**Editor’s Choice**

**Salmonella** methylglyoxal detoxification by STM3117-encoded lactoylglutathione lyase affects virulence in coordination with *Salmonella* pathogenicity island 2 and phagosomal acidification

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Intracellular pathogens such as *Salmonella enterica* serovar Typhimurium (S. Typhimurium) manipulate their host cells through the interplay of various virulence factors. A multitude of such virulence factors are encoded on the genome of S. Typhimurium and are usually organized in pathogenicity islands. The virulence-associated genomic stretch of STM3117–3120 has structural features of pathogenicity islands and is present exclusively in non-typhoidal serovars of *Salmonella*. It encodes metabolic enzymes predicted to be involved in methylglyoxal metabolism. STM3117-encoded lactoylglutathione lyase significantly impacts the proliferation of intracellular *Salmonella*. The deletion mutant of STM3117 (Δlgl) fails to grow in epithelial cells but hyper-replicates in macrophages. This difference in proliferation outcome was the consequence of failure to detoxify methylglyoxal by Δlgl, which was also reflected in the form of oxidative DNA damage and upregulation of kefB in the mutant. Within macrophages, the toxicity of methylglyoxal adducts elicits the potassium efflux channel (KefB) in the mutant which subsequently modulates the acidification of mutant-containing vacuoles (MCVs). The perturbation in the pH of the MCV milieu and bacterial cytosol enhances the *Salmonella* pathogenicity island 2 translocation in Δlgl, increasing its net growth within macrophages. In epithelial cells, however, the maturation of Δlgl-containing vacuoles were affected as these non-phagocytic cells maintain less acidic vacuoles compared to those in macrophages. Remarkably, ectopic expression of Toll-like receptors 2 and 4 on epithelial cells partially restored the survival of Δlgl. This study identified a novel metabolic enzyme in S. Typhimurium whose activity during intracellular infection within a given host cell type differentially affected the virulence of the bacteria.

**INTRODUCTION**

The genus *Salmonella* comprises facultative intracellular pathogens (Haraga *et al.*, 2008) notorious for causing persistent gastrointestinal infections in a number of hosts that often lead to fatal systemic diseases (Jantsch *et al.*, 2011). The intracellular life of the pathogen exists within a phagosomal/endosomal vacuole called a *Salmonella*-containing vacuole (SCV) (Dandekar *et al.*, 2012) whose maturation dynamics are similar to those of the canonical endocytic pathway and are modulated by several bacterial virulence factors (Drecktrah *et al.*, 2008; Jantsch *et al.*, 2011). Typically, combinations of virulence factors are employed to combat the attack mounted by the host. For instance, metabolic and detoxifying enzymes are utilized for rigorous adaptation in nutrient-limiting conditions and exploitation of host resources, thereby favouring pathogen persistence.

Metabolism of *Salmonella enterica* serovar Typhimurium (S. Typhimurium) is remarkably different in epithelial cells and macrophages due to modulation in the expression of key metabolic enzymes in the two host cell types (Eisenreich *et al.*, 2010). Pathogens generally adapt themselves as per their host cell milieu and their metabolic enzymes play a pivotal role in this adaptation process. Pathogens such as *S. Typhimurium*, *Shigella flexneri*, *Mycobacterium tuberculosis* and *Listeria monocytogenes* utilize different substrates, e.g. glucose, C1 compounds, C2 units and glycerol, respectively (Eisenreich *et al.*, 2010). Pathogen-specific metabolic adaptations subsequently activate those virulence factors in the

**Abbreviations:** AMP, antimicrobial peptide; BAF, baflomycin A1; BMDM, bone-marrow-derived macrophage; DiBAC4(3), bis-(1,3-dibutylbarbituric acid)-trimethine oxonol; EEA1, early endosome autoantigen 1; HBD, human β-defensin; LAMP1, lysosome-associated membrane protein 1; MCV, mutant-containing vacuole; PCA, perchloric acid; p.i., post-infection; q, quantitative; RT, real-time; SCV, *Salmonella*-containing vacuole; SPI, *Salmonella* pathogenicity island; TLR, Toll-like receptor; TTSS, type III secretion system.

Five supplementary figures are available with the online version of this paper.
pathogen which are required for its intracellular lifestyle. Intracellular Salmonella within epithelial cells and macrophages uses glucose as the preferred carbon source, and mutants defective in glycolysis or glucose uptake are impaired in their replication capacity. However, in situations of limited glycolytic carbon sources, e.g. in vivo infections, survival of S. Typhimurium is strongly dependent on the acquisition of anabolic monomers such as purines and amino acids from the host cell into the SCVs (Fields et al., 1986; Mercado-Lubo et al., 2009). Both glycolysis and amino acid degradation have been shown to contribute to the intracellular pool of methylglyoxal (Ferguson et al., 1998). Considering the dissimilar preference of metabolic pathways in different host cells, it was interesting to investigate the consequences of the methylglyoxal pathway within macrophages and epithelial cells. Currently, very little information exists on methylglyoxal catabolism in intracellular pathogens. Elevated levels of mycobacteria-induced methylglyoxal and advanced glycation end-products in granulomatosus lesions have been demonstrated to induce apoptosis of the resident macrophages as well as alter immune response genes (Rachman et al., 2006). The enhanced level of lactoylglutathione lyase (methylglyoxal detoxifying enzyme) has also been implicated in contributing to Streptococcus mutans aciduricy in dental plaques (Korithoski et al., 2007). Uprogulation of other bacterial methylglyoxal-detoxifying genes or level of endogenous methylglyoxal during infection implies that pathogens exploit the methylglyoxal pathway differently to persist in host environs. Glyoxalase I (lactoylglutathione lyase) and glyoxalase II are the detoxifying enzymes which are pivotal in contributing to survival against toxic effects of electrophiles such as methylglyoxal (Booth et al., 2003; MacLean et al., 1998; Thornalley, 2008).

