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DNA base excision repair potentiates the protective effect of *Salmonella* Pathogenicity Island 2 within macrophages

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Reactive oxidants are a primary weapon of the macrophage antibacterial arsenal. The ability of virulent *Salmonella* to repair oxidative DNA lesions via the base-excision repair system (BER) enables its survival and replication within the macrophage, but is not required for extracellular growth. *Salmonella* also inhibits the targeting of oxidant generators to the *Salmonella*-containing vacuole (SCV) via *Salmonella* Pathogenicity Island 2 (SPI2). Accordingly, the relative contributions of these two discrete systems to *Salmonella* resistance to both oxidative mutagenesis and lethality within RAW 264.7 macrophages were investigated. A mutant unable to initiate BER was constructed by deleting all three BER bifunctional glycosylases (Δ*fpg*/*nth*/*nei*), and was significantly impaired for early intramacrophage survival. Mutations in various SPI2 effector (*sifA* and *sseEFG*) and structural (*ssaV*) genes were then analysed in the BER mutant background. Loss of SPI2 function alone appeared to increase macrophage-induced mutation. Statistical analyses of the reduced intramacrophage survival of SPI2 mutants and the corresponding SPI2/BER mutants indicated a synergistic interaction between BER and SPI2, suggesting that SPI2 promotes intramacrophage survival by protecting *Salmonella* DNA from exposure to macrophage oxidants. Furthermore, this protection may involve the SseF and SseG effectors. In contrast, the SifA effector did not seem to play a major role in oxidant protection. It is speculated that *Salmonella* initially stalls oxidative killing by preserving its genomic integrity through the function of BER, until it can upregulate SPI2 to limit its exposure to macrophage oxidants.

INTRODUCTION

Reactive oxygen and nitrogen intermediates (ROI and RNI) are generated as by-products of metabolism, but can readily react with DNA and cause a variety of mutations. DNA repair systems thus serve a housekeeping role in maintaining genomic fidelity. However, the ability to repair oxidized DNA may be especially critical in the context of bacterial pathogenesis, since a primary antibacterial mechanism of host macrophages is oxidative stress mediated by phagocyte NADPH oxidase (phox) and inducible nitric oxide synthase (iNOS) (reviewed by Babior, 2000; Nathan & Shiloh, 2000). Mutants in the DNA base excision repair (BER) pathway of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) were impaired for survival within wild-type macrophages, but were restored in macrophages lacking both phox and iNOS (Suvarnapunya *et al.*, 2003b). Thus, a critical mechanism of *Salmonella* persistence in macrophages is the specific repair of DNA bases oxidized by macrophage oxidants.

BER is dedicated to the repair of oxidative DNA damage and occurs in two steps, followed by repair polymerization (reviewed by Wallace, 1997). The first step is mediated by bifunctional glycosylases which have both DNA *N*-glycosylase and apurinic/apyrimidinic (AP) lyase activity (Fig. 1a). The second step is mediated by AP endonucleases (Fig. 1a), which can also repair single-strand breaks. The overlapping substrate specificities of the bifunctional glycosylases impart significant functional redundancy at the initial step of BER, and the same is true for the AP endonucleases at the second step. The redundancy in BER

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Abbreviations: AP, apurinic/apyrimidinic; BER, base excision repair; CRAMP, cathelicidin-related antimicrobial peptide; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; SCV, *Salmonella*-containing vacuole; phox, phagocyte NADPH oxidase; RNI, reactive nitrogen intermediate; ROI, reactive oxygen intermediate; SPI2, *Salmonella* Pathogenicity Island 2; STE, *Salmonella*-translocated effector.

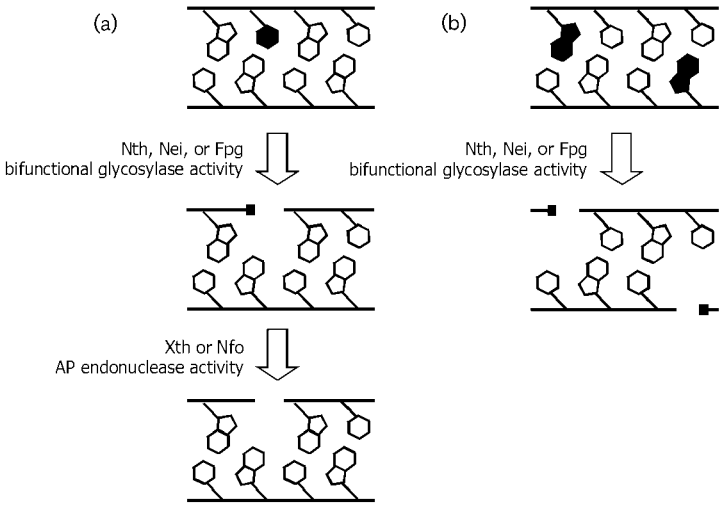

Fig. 1. (a) Base excision repair pathway of oxidative DNA damage. Exposure of DNA to ROI or RNI leads to base damage (filled base). Damaged pyrimidines are removed by one of two bifunctional DNA glycosylases (Nth or Nei), and damaged purines by a third bifunctional glycosylase, Fpg, resulting in an abasic site with a 3′ α,β-unsaturated aldehyde (black square). Single-strand breaks formed by glycosylase activity are then processed by one of two AP endonucleases, creating a suitable substrate for Pol I. (b) Abortive base excision repair pathway. A double-strand break is created when glycosylases act on closely opposed oxidative lesions on opposite strands, precluding repair.

is greatest in *S. typhimurium*, *Escherichia coli* and *Mycobacterium tuberculosis*, which are the only bacteria that possess a homologue of every BER enzyme identified (Mizrahi & Andersen, 1998; Eisen & Hanawalt, 1999). Additionally, the role of bifunctional BER glycosylases in *Salmonella* pathogenesis is consistent with that reported for *Helicobacter pylori*. *H. pylori* encodes only one oxidative BER glycosylase, Nth, and *H. pylori nth* mutants are reduced for macrophage survival and are inhibited for colonization in the murine model (O'Rourke *et al.*, 2003).

In addition to repairing the DNA damage caused by macrophage oxidants, a key survival strategy employed by *S. typhimurium* may be the inhibition of the delivery of macrophage oxidant generators to the SCV. The Type III secretion system encoded by *Salmonella* Pathogenicity Island 2 (SPI2; reviewed by Holden, 2002) is required to divert both phox and iNOS from the SCV (Vazquez-Torres *et al.*, 2000a; Gallois *et al.*, 2001; Chakravortty *et al.*, 2002), although the SPI2 effector proteins that are responsible for manipulating this aspect of intracellular trafficking are currently unknown. Because the proximity of oxidant generators to their targets is assumed to directly correlate to oxidant exposure, it is thought that SPI2 protects *Salmonella* from direct oxidant cytotoxicity. However, this premise is somewhat controversial (reviewed by Linehan & Holden, 2003).

For example, the effect of SPI2 on the trafficking of oxidant generators could interfere with other important functions of oxidants, rather than reducing direct exposure to the oxidants themselves. Indeed, it has been found that the cathelicidin-related antimicrobial peptide (CRAMP) of murine macrophages is processed in a phox-dependent manner, and that CRAMP has bactericidal activity against *S. typhimurium* (Rosenberger *et al.*, 2004). Thus, in murine models of infection, the primary function of SPI2 may be to inhibit the processing of CRAMP proximal to the SCV, thereby protecting *S. typhimurium* against peptide-mediated killing.

