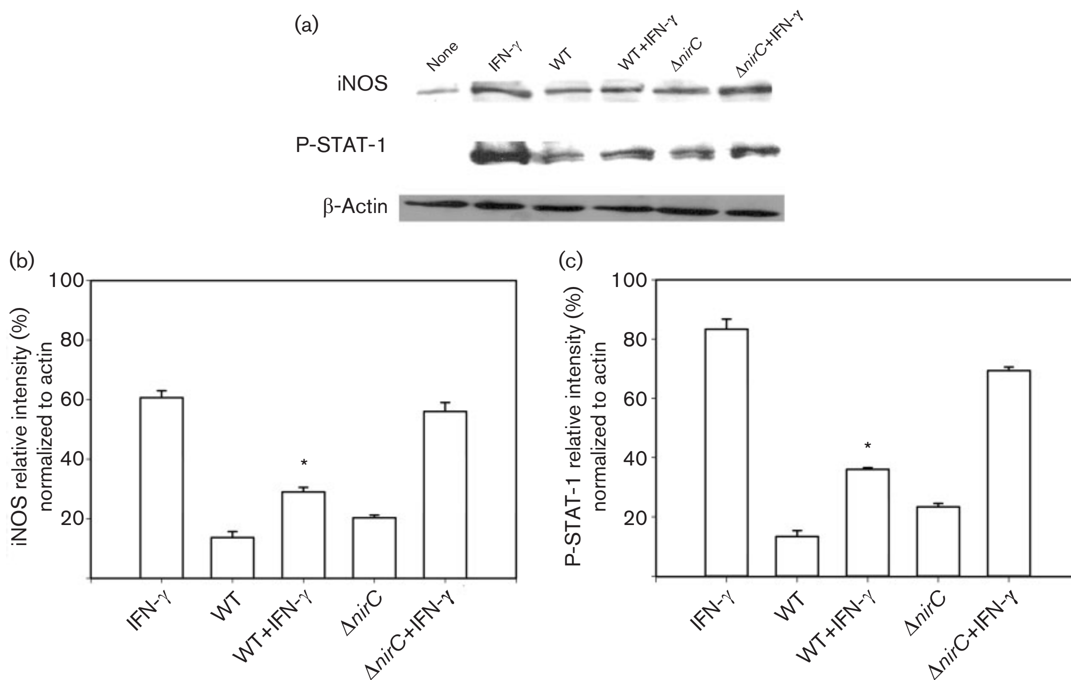

Fig. 5. Live *Salmonella* Typhimurium infection downregulates STAT-1 phosphorylation and iNOS protein expression in activated RAW264.7 macrophages in a *nirC*-dependent manner. RAW264.7 cells were infected with either the WT or *nirC* mutant *Salmonella* for 8 h. Then total protein was extracted from the cells and a 100 μg sample was subjected to Western blot analysis to check the levels of iNOS level (a, upper panel) and phosphorylated STAT-1 (a, middle panel) (b). Relative intensities of the iNOS (b) and P-STAT (c) blots obtained by densitometric image analysis in which the density values of the test proteins were normalized to those of β-actin and plotted as means of three independent experiments. Statistical significance was defined as follows: *, $P<0.05$ (Student's *t* test).

NO inhibits the expression of *spiC* in a *nirC*-dependent manner

Consistent with previous findings (McCollister *et al.*, 2005), we also observed the NO-mediated suppression of *spiC* promoter activity by β-galactosidase assay in the WT *Salmonella*. However, the downregulation of *spiC* under acidified nitrite conditions when compared to a no-stress control was much more pronounced in the Δ*nirC* *Salmonella*: 500 μM $NaNO_2$ under acidic conditions reduced *spiC* transcription by 80-fold in the Δ*nirC* strain but only 45-fold in case of the WT strain (Fig. 6a). *spiC* transcription was also checked independently by RT-PCR. As shown in Fig. 6(b, c), in accordance with β-galactosidase activity, PCR amplification revealed more reduction in *spiC* transcription in the Δ*nirC* strain than in the WT after 3 h of growth in the SPI2-inducing low-osmolarity N salts medium. In contrast, the expression of the housekeeping gene encoding 16S rRNA was similar after 3 h of culture and was not affected by the addition of $NaNO_2$.