The timing of SPI2 expression compared with the kinetics of oxidative killing in macrophages also does not fully coincide. SPI2 is not fully induced in *S. typhimurium* until several hours after phagocytosis (e.g. Eriksson *et al.*, 2003a), whereas the oxidative burst in response to *S. typhimurium* occurs essentially immediately (e.g. Vazquez-Torres *et al.*, 2000b). Therefore, other oxidant defence systems that are rapidly induced or constitutive, such as BER, could conceivably handle any direct oxidant exposure that occurs outside of the time-frame in which SPI2 acts.

Clearly, further investigation into whether and how SPI2 participates in oxidant defence is warranted. To define the putative role of SPI2 in direct protection against macrophage oxidants, we tested whether SPI2 function shields the critical target of *Salmonella* DNA from oxidant exposure. We exploited the specific sensitivity of a defined BER mutant to macrophage oxidants as an oxidant 'sentinel' to probe the unique microenvironment of the SCV.

METHODS

Bacterial strains. Internal, unmarked deletions of BER bifunctional glycosylases were made in *S. typhimurium* using positive selection and allelic exchange methodologies described previously (Guy *et al.*, 2000; Suvarnapunya *et al.*, 2003b). Briefly, a ~2 kb deletion allele was made by joining PCR products that contain a portion of the *fpg* gene (also known as *mutM*) flanked by upstream and downstream sequence (Table 1). The resulting *fpg* deletion allele is in-frame and encodes amino acids 1–23 and 253–269 of the Fpg glycosylase. An additional three amino acids (RLN) were introduced between the N- and C-terminal portions of Fpg by the *Pme*I site used to construct the deletion allele. The wild-type copy of *fpg* in a *S. typhimurium* Δ*nth*/*nei* mutant (Suvarnapunya *et al.*, 2003b) and in the parental wild-type SL1344 strain was replaced by the *fpg* deletion allele, resulting in the Δ*fpg*/*nth*/*nei* and Δ*fpg* mutants (Table 1).

The *ssaV*::Mu*dJ*, *sifA*::Tn10*d*Cm, *sseE*::Mu*dJ*, and *sseF*::Mu*dJ*-marked insertional mutations were transduced into the Δ*fpg*/*nth*/*nei* background by P22 phage, according to standard methodologies (Schmieger, 1972). The *sseE*::Mu*dJ* mutation exerts a polar effect

Table 1. Bacterial strains and oligonucleotide primers

The parental strains of all *E. coli* and *S. typhimurium* strains used were K-12 and SL1344, respectively.

Strain/primer	Source/reference	Genotype/nucleotide sequence	Description
E. coli			
Top10	Invitrogen	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) (80*lacZ*ΔM15 Δ*lacX*74 *recA*1 *deoR araD*139 Δ(*ara-leu*)7697 *galU galK rpsL* (Str^R) *endA*1 *nupG*	Host for cloned PCR products
DH5α	Laboratory collection	*endA*1 *hsdR*17(r$^-$m$^+$) *supE*44 *thi-1 recA*1 *gyrA*1(Nal^R) *relA*1 Δ(*lacIZYA-argF*)U169 *deoR* ((80*dlac*Δ(*lacZ*)M15)	Host for routine genetic constructions
DH5α*λ*pir	Laboratory collection	*endA*1 *hsdR*17(r$^-$m$^+$) *supE*44 *thi-1 recA*1 *gyrA*1(Nal^R) *relA*1 Δ(*lacIZYA-argF*)U169 *deoR* ((80*dlac*Δ(*lacZ*)M15) *λ*pir	Host for allelic exchange constructions
SM10*λ*pir	Guy *et al.* (2000)	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km pir R6K	Permissive host for allelic exchange
S. typhimurium			
SL1344	Guy *et al.* (2000)	*hisG*46	Virulent wild-type
ST90	Stein *et al.* (1996)	*sifA*::Tn10*d*Cm	SPI2 STE mutant
ST229	Suvarnapunya *et al.* (2003b)	Δ*nth*/*nei*	BER double glycosylase mutant
ST244	Guy *et al.* (2000)	Δ*ssaV*	SPI2 loss-of-function mutant
ST254	Guy *et al.* (2000)	*ssaV*::Mu*dJ*	SPI2 loss-of-function mutant
ST258	Guy *et al.* (2000)	*sseE*::Mu*dJ*	SPI2 effector region mutant (polar)
ST364	Present study	Δ*fpg*	BER single glycosylase mutant
ST365	Present study	Δ*fpg*/*nth*/*nei*	BER triple glycosylase mutant
ST390	Present study	Δ*fpg*/*nth*/*nei ssaV*::Mu*dJ*	SPI2/BER oxidant sentinel
ST392	Present study	Δ*fpg*/*nth*/*nei sifA*::Tn10*d*Cm	SPI2/BER oxidant sentinel
ST394	Present study	Δ*fpg*/*nth*/*nei sseE*::Mu*dJ*	SPI2/BER oxidant sentinel
Primers			
UFPG1		GCGTGAGATTGTTCGGGAAGC	*fpg* upstream
UFPG2*		TATGTTTAAACGGATAGTAGCGCCAACTAAATG	*fpg* N-terminus
LFPG1*		TATGTTTAAACGCAACGAAACACGCACAG	*fpg* C-terminus
LFPG2		TCCCGGATGCTATCAATCTT	*fpg* downstream

*Introduced *Pme*I site is underlined.

on the downstream genes *sscB*, *sseF* and *sseG* (Guy *et al.*, 2000; Suvarnapunya & Stein, unpublished results), and is therefore essentially a mutation of the translocated effectors encoded in the effector region of SPI2. As suggested by the work of Guy *et al.* (2000), it has recently been experimentally demonstrated that SscB is a chaperone for SseF (Dai & Zhou, 2004).

Cell culture. RAW 264.7 murine macrophages (ATCC TIB-71) were grown in Dulbecco's Modified Eagle's Medium containing 0·15 % sodium bicarbonate (DMEM; ATCC 30-2002), supplemented with 10 % fetal bovine serum (FBS; ATCC 30-2020), at 37 °C and 5 % CO_2. All RAW 264.7 macrophages were used between passages 3 and 20 of the ATCC stock, and were periodically checked for respiratory burst capability using the NBT reduction assay (Damiani *et al.*, 1980). For gentamicin-protection assays, RAW 264.7 macrophages were seeded into 24-well plates and activated by incubation overnight in the presence of 20 U ml^{-1} interferon-γ (IFN-γ) that contained a final concentration of <1 pg ml^{-1} LPS (Invitrogen) as described elsewhere (Vazquez-Torres *et al.*, 2000b).

Ionizing radiation assay. Assays for ionizing radiation exposure were carried out as described previously (Suvarnapunya *et al.*, 2003b). Briefly, overnight cultures in LB broth (Lennox formulation) were subcultured and grown to mid-exponential phase. After washing and resuspending in PBS, 2 ml aliquots were exposed to various doses of ionizing radiation at 50 kV and 2 mA, with stirring. Dilutions of exposed cultures were then plated and colony-forming units (c.f.u.) were enumerated.

Gentamicin-protection assay. Survival within IFN-γ-activated RAW 264.7 macrophages was assessed using a gentamicin-protection assay (6 μg ml^{-1}), as described previously (Suvarnapunya *et al.*, 2003b). RAW 264.7 macrophages activated by IFN-γ mount a robust oxidative burst (Rosenberger & Finlay, 2002). Macrophages were seeded into 24-well plates and incubated overnight with 20 U ml^{-1} IFN-γ in DMEM, followed by infection with stationary-phase bacteria that had been grown in LB (Lennox formulation), at a multiplicity of infection of 10 bacteria per macrophage. At desired time-points, the infected monolayers were lysed in 1 % Triton X-100 in PBS, and dilutions of the macrophage lysates were enumerated for c.f.u.

Forward mutation assay of macrophage-resident *Salmonella*. Sublethal exposure to macrophage oxidants was determined from the rate of forward mutation to rifampicin-resistance, essentially as described elsewhere (Schlosser-Silverman *et al.*, 2000). Briefly, from a 24-well gentamicin-protection assay, 400 μl aliquots of lysates from infected macrophage monolayers were pooled from each of three

replicate wells per strain, to give a final volume of 1·2 ml per strain tested. The pooled lysates were gently pelleted and resuspended in the same volume of fresh LB (Lennox formulation) and statically incubated overnight at 37 °C. Dilutions of the overnight culture were then plated on LB agar plates with or without rifampicin (100 μg ml^{-1}) and incubated overnight at 37 °C. The forward mutation rate was calculated as the total c.f.u. ml^{-1} divided by the Rif^R c.f.u. ml^{-1}.

Statistical analysis. Statistical analysis was carried out using MINITAB 14 for Windows. Three independent intramacrophage replication assays were carried out, except where noted, in triplicate for each strain. Data from all mutant strains were normalized to the wild-type parental strain SL1344, yielding fold survival values. A one-way ANOVA was then performed on this normalized data. Subsequently, planned pairwise comparisons were made between a given SPI2 or BER (Δ*fpg*/*nth*/*nei*) mutant and the corresponding double SPI2/BER mutant using a protected Fisher's Least Significant Difference (LSD) procedure. The α-level for all comparisons was 0·05, except where indicated.

RESULTS

The Δ*fpg*/*nth*/*nei* mutant is unable to initiate BER

A *S. typhimurium* Δ*fpg*/*nth*/*nei* triple deletion mutant, which would be unable to initiate BER via bifunctional glycosylases, was constructed to serve as a potential probe to measure the oxidative stress that occurs within the specific and unique conditions of the SCV. A Δ*fpg* mutant was also constructed to enable comparison with the triple glycosylase mutant and the Δ*nth*/*nei* double pyrimidine-glycosylase mutant (Suvarnapunya *et al.*, 2003b). Neither the Δ*fpg* mutant nor the Δ*fpg*/*nth*/*nei* mutant displayed any generalized growth defects (data not shown). This is consistent with all other *S. typhimurium* BER mutants (Suvarnapunya *et al.*, 2003b).

To verify that the *S. typhimurium* Δ*fpg*/*nth*/*nei* mutant behaves like the previously characterized *E. coli nth nei fpg* mutant (Blaisdell & Wallace, 2001), Δ*fpg*/*nth*/*nei* was exposed to increasing doses of ionizing radiation. The Δ*fpg*/*nth*/*nei* mutant was fivefold more resistant at the highest dose tested (270 Gy) than the wild-type SL1344 parental strain (Fig. 2), which was statistically significant by a one-tailed *t* test ($P<0·05$). The Δ*fpg* mutant was also significantly more resistant than wild-type by the same criteria, but less resistant than the triple glycosylase mutant (Fig. 2). The increase in radiation resistance of *S. typhimurium* Δ*fpg*/*nth*/*nei* approximates the sixfold increase observed for *E. coli nth nei fpg* (Blaisdell & Wallace, 2001). The enhanced radiation resistance is attributed to the inability to initiate the BER pathway via bifunctional glycosylases and the corresponding lack of lethal double-strand DNA breaks produced by the bifunctional glycosylases under conditions of clustered oxidative damage (Fig. 1b; Blaisdell & Wallace, 2001). The radiation resistance of *S. typhimurium* Δ*fpg*/*nth*/*nei* thus indicates the inability of this mutant to initiate BER, as shown for *E. coli*.

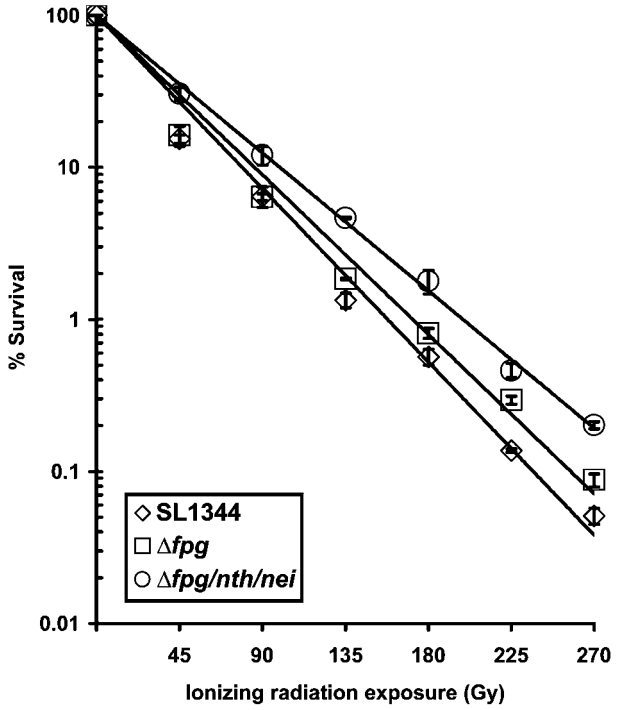

Fig. 2. Ionizing radiation sensitivity of *S. typhimurium* oxidative BER glycosylase mutants. Survival curves were plotted following exposure to increasing doses of ionizing radiation. The means of two independent experiments, each done in triplicate, were plotted using a best-fit curve ($R^2>0·95$). Error bars represent one standard deviation from the mean.

The inability to initiate BER significantly impairs intramacrophage survival of *Salmonella*

The Δ*fpg*/*nth*/*nei* mutant was tested for its ability to survive within macrophages by a gentamicin-protection assay. RAW 264.7 macrophages that had been activated with IFN-γ were infected with single (Δ*fpg*), double (Δ*nth*/*nei*) or triple (Δ*fpg*/*nth*/*nei*) BER glycosylase deletion mutants. A SPI2 loss-of-function mutant (Δ*ssaV*) was also included for comparison, since SPI2 has been well-established as a requirement for survival within macrophages (Hensel *et al.*, 1998).

At the 18 h time-point, *Salmonella* c.f.u. were enumerated from macrophage lysates, and fold survival was calculated as the ratio of mutant c.f.u. to wild-type SL1344 c.f.u. (Fig. 3). The Δ*ssaV* mutant was reduced for survival in activated macrophages to about 0·17-fold of wild-type (Fig. 3). The Δ*fpg*/*nth*/*nei* mutant was as sensitive to macrophage-mediated killing as Δ*ssaV* (~0·21-fold of wild-type; Fig. 3). The Δ*nth*/*nei* mutant was about 0·48-fold of wild-type, and the Δ*fpg* mutant was about 0·90-fold of wild-type (Fig. 3). This demonstrates an additive effect, within macrophages, of the loss of all three oxidative BER glycosylases, and highlights the importance to phagocytosed *Salmonella* of redundancy in BER glycosylases.