Supplying *spiC in trans* abrogates the *nirC*-dependent enhanced nitrite production

Our previous results suggested that in the Δ*nirC* strain, the amount of *spiC* was significantly less than in the WT under acidified nitrite stress. To check the relevance of this finding in the cell culture model, we infected RAW264.7 cells with either the WT or the Δ*nirC* strain, each harbouring the *spiC* promoter cloned in a promoterless plasmid. It was found that in the presence of IFN-γ the level of *spiC* was significantly downregulated in both the strains. However, the *spiC* promoter activity was significantly lower in the Δ*nirC* strain than in the WT in the activated macrophage population (Fig. 7a).

spiC cloned strains were further evaluated for their nitrite production in BMDM. The WT strain with the *spiC* clone produced a significantly lower amount of nitrite in the activated cell supernatant when compared to the WT control. Similar results were obtained in the case of the Δ*nirC* strain. The enhanced nitrite production by the Δ*nirC* strain when compared to cells treated with only IFN-γ was abrogated in the Δ*nirC*/pQE60-spiC$^+$ strain (Fig. 7b).

The intracellular growth in activated BMDM was also in accordance with the nitrite values (Fig. 7c). Interestingly, the attenuation of the Δ*nirC* strain was completely reverted in the Δ*nirC*/pQE60-*spiC*$^+$ strain. These data clearly indicate that the decreased *spiC* level actually leads to the enhanced nitrite production and growth retardation in the Δ*nirC* strain.

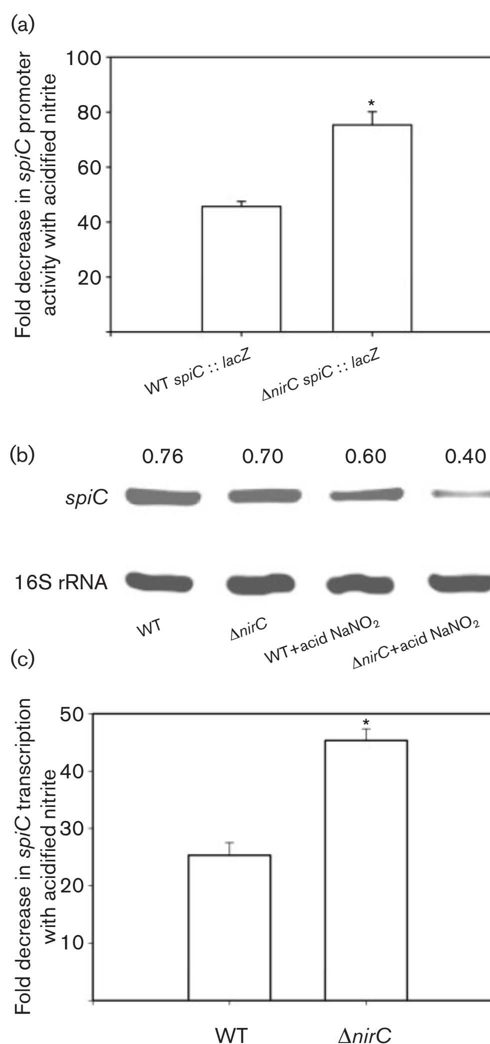

Fig. 6. Increased inhibition of SPI2 transcription in *nirC* mutant *Salmonella* by NO. (a) SPI2 was quantified by measuring β-galactosidase activity of the strain expressing a *spiC*::*lacZ* promoter construct. The bacteria were grown in low-osmolarity N salts medium in the presence of the nitrite donor acidified $NaNO_2$. (b) Transcription of *spiC* and 16S rRNA genes was quantified by RT-PCR amplification of RNA samples isolated from the WT and Δ*nirC* strains cultured in 8 μM $MgCl_2$ N salts medium in the presence or absence of 500 μM acidified $NaNO_2$. The abundance of *spiC* transcript relative to internal 16S rRNA levels, determined by densitometric analysis, is shown above the respective lanes. (c) Mean fold decrease in *spiC* transcription from three independent experiments detemined by densitometric analysis and normalization with 16S rRNA.