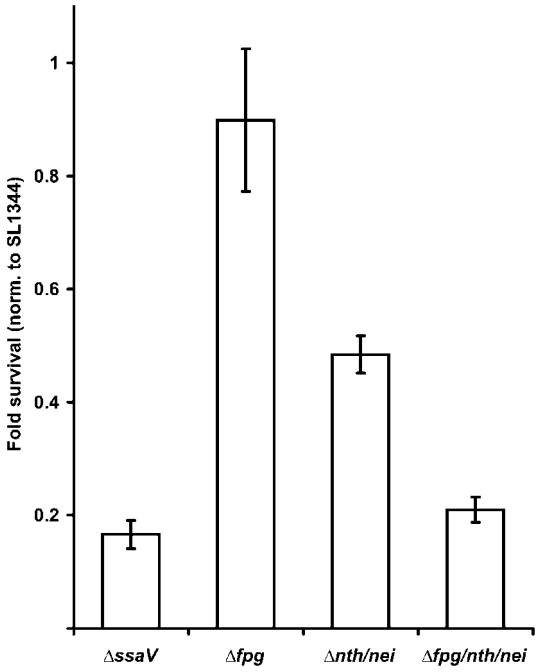

Fig. 3. Survival of *S. typhimurium* bifunctional BER glycosylase mutants within activated RAW 264.7 macrophages. Fold survival was calculated as the ratio of mutant c.f.u. to wild-type c.f.u. at 18 h post-infection. Data presented are means of three independent experiments, each done in triplicate. Error bars, 1 SD.

Because, like all other *Salmonella* BER mutants, Δ*fpg*/*nth*/*nei* does not display any generalized growth defects (data not shown; Suvarnapunya *et al.*, 2003b), and is specifically sensitive to exogenous oxidative stress, this mutant is suitable for use as a 'sentinel' of oxidized bases within macrophages to address the role of SPI2 in protecting DNA from oxidative damage.

SPI2 protects the DNA of macrophage-resident *Salmonella* from oxidative damage

Our initial approach to addressing if SPI2 protects *Salmonella* DNA from macrophage oxidants was to evaluate the mutagenicity of macrophage-resident *Salmonella*. Mutation rates of the *ssaV*::Mu*dJ* SPI2 loss-of-function mutant within macrophages were measured by the acquisition of rifampicin resistance (e.g. Schlosser-Silverman *et al.*, 2000), which indicates mutagenic DNA damage. *Salmonella* were assayed for rifampicin resistance at 10 h post-infection. This is considered a late time-point of macrophage infection, by which significant macrophage oxidant exposure has occurred in BER mutants (Suvarnapunya *et al.*, 2003b) and SPI2 is active (Eriksson *et al.*, 2003a).

As expected, the forward mutation rate for Δ*fpg*/*nth*/*nei* was approximately 18-fold higher than that of the parental wild-type (Table 2). The forward mutation rate of *ssaV*::Mu*dJ* was also significantly higher than that of the wild-type (~3·8-fold), but much less than that observed for Δ*fpg*/*nth*/*nei* (Table 2). The effect of the loss of SPI2 function on the Δ*fpg*/*nth*/*nei* sentinel was then analysed by introducing the *ssaV*::Mu*dJ* mutation via P22 transduction. The forward mutation rate of the resulting Δ*fpg*/*nth*/*nei ssaV*::Mu*dJ* mutant was about 19-fold higher than wild-type (Table 2), which is similar to the Δ*fpg*/*nth*/*nei* mutant. *Salmonella* were also assayed 4 h post-infection, an earlier time-point by which macrophage oxidants are active (Vazquez-Torres *et al.*, 2000b) and SPI2 has been upregulated (Eriksson *et al.*, 2003a). The forward mutation rates of both Δ*fpg*/*nth*/*nei* and Δ*fpg*/*nth*/*nei ssaV*::Mu*dJ* were again approximately equal, about 11-fold higher than wild-type (data not shown).

Thus, the increase in mutability supports the hypothesis that SPI2 shields *Salmonella* DNA from macrophage oxidants, since the lack of SPI2 function results in a clear trend towards a higher mutation rate than wild-type. This shows that SPI2 function directly protects a cytosolic *Salmonella* target from damage caused by macrophage oxidants. However, no additive effect was observed when SPI2 function was genetically abrogated in the BER mutant background. Since measurements of rifampicin resistance only apply to the surviving subpopulation, it was possible that the exposure of Δ*fpg*/*nth*/*nei ssaV*::Mu*dJ* to macrophage oxidants resulted in lethality that was not apparent by this assay. This possibility was therefore addressed by evaluating the effect of SPI2 on Δ*fpg*/*nth*/*nei* viability in macrophages.

SPI2 provides direct protection against lethality of macrophage oxidants

We tested the hypothesis that SPI2 protects *Salmonella* from effective (i.e. lethal) exposure to macrophage oxidants. The

Table 2. Forward mutation rates of *S. typhimurium* BER and SPI2 mutants within activated macrophages

Mutant	Forward mutation rate to Rif^R (10^{-8}) in experiment no.:						Mutation rate relative to SL1344
	1	2	3	4	5	Mean	
SL1344	0·008	0·053	0·044	0·044	2·80	0·590	1·00
ssaV::Mu*dJ*	0·410	5·80	0·930	0·160	4·00	2·26	3·83
Δ*fpg*/*nth*/*nei*	1·40	17·0	5·00	27·0	1·50	10·4	17·6
Δ*fpg*/*nth*/*nei ssaV*::Mu*dJ*	13·0	16·0	8·40	8·50	9·00	11·0	18·6

gentamicin-protection assay was used to determine if SPI2 and BER act synergistically to facilitate survival in the intensely oxidative environment within IFN-γ-activated RAW 264.7 macrophages. The question of synergy between the two systems was addressed by determining whether the fold survival values of the Δ*fpg*/*nth*/*nei* or *ssaV*::Mu*dJ* mutants were significantly different from that of the Δ*fpg*/*nth*/*nei ssaV*::Mu*dJ* mutant using a protected (one-way ANOVA) Fisher's LSD test. Fold survival values were calculated 18 h post-infection, relative to the wild-type SL1344 strain.

If BER and SPI2 cooperate to inhibit the lethality of macrophage oxidants, the fold survival of Δ*fpg*/*nth*/*nei ssaV*::Mu*dJ* will be significantly less than either Δ*fpg*/*nth*/*nei* or *ssaV*::Mu*dJ*. As shown in Fig. 4, statistical analysis indicated that the 0·04-fold survival of the Δ*fpg*/*nth*/*nei ssaV*::Mu*dJ* mutant is significantly less than the 0·22-fold survival of the *ssaV*::Mu*dJ* mutant, as well as the 0·24-fold survival of the Δ*fpg*/*nth*/*nei* mutant (Fig. 4). This result indicates that the ability of macrophage-resident *Salmonella* to repair oxidative DNA lesions is necessary for survival when macrophage oxidant generators are targeted to the SCV.

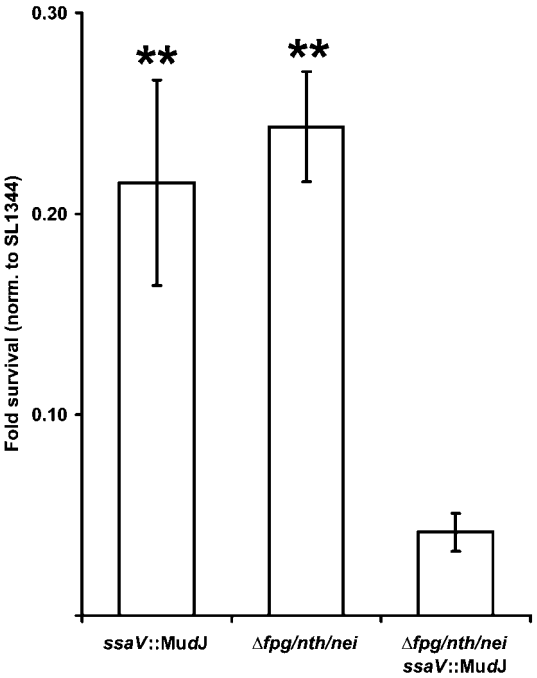

Fig. 4. Effect of SPI2 loss of function on the survival of the *S. typhimurium* BER oxidative sentinel within activated RAW 264.7 macrophages. Fold survival was calculated as the ratio of mutant c.f.u. to wild-type c.f.u. at 18 h post-infection. Data presented are means of three independent experiments, each done in triplicate. Error bars, 1 SD. A Fisher's protected LSD test was performed on planned pairwise comparisons between each of the single mutants and the double mutant. Asterisks indicate a significant difference from the double mutant. Significance was achieved only where indicated by asterisks. **, $P<0{\cdot}05$.

Thus, the fundamentally different oxidant defence strategies of oxidative damage repair (BER) and oxidant avoidance (SPI2) appear to function synergistically in macrophage-resident *Salmonella*. Furthermore, these data are the first experimental evidence that the direct protection of a specific *Salmonella* target by SPI2 enables *Salmonella* survival within the highly oxidative environment of macrophages.

The roles of specific SPI2 effectors in protection of *Salmonella* against lethality of macrophage oxidants

We then used the BER oxidant sentinel (Δ*fpg*/*nth*/*nei*) to address the potential contributions of representative SPI2 effector proteins. Effector proteins of SPI2 are divided into two classes, based on whether they are encoded within the SPI2 operon or not. The Effector region of SPI2 encodes export targets that include the translocon module (SseBCD) and the putative SseE, SseF and SseG effectors. SseE is a protein of unknown function, while SseF and SseG are both required for the aggregation of host compartments into *Salmonella*-induced filaments (Sif; Guy *et al.*, 2000). SseG also directs the recruitment of Golgi membranes to the SCV (Salcedo & Holden, 2003). There are several distally encoded SPI2 effectors, termed *Salmonella*-translocated effectors (STE; Miao & Miller, 2000). A major STE is SifA, which is required for Sif formation (Stein *et al.*, 1996) and the maintenance of SCV integrity (Beuzón *et al.*, 2000), and is also a critical determinant of *Salmonella* replication within both epithelial cells (Stein *et al.*, 1996) and macrophages (Guy *et al.*, 2000; Beuzón *et al.*, 2002).

Gentamicin-protection assays were performed as before, with a polar *sseE*::Mu*dJ* SPI2-encoded effector mutant, a non-polar *sifA*::Tn10*d*Cm STE mutant (Stein *et al.*, 1996) and the corresponding SPI2/BER sentinel mutants. We have previously shown that the *sseE*::Mu*dJ* mutation exerts a polar effect on the downstream *sseF* and *sseG* effector genes (Guy *et al.*, 2000; Suvarnapunya & Stein, unpublished results). The *sscB* gene is directly downstream of *sseE*, and SscB has now been experimentally demonstrated to be a chaperone for SseF (Dai & Zhou, 2004). Therefore, the *sseE*::Mu*dJ* mutant is defective for translocated effectors encoded in the SPI2 Effector region. The fold survival of these mutants is shown in Fig. 5. The data were analysed as before with a protected Fisher's LSD test.

SifA does not seem to serve a role in the diversion of oxidant generators. The 0·42-fold survival of *sifA*::Tn10*d*Cm was not significantly different from that of the 0·25-fold survival of Δ*fpg*/*nth*/*nei sifA*::Tn10*d*Cm (Fig. 5a). The 0·40-fold survival of Δ*fpg*/*nth*/*nei* in this set of experiments was also not significantly different from that of the Δ*fpg*/*nth*/*nei sifA*::Tn10*d*Cm mutant (Fig. 5a).

The *sseE*::Mu*dJ* mutant is 0·88-fold reduced for survival compared to wild-type (Fig. 5b). However, Δ*fpg*/*nth*/*nei sseE*::Mu*dJ* is 0·13-fold reduced (Fig. 5b), and this is

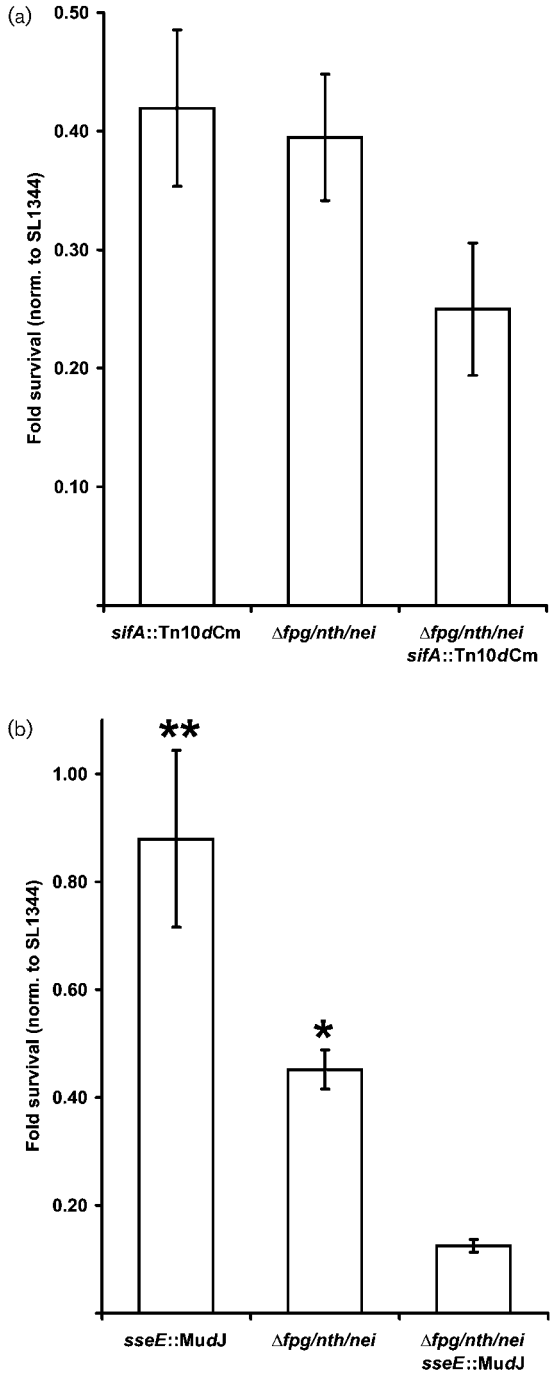

Fig. 5. Effect of SPI2 effectors on the survival of the *S. typhimurium* BER oxidative sentinel within activated RAW 264.7 macrophages. (a) Effect of the SifA STE, and (b) effect of the SPI2 effector region. Fold survival was calculated as the ratio of mutant c.f.u. to wild-type c.f.u. at 18 h post-infection. Data presented are means of four independent experiments (a), or three independent experiments (b), each done in triplicate. Error bars, 1 SD. A Fisher's protected LSD test was performed on planned pairwise comparisons between each of the single mutants and the double mutant. Asterisks indicate a significant difference from the double mutant. Significance was only achieved where indicated by asterisks. **, $P<0{\cdot}05$; *, $P<0{\cdot}08$.

significantly less than that observed for the *sseE*::Mu*dJ* mutant. In this set of experiments, the 0·13-fold survival of Δ*fpg*/*nth*/*nei sseE*::Mu*dJ* was also significantly less than the 0·45-fold survival of Δ*fpg*/*nth*/*nei* ($\alpha=0{\cdot}08$; Fig. 5b). Thus, while SifA does not appear to be involved in oxidant defence, SseF and SseG are implicated as SPI2 mediators of oxidant defence.