Lower burden of the Δ*nirC* strain in the organs of mice

It has been reported previously that *Salmonella* infection upregulates IFN-γ production in mice lymphoid tissues and spleen (Ramarathinam *et al.*, 1991). As our previous results suggested increased susceptibility of the Δ*nirC* strain in IFN-γ-activated macrophages, we next sought to correlate our findings in the mouse model of infection. We infected groups of 10 mice with WT or Δ*nirC* bacteria. As shown in Fig. 8(a), the bacterial burden in spleens of Δ*nirC*-infected mice after 5 days of infection was signific-

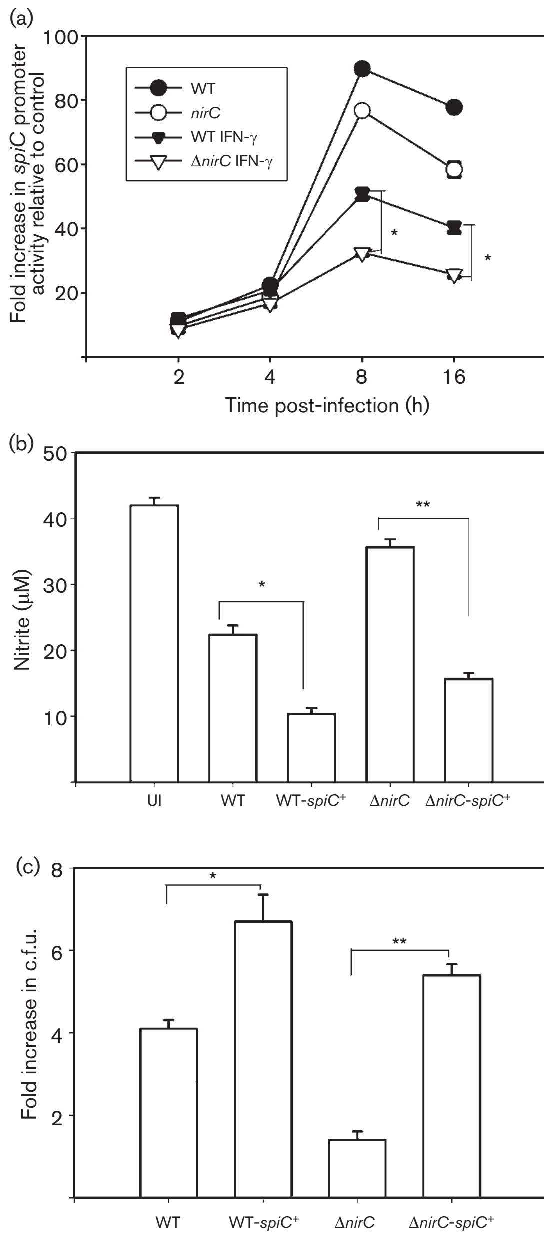

Fig. 7. (a) Analysis of promoter activity of *spiC*. RAW264.7 cells were infected at an m.o.i. of 10 with WT and *nirC* mutant *Salmonella* carrying *spiC*::*lacZ* promoter construct or with WT *Salmonella* carrying empty vector, with or without IFN-γ. At selected time points post-infection cell lysates were collected and β-galactosidase activity was determined. (b, c) Nitrite production and growth in BMDM. (b) BMDM were infected at an m.o.i. of 10 with WT *Salmonella*, with the *nirC* mutant or with each strain carrying the *spiC*$^+$ plasmid pQE60. IFN-γ was added at the time of infection. Production of nitrite was determined in culture supernatants by Griess reaction after 12 h of infection. Values are expressed as mean ± SD of one of three independent experiments performed in triplicate. UI, uninfected. (c) BMDM were infected as in (b). IFN-γ was added at the time of infection. The fold increase in c.f.u. in BMDM between 2 h and 16 h from three independent experiments is plotted. Statistical significance was defined as follows: *, $P<0.05$; **, $P<0.01$ (Student's *t* test).