DISCUSSION

The ability of *Salmonella* to survive and replicate within macrophages is well-established as being essential for the systemic disease process (Fields *et al.*, 1986; Richter-Dahlfors *et al.*, 1997). In order to thrive within host macrophages, *Salmonella* must overcome several formidable obstacles, including the highly reactive oxidants that are rapidly and steadily produced upon internalization. It is perhaps not surprising then that recent studies have shown that *Salmonella* has at least two solutions: the constitutive highly redundant BER system for repair of oxidative DNA damage (Suvarnapunya *et al.*, 2003b), and the inducible SPI2-encoded Type III secretion system for redirection of the enzymic generators of oxidants (Vazquez-Torres *et al.*, 2000a; Gallois *et al.*, 2001; Chakravortty *et al.*, 2002). However, some dissenting results for a direct oxidant protection role for SPI2 have recently emerged. The present study was therefore aimed at evaluating the putative SPI2 oxidant defence function via oxidative DNA damage criteria and determining how these two distinct systems of BER and SPI2, seemingly unrelated in function until very recently, interact as an integrated *Salmonella* response against macrophage oxidants.

Utility of the *Salmonella* oxidative sentinel

Our earlier study identifying BER as important for *Salmonella* pathogenesis (Suvarnapunya *et al.*, 2003b) indicated that this defined DNA repair system could be used to functionally address the role of SPI2 in oxidant defence. The BER system is specific for oxidized bases and not other unrelated types of DNA damage. Furthermore, BER mutants grow normally in the absence of exogenous oxidative stress. These features were thus exploited to create a mutant that could present a known and consequential target of macrophage oxidants within the unique conditions of the SCV in an *ex vivo* system and could 'report' any oxidative stress encountered.

To this end, we constructed a mutant that is unable to initiate BER via bifunctional glycosylases, in order to avoid the small but real possibility that abortive BER (Fig. 1b) or other similar phenomena related to toxic repair intermediates may preclude accurate assessments. Radiation resistance is the hallmark phenotype of the lack of BER bifunctional glycosylase activity, since ionizing radiation produces the type of clustered oxidative DNA damage that prematurely aborts BER.

The *S. typhimurium* Δ*fpg* mutant was significantly more

radiation resistant than wild-type (Fig. 2), and thus qualitatively consistent with its analogous *E. coli* mutant. More importantly, the resulting Δ*fpg*/*nth*/*nei* mutant was found to have a similar degree of radiation resistance to its analogous *E. coli* mutant (Fig. 2), previously characterized by Blaisdell & Wallace (2001). Thus, we concluded that the *S. typhimurium* Δ*fpg*/*nth*/*nei* mutant had the desired lack of BER bifunctional glycosylase activity.

Oxidative stress upon macrophage-resident *Salmonella* has mainly lethal, not mutagenic, consequences

Earlier studies of pleiotropic *recA* mutants have suggested the importance of DNA repair in *Salmonella* pathogenesis (Buchmeier *et al.*, 1993, 1995), and we have shown that it is the repair of oxidative DNA damage specifically by the dedicated BER system that is a major mechanism of *Salmonella* intramacrophage survival (Suvarnapunya *et al.*, 2003b). The increased mutability and reduced viability of the Δ*fpg*/*nth*/*nei* mutant within macrophages in the present study confirms our earlier conclusion that host macrophages attempt to control phagocytosed *Salmonella* by causing oxidative damage to its bacterial DNA, and that this damage is repaired by BER. Therefore, although oxidative damage can occur to a variety of targets, including lipids and proteins (reviewed by Marnett *et al.*, 2003), it is quite clear that *Salmonella* DNA is the critical target of macrophage oxidants. It is therefore also apparent that macrophage oxidants do gain access to the *Salmonella* cytosol and retain sufficient reactivity to produce oxidative DNA damage.

While the mutation rate of Δ*fpg*/*nth*/*nei* is increased by an order of magnitude within macrophages (Table 2), *ssaV*::Mu*dJ* also displays a hypermutation phenotype in macrophages (Table 2). Because *ssaV* mutants are defective for SPI2 function, due to the inability to secrete or translocate SPI2 effectors (e.g. Zurawski & Stein, 2003), the hypermutation of *ssaV*::Mu*dJ* is evidence that SPI2 function is required to inhibit direct exposure of *Salmonella* DNA to mutagenic compounds within macrophages. While no additive effect was observed upon mutation rate when SPI2 function was abrogated in the Δ*fpg*/*nth*/*nei* mutant, it is important to note that the mutation rates observed would only apply to the surviving bacterial subpopulation, and not to those bacteria that had already been killed. Thus, it is likely the full brunt of macrophage oxidative stress experienced by *Salmonella* when neither BER nor SPI2 are functioning results mainly in lethality, rather than mutagenicity. While the concept of adaptive mutation is still currently being debated (reviewed by Rosenberg, 2001; Wright, 2004), earlier results with *Salmonella* DNA-mismatch repair mutants indicate that hypermutability alone does not reduce *Salmonella* virulence (Zahrt *et al.*, 1999; Campoy *et al.*, 2000; Suvarnapunya *et al.*, 2003a).

SPI2 promotes intramacrophage survival by protecting *Salmonella* DNA against macrophage oxidants

The significant intramacrophage survival defect of Δ*fpg*/*nth*/*nei* (Fig. 3), as with other BER mutants (Suvarnapunya *et al.*, 2003b), is observed despite the function of SPI2. Likewise, *ssaV* mutants show increased mutation rates within macrophages (Table 2) and are significantly reduced for intramacrophage survival (e.g. Hensel *et al.*, 1998), despite the function of BER. Thus, we considered the possibility that the two systems may not function in parallel, but function synergistically. An alternative possibility was that SPI2 does not affect direct oxidant exposure, but instead affects other oxidant-dependent antibacterial mechanisms such as CRAMP processing (Rosenberger *et al.*, 2004). We reasoned that either possibility would become apparent in gentamicin-protection assays when SPI2 function is removed from a mutant unable to repair DNA bases that are damaged by macrophage oxidants.

The loss of SPI2 function in the BER oxidant sentinel (Δ*fpg*/*nth*/*nei ssaV*::Mu*dJ*) imparts a significant and dramatic decrease in intramacrophage survival (Fig. 4), providing the first evidence that SPI2 promotes *Salmonella* survival within macrophages by directly inhibiting the oxidation of *Salmonella* DNA bases. We also observe a similar phenotype in an AP endonuclease-deficient background (Δ*xth*/*nfo ssaV*::Mu*dJ*; data not shown), suggesting that single-strand breaks are inhibited by SPI2 as well. Additionally, our data suggest a synergistic relationship between BER and SPI2. The involvement of SPI2 in macrophage oxidant defence does not agree with a study by Rosenberger *et al.* (2004), which used bacterial filamentation as a non-specific indicator of oxidative stress.

The loss of SifA in the BER oxidant sentinel did not significantly alter its survival in macrophage (Fig. 5a). The apparent dispensability of SifA function with regard to oxidant defence is consistent with the findings of Vazquez-Torres *et al.* (2000b) that phox is the predominant source of oxidants that are bactericidal to *Salmonella*. Because the phox complex must be assembled on the phagosomal membrane for full activity, loss of the membrane in *sifA* mutants would likely preclude phox-mediated killing. Furthermore, since *sifA* mutants are released into the host cytosol, our data suggest that there is no significant cytosolic oxidative killing mechanism in macrophages, such as the cytosolic iNOS (Vodovotz *et al.*, 1995; Webb *et al.*, 2001).

Other SPI2 effectors that may mediate the disruption of phox and iNOS targeting to the SCV are SseF and SseG, the first SPI2-encoded effectors shown to alter endocytic traffic (Guy *et al.*, 2000). Additionally, SseG was found to mediate the recruitment of Golgi membranes to the SCV (Salcedo & Holden, 2003). We have shown here that the polar effect of the *sseE*::Mu*dJ* mutation on *sscB*, *sseF* and *sseG* significantly enhances the sensitivity of the

BER oxidative sentinel to macrophage oxidants (Fig. 5b). SscB has recently been shown to be a chaperone for SseF (Dai & Zhou, 2004), so polarity on *sscB* is not envisioned to have an independent effect. Therefore, our results suggest a role for SseF and/or SseG in control of phox and iNOS trafficking.

A model for BER and SPI2 synergy in *Salmonella* macrophage oxidant defence

The expression of SPI2 is tightly regulated and induced only by specific conditions, such as low pH or low osmolarity, that are characteristic of the intracellular environment. Induction and expression of SPI2 seem to occur between 2 and 6 h post-infection (Garmendia *et al.*, 2003). Therefore, it would seem that *Salmonella* requires another means of at least stalling oxidative killing until the SPI2 secretion apparatus can be assembled and appropriate SPI2 effectors can be secreted and translocated. We speculate that the constitutive function of BER allows *Salmonella* to stall oxidative killing by preserving its genomic integrity, until SPI2 can be fully upregulated to limit direct exposure to macrophage oxidants.

We have previously observed that most of the macrophage-mediated killing of *S. typhimurium* BER mutants occurs during the first 10 h of infection, then tapers off (Suvarnapunya *et al.*, 2003b). This suggests that BER function is most crucial to *Salmonella* during relatively early time-points of macrophage colonization, although the possibility that BER contributes to oxidant resistance throughout intramacrophage residence cannot be excluded. It also suggests that SPI2 disruption of phox targeting, while detectable as early as 1 h post-infection (Vazquez-Torres *et al.*, 2000a), may not take full effect until several hours after infection. Additionally, induction of SPI2 prior to macrophage infection dramatically enhances the survival of BER mutants, as well as wild-type *Salmonella* (Suvarnapunya & Stein, unpublished results). These observations collectively suggest that the previously reported function of BER in *Salmonella* pathogenesis (Suvarnapunya *et al.*, 2003b) may be most important in countering the lethality of the oxidative burst, when the replication-permissive SCV has not yet been established by SPI2.

The potential importance of also dealing with iNOS-derived oxidants should be considered as well, as it has recently been shown that nitric oxide (NO) is bactericidal for *Salmonella* in dendritic cells (Eriksson *et al.*, 2003b), in contrast to being bacteriostatic in macrophages (Vazquez-Torres *et al.*, 2000b). Investigations into the mechanisms of NO-based *Salmonella* cytotoxicity have also pointed towards DNA as a critical target, via interference with the DNA replication process (Schapiro *et al.*, 2003). Preliminary studies of *S. typhimurium* BER mutant survival within iNOS-deficient primary macrophages suggest that iNOS-derived oxidants may also cause lethal DNA damage in the absence of BER function (Suvarnapunya & Stein, unpublished results).

An intriguing hypothesis emerging from these studies is that *Salmonella* may actually require exposure to oxidants in order to activate SPI2 and other operons that contribute to survival in phagocytic cells. Supporting the idea that *Salmonella* modulates, rather than strictly inhibits, the oxidative stress encountered within macrophages is the recent report that the invasion-associated SPI1 effector, SopB, actually promotes NO production from iNOS in macrophages (Drecktrah *et al.*, 2004). The constitutive BER system would thus protect *Salmonella* DNA integrity under the requisite and prolonged oxidative stress.

Clearly, BER and SPI2 do not represent the sum of the *Salmonella* defensive repertoire against macrophage oxidants. The remarkable detoxification and scavenging mechanisms of *Salmonella*, exemplified by the presence of five superoxide dismutases (Fang *et al.*, 1999; Figueroa-Bossi *et al.*, 2001) and three catalases (Buchmeier *et al.*, 1995; Robbe-Saule *et al.*, 2001) that are differentially localized and co-factored, are beyond the scope of this study. Furthermore, oxidative damage alone is not the entire basis of the macrophage–*Salmonella* interaction. Nevertheless, SPI2 is evidently the primary means by which *Salmonella* is able to survive within the normally hostile environment of the macrophage phagosome, and the model suggested by this study may clarify how SPI2 enables *Salmonella* persistence in the host and the subsequent establishment of systemic disease.

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REFERENCES

Babior, B. M. (2000). Phagocytes and oxidative stress. *Am J Med* **109**, 33–44.

Beuzón, C. R., Meresse, S., Unsworth, K. E., Ruiz-Albert, J., Garvis, S., Waterman, S. R., Ryder, T. A., Boucrot, E. & Holden, D. W. (2000). *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. *EMBO J* **19**, 3235–3249.

Beuzón, C. R., Salcedo, S. P. & Holden, D. W. (2002). Growth and killing of a *Salmonella enterica* serovar Typhimurium *sifA* mutant strain in the cytosol of different host cell lines. *Microbiology* **148**, 2705–2715.

Blaisdell, J. O. & Wallace, S. S. (2001). Abortive base-excision repair of radiation-induced clustered DNA lesions in *Escherichia coli*. *Proc Natl Acad Sci U S A* **98**, 7426–7430.

Buchmeier, N. A., Lipps, C. J., So, M. Y. & Heffron, F. (1993). Recombination deficient mutants of *Salmonella typhimurium* are avirulent and sensitive to the oxidative burst of macrophages. *Mol Microbiol* **7**, 933–936.

Buchmeier, N. A., Libby, S. J., Xu, Y., Loewen, P. C., Switala, J. & Guiney, D. G. (1995). DNA repair is more important than catalase for *Salmonella* virulence in mice. *J Clin Invest* **95**, 1047–1053.

Campoy, S., Perez de Rozas, A. M., Barbe, J. & Badiola, I. (2000). Virulence and mutation rates of *Salmonella typhimurium* strains with increased mutagenic strength in a mouse model. *FEMS Microbiol Lett* **187**, 145–150.

Chakravortty, D., Hansen-Wester, I. & Hensel, M. (2002). *Salmonella* Pathogenicity Island 2 mediates protection of intracellular *Salmonella* from reactive nitrogen intermediates. *J Exp Med* **195**, 1155–1166.

Dai, S. & Zhou, D. (2004). Secretion and function of *Salmonella* SPI-2 effector SseF require its chaperone, SscB. *J Bacteriol* **186**, 5078–5086.

Damiani, G., Kiyotaki, C., Soeller, W., Sasada, M., Peisach, J. & Bloom, B. R. (1980). Macrophage variants in oxygen metabolism. *J Exp Med* **152**, 808–822.

Drecktrah, D., Knodler, L. A., Galbraith, K. & Steele-Mortimer, O. (2005). The *Salmonella* SPI1 effector SopB stimulates nitric oxide production long after invasion. *Cell Microbiol* (in press). (doi:10.1111/j.1462-5822.2004.00436.x)

Eisen, J. A. & Hanawalt, P. C. (1999). A phylogenomic study of DNA repair genes, proteins, and processes. *Mutat Res* **435**, 171–213.

Eriksson, S., Lucchini, S., Thompson, A., Rhen, M. & Hinton, J. C. D. (2003a). Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol Microbiol* **47**, 103–118.

Eriksson, S., Chambers, B. J. & Rhen, M. (2003b). Nitric oxide produced by murine dendritic cells is cytotoxic for intracellular *Salmonella enterica* sv. Typhimurium. *Scand J Immunol* **58**, 493–502.