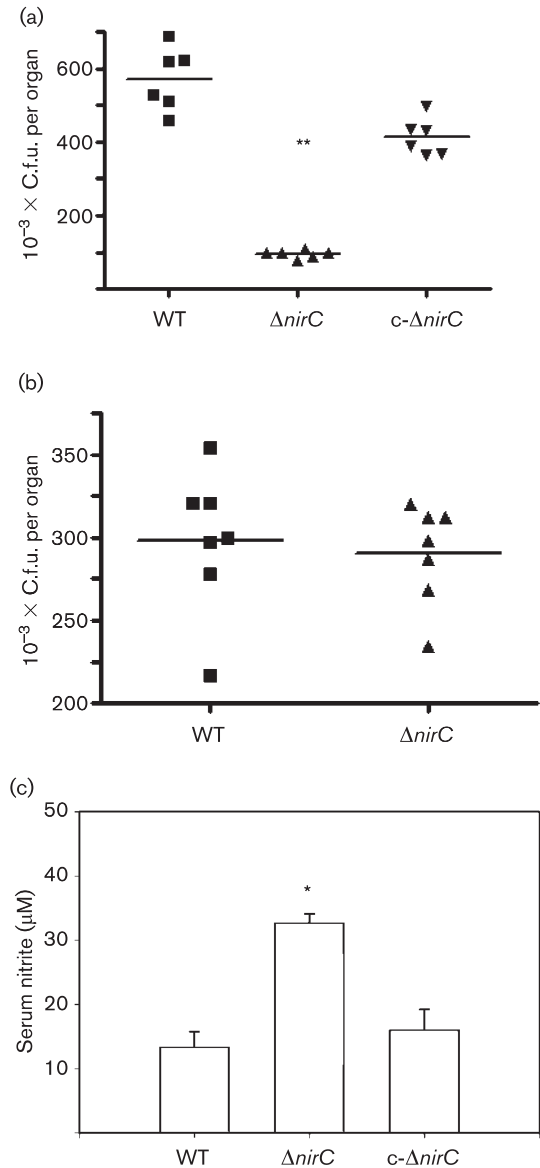

Fig. 8. (a, b) Δ*nirC Salmonella* is attenuated for virulence in BALB/c mice and proliferates like the WT strain in various organs of congenic iNOS$^{-/-}$ mice. After 5 days of infection, homogenized samples of spleens from BALB/c mice (a) and iNOS$^{-/-}$ mice (b) were plated on antibiotic plates and the colonies were counted. The results presented are from one of three independent experiments. (c) Serum nitrite from BALB/c mice infected with the WT, Δ*nirC* or complemented Δ*nirC* strain (three mice per group) was measured as described in Methods. Statistical significance was defined as follows: *, $P<0.05$, **; $P<0.01$ (Mann–Whitney *U* test).

antly less than that in the WT-infected mice. The complemented strain (c-Δ*nirC*) showed significant reversion from the mutant strain in *in vivo* growth. Production of serum nitrite was further compared between the infected mice groups. In accordance with our cell culture data, we

also observed an increased production of nitrite in the serum of the Δ*nirC*-infected mice (Fig. 8c).

Further, competitive index assay in BALB/c mice (Supplementary Table S3) showed that the Δ*nirC* strain exhibited a virulence defect. The WT strain outcompeted the Δ*nirC* strain in all the organs analysed (liver, spleen and MLN) in mixed-infected mice. The complemented Δ*nirC* strain was found to compete equally against the WT strain.

In iNOS$^{-/-}$ mice the Δ*nirC* strain grows just as well as the WT strain

In order to confirm our findings, we next challenged congenic iNOS$^{-/-}$ mice orally with either the WT or the knockout bacteria. After 5 days of infection, the load of the Δ*nirC* strain from all the organs examined was similar to that of the WT bacteria. As shown in Fig. 8(b), there was no significant difference in the bacterial burden after 5 days of infection in the spleens of Δ*nirC*-infected iNOS$^{-/-}$ mice versus WT-infected iNOS$^{-/-}$ mice.

DISCUSSION

Survival of *Salmonella* Typhimurium inside macrophages depends on the ability of the bacteria to evade various host defence mechanisms (Fields *et al.*, 1986). We have shown that IFN-γ-treated RAW264.7 cells infected with WT *Salmonella* showed inhibition of iNOS induction and NO production. Inhibition of NO production as a survival strategy is used by various other pathogens such as *Cryptococcus neoformans* (Kawakami *et al.*, 1997), *Trypanosoma cruzi* (Pakianathan & Kuhn, 1994) and *Leishmania major* (Balestieri *et al.*, 2002; Proudfoot *et al.*, 1995, 1996). Interferons are pro-inflammatory cytokines involved in both innate and acquired immune responses and are known to enhance iNOS expression by macrophages (Boehm *et al.*, 1997; MacMicking *et al.*, 1997; Rottenberg *et al.*, 2000). In addition, several lines of evidence have shown that many pathogenic bacteria are rapidly killed by interferon-activated macrophages by various means (Miyagi *et al.*, 1997; Nairz *et al.*, 2008). We observed that infection with live bacteria is required for the manifestation of the NO inhibition phenotype, as heat-killed *Salmonella* was unable to inhibit NO synthesis. The inhibition of IFN-γ stimulated NO production was also found to be dependent on the m.o.i. used. No inhibition was observed in the macrophages infected with an m.o.i. of less than 1. Prokaryotic protein synthesis was required for the manifestation of this phenotype, as it was not seen in the presence of chloramphenicol.

This work further elucidates the role of NirC protein in *Salmonella* virulence. Our bioinformatics study predicted that NirC of *Salmonella* indeed is a polytopic membrane protein similar to its *E. coli* homologue, having six transmembrane helices. The high homology of this protein amongst various *Salmonella* serovars and many other known nitrite transporters from diverse bacterial species also confirms the role of NirC in nitrite transport. Various transcriptional regulators and enzymes that detoxify NO in many diverse micro-organisms, particularly pathogenic bacteria including *Salmonella*, are well documented (Gilberthorpe *et al.*, 2007; Mills *et al.*, 2008). However, to our knowledge no study has previously addressed the role of nitrite transporters in *Salmonella*. The novel function of NirC is accomplished by its nitrite transporter activity as opposed to the NO-detoxifying mechanisms used by HmpA and NorV. NarK also serves as a nitrite importer but NirC can maintain nitrite uptake at the normal rate in the absence of NarK (Clegg *et al.*, 2002). A non-polar deletion mutant of the nitrite transporter *nirC* in *Salmonella* was constructed and our data showed that a higher level of nitrite was induced in the supernatant of the *nirC* mutant *Salmonella*-infected macrophages than that of the WT-infected macrophages. Furthermore, *nirC* transcription was demonstrated to be upregulated in the presence of nitrite stress and under anaerobic conditions, but hydrogen peroxide stress had no effect. This suggests that the transcription of *nirC* is upregulated in the presence of high nitrite and it then acts as a nitrite transporter and might help the bacteria to adapt to RNS stress. Our data strongly indicate that NirC is the principal nitrite transporter in *Salmonella*, as the deficiency of NirC reduced the nitrite uptake significantly and its complementation fully restored it.

The *nirC* mutant was attenuated in the mouse macrophage cell line RAW264.7 and in BMDM both with and without IFN-γ activation, and in an *in vivo* mouse model. L-NIL treatment abrogated this attenuation, indicating that the *nirC* growth defect is solely due to NO stress. The reaction of RNS with ROI generates peroxynitrite (Pacher & Szabo, 2006), a reactive molecule with potent antimicrobial activity against *Salmonella* Typhimurium *in vitro* and rarely co-localizing with WT *Salmonella* (Chakravortty *et al.*, 2002). In contrast, nitrotyrosine residues were frequently located in the vicinity of as well as co-localized with the *nirC* mutant bacteria. Thus, in the *nirC*-infected cells, an increased level of RNS leads to enhanced production of peroxynitrite and might be responsible for the observed attenuation. We can speculate that the intracellular survival of *Salmonella* even after IFN-γ treatment is due to our proposed mechanism of NirC-mediated NO quenching, which makes infected cells less responsive to cytokine activation. In the mouse model of infection, the knockout bacteria were attenuated when compared to the WT strain in all the organs tested. Similar results were obtained in the competitive index experiment: the WT bacteria could outcompete the knockout bacteria in a mixed infection. A previous study reported that IFN-γ plays a crucial role in *Salmonella* infection (Bao *et al.*, 2000). The increased production of serum nitrite after Δ*nirC* infection also leads to the same conclusion, further validating our cell culture data. However, in the iNOS$^{-/-}$ mouse model of infection the attenuation of the Δ*nirC* was abrogated.

To arrive at a rationale for this heightened NO response in the *nirC* mutant *Salmonella,* we examined the iNOS expression and JAK/STAT-1 signalling pathway upon IFN-γ stimulation. Surprisingly, the levels of P-STAT-1 and iNOS induction with IFN-γ treatment in the Δ*nirC*-infected macrophages were significantly higher than that of the WT-infected macrophages. Functioning of the type III secretion system encoded by SPI2 is essential for the ability of *Salmonella* to cause systemic infections and for intracellular survival (Hensel, 2000; Ochman *et al.*, 1996; Shea *et al.*, 1996; Waterman & Holden, 2003). Studies have indicated that SPI2 is used by intracellular *Salmonella* to actively modify functions of the host cells and one of the effector proteins, SpiC, is important in this regard (Uchiya *et al.*, 1999). SpiC was found to be required for SPI2-mediated secretion of many effector proteins (Yu *et al.*, 2002). Also, the NO-mediated inhibition of SPI2 transcription in activated macrophages is well known (McCollister *et al.*, 2005). Additionally, the SPI2 protein SpiC plays a critical role in reducing JAK/STAT signalling. SpiC blocks the phosphorylation of STAT-1 proteins by inducing SOCS-3 (Uchiya & Nikai, 2005). Inhibition of SPI2 function by NO is critical for the anti-*Salmonella* activity of IFN-γ-treated macrophages (McCollister *et al.*, 2005) and in turn SPI2 effector proteins can inhibit IFN-γ signalling (Uchiya & Nikai, 2005). We provide an important connecting link between these two interesting findings regarding IFN-γ and *Salmonella* biology. When *spiC* was cloned *in trans* in a low-copy-number plasmid, the *nirC*-dependent high nitrite production and attenuation was abrogated in the knockout strain.