Fang, F. C., DeGroote, M. A., Foster, J. W. & 8 other authors (1999). Virulent *Salmonella typhimurium* has two periplasmic Cu, Zn-superoxide dismutases. *Proc Natl Acad Sci U S A* **96**, 7502–7507.

Fields, P. I., Swanson, R. V., Haidaris, C. G. & Heffron, F. (1986). Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci U S A* **83**, 5189–5193.

Figueroa-Bossi, N., Uzzau, S., Maloriol, D. & Bossi, L. (2001). Variable assortment of prophages provides a transferable repertoire of pathogenic determinants in *Salmonella*. *Mol Microbiol* **39**, 260–271.

Gallois, A., Klein, J. R., Allen, L. A., Jones, B. D. & Nauseef, W. M. (2001). *Salmonella* pathogenicity island 2-encoded type III secretion system mediates exclusion of NADPH oxidase assembly from the phagosomal membrane. *J Immunol* **166**, 5741–5748.

Garmendia, J., Beuzon, C. R., Ruiz-Albert, J. & Holden, D. W. (2003). The roles of SsrA-SsrB and OmpR-EnvZ in the regulation of genes encoding the *Salmonella typhimurium* SPI-2 type III secretion system. *Microbiology* **149**, 2385–2396.

Guy, R. L., Gonias, L. A. & Stein, M. A. (2000). Aggregation of host endosomes by *Salmonella* requires SPI2 translocation of SseFG and involves SpvR and the *fms-aroE* intragenic region. *Mol Microbiol* **37**, 1417–1435.

Hensel, M., Shea, J. E., Waterman, S. R. & 7 other authors (1998). Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. *Mol Microbiol* **30**, 163–174.

Holden, D. W. (2002). Trafficking of the *Salmonella* vacuole in macrophages. *Traffic* **3**, 161–169.

Linehan, S. A. & Holden, D. W. (2003). The interplay between *Salmonella typhimurium* and its macrophage host – what can it teach us about innate immunity? *Immunol Lett* **85**, 183–192.

Marnett, L. J., Riggins, J. N. & West, J. D. (2003). Endogenous generation of reactive oxidants and electrophiles and their reactions with DNA and protein. *J Clin Invest* **111**, 583–593.

Miao, E. A. & Miller, S. I. (2000). A conserved amino acid sequence directing intracellular type III secretion by *Salmonella typhimurium*. *Proc Natl Acad Sci U S A* **97**, 7539–7544.

Mizrahi, V. & Andersen, S. J. (1998). DNA repair in *Mycobacterium tuberculosis*. What have we learnt from the genome sequence? *Mol Microbiol* **29**, 1331–1339.

Nathan, C. & Shiloh, M. U. (2000). Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A* **97**, 8841–8848.

O'Rourke, E. J., Chevalier, C., Pinto, A. V., Thiberge, J. M., Ielpi, L., Labigne, A. & Radicella, J. P. (2003). Pathogen DNA as target for host-generated oxidative stress: role for repair of bacterial DNA damage in *Helicobacter pylori* colonization. *Proc Natl Acad Sci U S A* **100**, 2789–2794.

Richter-Dahlfors, A., Buchan, A. M. J. & Finlay, B. B. (1997). Murine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes *in vivo*. *J Exp Med* **186**, 569–580.

Robbe-Saule, V., Coynault, C., Ibanez-Ruiz, M., Hermant, D. & Norel, F. (2001). Identification of a non-haem catalase in *Salmonella* and its regulation by RpoS (σ^S). *Mol Microbiol* **39**, 1533–1545.

Rosenberg, S. M. (2001). Evolving responsively: adaptive mutation. *Nat Rev Genet* **2**, 504–515.

Rosenberger, C. M. & Finlay, B. B. (2002). Macrophages inhibit *Salmonella* Typhimurium replication through MEK/ERK kinase and phagocyte NADPH oxidase activities. *J Biol Chem* **277**, 18753–18762.

Rosenberger, C. M., Gallo, R. L. & Finlay, B. B. (2004). Interplay between antibacterial effectors: a macrophage antimicrobial peptide impairs intracellular *Salmonella* replication. *Proc Natl Acad Sci U S A* **101**, 2422–2427.

Salcedo, S. P. & Holden, D. W. (2003). SseG, a virulence protein that targets *Salmonella* to the Golgi network. *EMBO J* **22**, 5003–5014.

Schapiro, J. M., Libby, S. J. & Fang, F. C. (2003). Inhibition of bacterial DNA replication by zinc mobilization during nitrosative stress. *Proc Natl Acad Sci U S A* **100**, 8496–8501.

Schlosser-Silverman, E., Elgrably-Weiss, M., Rosenshine, I., Kohen, R. & Altuvia, S. (2000). Characterization of *Escherichia coli* DNA lesions generated within J774 macrophages. *J Bacteriol* **182**, 5225–5230.

Schmieger, H. (1972). Phage P22-mutants with increased or decreased transduction abilities. *Mol Gen Genet* **119**, 75–88.

Stein, M. A., Leung, K. Y., Zwick, M., Garcia-del Portillo, F. & Finlay, B. B. (1996). Identification of a *Salmonella* virulence gene required for formation of filamentous structures containing lysosomal membrane glycoproteins within epithelial cells. *Mol Microbiol* **20**, 151–164.

Suvarnapunya, A. E., Zurawski, D. V., Guy, R. L. & Stein, M. A. (2003a). Molecular characterization of the prototrophic *Salmonella* mutants defective for intraepithelial replication. *Infect Immun* **71**, 2247–2252.

Suvarnapunya, A. E., Lagassé, H. A. D. & Stein, M. A. (2003b). The role of DNA base excision repair in the pathogenesis of *Salmonella enterica* serovar Typhimurium. *Mol Microbiol* **48**, 549–559.

Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Holden, D. W., Lucia, S. M., Dinauer, M. C., Mastroeni, P. & Fang, F. C. (2000a). *Salmonella* Pathogenicity Island 2-dependent evasion of the phagocyte NADPH oxidase. *Science* **287**, 1655–1658.

Vazquez-Torres, A., Jones-Carson, J., Mastroeni, P., Ischiropoulos, H. & Fang, F. C. (2000b). Antimicrobial actions of the NADPH

phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J Exp Med* **192**, 227–236.

Vodovotz, Y., Russell, D., Xie, Q. W., Bogdan, C. & Nathan, C. (1995). Vesicle membrane association of nitric oxide synthase in primary mouse macrophages. *J Immunol* **154**, 2914–2925.

Wallace, S. S. (1997). Oxidative damage to DNA and its repair. In *Oxidative Stress and the Molecular Biology of Antioxidant Defenses*, pp. 49–90. Edited by J. G. Scandalios. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Webb, J. L., Harvey, M. W., Holden, D. W. & Evans, T. J. (2001). Macrophage nitric oxide synthase associates with cortical actin but is not recruited to phagosomes. *Infect Immun* **69**, 6391–6400.

Wright, B. E. (2004). Stress-directed adaptive mutations and evolution. *Mol Microbiol* **52**, 643–650.

Zahrt, T. C., Buchmeier, N. & Maloy, S. (1999). Effect of *mutS* and *recD* mutations on *Salmonella* virulence. *Infect Immun* **67**, 6168–6172.

Zurawski, D. V. & Stein, M. A. (2003). SseA acts as the chaperone for the SseB component of the *Salmonella* Pathogenicity Island 2 translocon. *Mol Microbiol* **47**, 1341–1351.