A model for the protective role of NirC based on our results is shown in Fig. 9. In this model, transporting nitrite inside the bacteria reduces the amount of toxic NO. As a result, the SPI2 inhibition by NO is significantly decreased. It has been reported that NO can be auto-oxidized to nitrite (Kharitonov *et al.*, 1994). Nitrite, which is not freely diffusible like NO, cannot be transported inside the bacteria in the *nirC* mutant strain. Hence, it can be speculated that the increased amount of nitrite surrounding the *Salmonella*-containing vacuole or individual bacteria in the acidic condition might be reconverted to the freely diffusible NO and upon entering the bacteria can suppress SpiC expression. Our study has shown that in activated macrophages, the amount of SpiC repression is much more pronounced in the NirC knockout bacteria than in the WT counterpart. This reduced level of SpiC in the NirC-null background thereby cannot effectively block IFN-γ signalling, unlike in the WT strain (Fig. 9). The upregulation of iNOS expression occurs due to enhanced phosphorylation of STAT-1 leading to an increase in NO production in the knockout strain. The ability of a nitrite

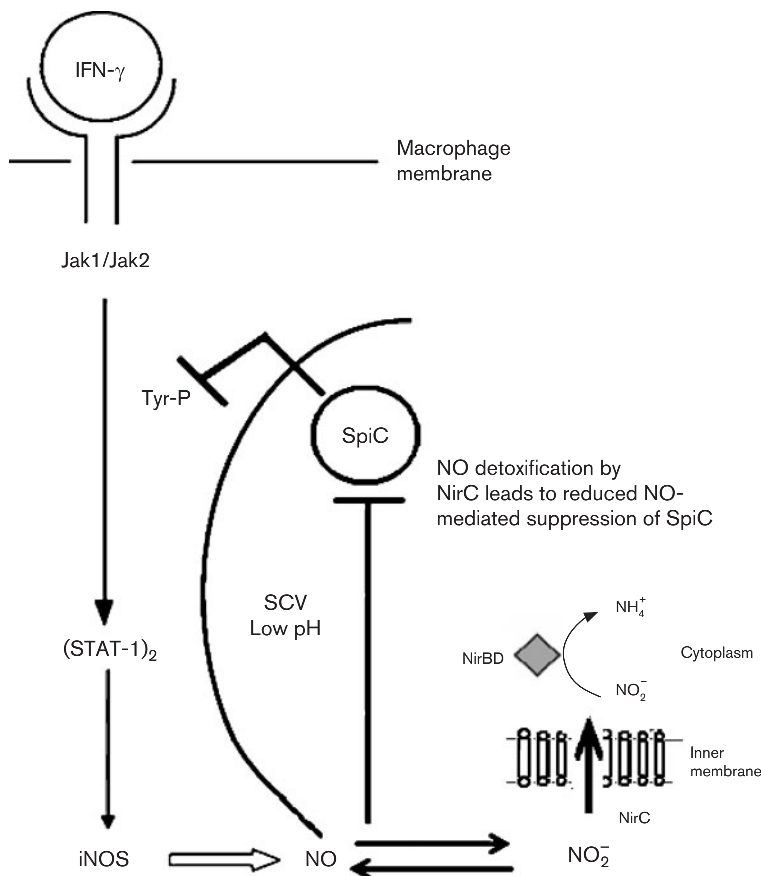

Fig. 9. Schematic summary of the NirC-mediated NO quenching and SPI2-dependent iNOS inhibition. Left: IFN-γ-induced iNOS induction and NO production. Centre: NO-mediated inhibition of SpiC and SpiC-mediated inhibition of STAT-1 phosphorylation. Right: nitrite produced is transported into the bacteria via NirC and detoxified, resulting in a reduced amount of NO to suppress SpiC. For details, see the text.

transporter to play a pivotal role in *Salmonella* pathogenesis as shown in our study represents an exciting area for future research.

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