Unlike other bacteria which strictly harbour single copies of each glxl and glxII, S. Typhimurium has two genomic loci encoding putative glyoxalase I apart from the canonical glyoxalase I gene (glxl) (Campos-Bermudez et al., 2007; Tötemeyer et al., 1998). glxO encodes the canonical Glxl, whilst STM3117 and yaeK encode putative GlxI (Campos-Bermudez et al., 2007; Eriksson et al., 2003; Santiviago et al., 2009; Shi et al., 2006). STM3117 (Gene ID: 1254640) shares only 24% homology with the Ni²⁺-dependent glyoxalase I of Escherichia coli. Curiously, STM3117 is part of the virulence-associated operon (STM3117–3120) (Haneda et al., 2009) which has three downstream coding regions and was predicted to be involved in the methylglyoxal pathway (Shi et al., 2006). The STM3117–3120 operon along with a stretch of 14 downstream ORFs, clustered adjacent to the tRNA pheV gene, have been denoted as Salmonella pathogenicity island (SPI)-13 due to the characteristic low G+C content, similar to typical pathogenicity islands (Shah et al., 2005). In accordance, the homologous region corresponding to STM3117–3120 in S. enteritidis was observed to be crucial for survival in chicken macrophages (Shah et al., 2005). In transcriptome analysis of S. Typhimurium from macrophages, seven- to 20-fold upregulation of the four members of the STM3117–3120 operon was observed (Eriksson et al., 2003). Based on the abundance of proteins in infected macrophages, it was demonstrated that STM3117 contributes to colonization within natural resistance-associated macrophage protein negative (NRAMP1⁻) macrophages (Shi et al., 2006) and the gene was essential for survival in vivo (Haneda et al., 2009; Santiviago et al., 2009). Contrary to this, the transcriptome of S. Typhimurium in epithelial cells did not show any appreciable induction in the expression of STM3117, STM3119 and STM3120 during infection (Hautefort et al., 2008).

It is known that defects in methylglyoxal detoxification induce expression of kefB (K⁺ efflux channel) in bacterial cells (Ferguson et al., 1997). The channel works by antiporting H⁺ against a K⁺ efflux. In this work, we show that within macrophages, absence of STM3117 elicits kefB in S. Typhimurium which antiports H⁺ from mutant-containing vacuoles (MCVs) into the bacterial cytosol, causing a decrease in the bacterial pH (pH Bact). The relative lowering of the bacterial pH with respect to MCVs enhances SPI-2 translocon secretion, increasing the proliferation of Δglg. By employing an STM3117-overexpressing strain, we showed a reversal in the trend with reduced SPI-2 secretion and proliferation. In epithelial cells, additional Toll-like receptor (TLR) 2 and 4 signalling could rescue the defective proliferation and maturation of Δglg containing vacuoles. Altogether, our results offer an insight on the possibilities of varied outcomes in the intracellular survival of S. Typhimurium in response to the activity of STM3117.

**METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. S. Typhimurium NCTC 12023 was used as the parental WT strain. Bacteria were routinely grown at 37 °C in LB medium containing appropriate antibiotics unless otherwise mentioned. Nalidixic acid, kanamycin and ampicillin were each used at 50 μg ml⁻¹. Generation of Δalg, ΔkefB and ΔalgΔkefB double-knockout strains with deletion of STM3117, kefB and both genes, respectively, was carried out using the one-step gene deletion strategy (Datsenko & Wanner, 2000; Eswarappa et al., 2008). For double knockout, the second gene was replaced with a chloramphenicol resistance cassette (pKD3). The knockout primers and confirmatory primers are listed in Table 2. For methylglyoxal sensitivity, 1 × 10⁵ exponential-phase bacteria were either exposed to 0.2 mM methylglyoxal in LB or left untreated and were plated every hour until 3 h to enumerate the number of c.f.u. surviving. Growth was calculated for both untreated and treated sets by dividing the c.f.u. ml⁻¹ at indicated time point by the c.f.u. ml⁻¹ at the initial time point. For immunoblot analyses of SPI-2 and SPI-1 secreted proteins, F-medium (Eswarappa et al., 2008) and LB with 0.3 M NaCl were used, respectively. Kinetic analyses of the STM3117 apoenzyme and holoenzyme with supplemented divalent metal ions were performed as described previously (Clugston et al., 2004, Korithoski et al., 2007). The method for determination of methylglyoxal in bacterial cells was adapted with modifications from Cordeiro et al. Briefly, 100 ml exponential-phase culture (OD 600 0.5) corresponding to 0.01 g bacterial wet weight was used. Cells were deproteinized with 3 ml 5 M perchloric acid (PCA), stirred and immediately frozen in liquid nitrogen. The frozen cultures were centrifuged at 13000 g for 30 min to remove the PCA precipitate. The supernatant was used for quantification of endogenous methylglyoxal. To 800 μl supernatant, 100 μl...
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Background</th>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>S. Typhimurium</td>
<td>NCTC 12023</td>
<td>WT STM (Nal&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Chakravortty et al. (2005)</td>
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<tr>
<td>ΔIgl</td>
<td>WT 12023 ΔIgl::Kan (Km&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>This study</td>
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<tr>
<td>ΔIgl-pQE::lgl</td>
<td>ΔIgl pQE60::lgl</td>
<td>This study</td>
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<td>ΔIgl-pTrc::lgl</td>
<td>ΔIgl pTrc99c::lgl</td>
<td>This study</td>
<td></td>
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<tr>
<td>ΔkefB</td>
<td>WT 1203 ΔkefB::Kan (Km&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>This study</td>
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<tr>
<td>ΔIglΔkefB</td>
<td>WT 1203 ΔIgl::Kan::ΔkefB::Cat (Km&lt;sup&gt;+&lt;/sup&gt;, Chl&lt;sup&gt;+&lt;/sup&gt;)</td>
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<td>Plasmid</td>
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<td>pFPV25.1-mCherry</td>
<td>Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Chakravortty et al. (2005)</td>
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<tr>
<td>pKD4</td>
<td>pANTSy: derivative (Km&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Datsenko &amp; Wanner (2000)</td>
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<tr>
<td>pKD46</td>
<td>pBAD18 derivative (Amp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Datsenko &amp; Wanner (2000)</td>
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<td>pHG86</td>
<td>7 kb, promoterless reporter plasmid carrying lacZ gene (Amp&lt;sup&gt;+&lt;/sup&gt;)</td>
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<td>pTrc9c</td>
<td>S. Typhimurium lgl complementing vector (Amp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>This study</td>
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<td>pQE60</td>
<td>3.4 kb, low-copy-number vector; containing T5 promoter, T5 transcription start site and CoEl origin of replication (Amp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Das et al. (2009)</td>
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<td>pCMV1::hTLR2-FLAG</td>
<td>Human TLR2 without leader (N-terminal FLAG fusion) (Amp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Kind gift from Dr Schnare (University of Marburg, Germany)</td>
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<tr>
<td>pCMV1::hTLR4-FLAG</td>
<td>Human TLR4 without leader (N-terminal FLAG fusion) (Amp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Kind gift from Dr Schnare (University of Marburg, Germany)</td>
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<td>pCMV1::mTLR2(DN)</td>
<td>Mouse TLR2 (dominant negative) cloned in pCMV1 (Amp&lt;sup&gt;+&lt;/sup&gt;)</td>
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<td>pCDNA3.1::mTLR4(DN)</td>
<td>Mouse TLR4 (dominant negative) cloned in pCDNA3.1 (Amp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Kind gift from Dr Schnare (University of Marburg, Germany)</td>
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7.2 mM o-phenylenediamine and 100 µl 5 M PCA were added for derivatization to 2-methylquinoxaline. 5-Methylquinoxaline was added as an internal standard. The quinoxaline derivative of methylglyoxal and the internal standard 5-methylquinoxaline were analysed by a reverse-phase C18 column at 330 nm absorbance; 68 vol.% 10 mM KH<sub>2</sub>PO<sub>4</sub> (pH 2.5) and 32 vol.% HPLC-grade acetonitrile were used as the mobile phase at an isocratic flow rate of 1 ml min<sup>−1</sup>. Duplicate injections were made for each sample. The retention times of 2- and 5-methylquinoxaline were 6 and 8.75 min, respectively. A standard curve was constructed by making serial dilutions of methylglyoxal (0.2–20 nmol). The concentration of endogenous methylglyoxal was measured by calculating the peak heights of the analyte between WT and ΔIgl, and determining their corresponding concentrations from the 2-methylquinoxaline standard curve.

**Construction of plasmids.** Low-copy-number plasmid construct pQE60::Igl, which expresses STM3117 from a T5 promoter, was constructed by cloning the 435 bp STM3117 ORF from WT 12032 strain into the BanHI/HindIII site of pQE60 with the primers listed in Table 2. Overexpressing construct pTrc::Igl was generated similarly by cloning at the same restriction sites of the pTrc99c vector. The constructs were transformed into the ΔIgl background generating ΔIgl-pQE60::lgl and ΔIgl-pTrc::lgl clones, respectively. For analyzing the expression of SPI-1 genes (TTS5-1), promoters (~200 bp upstream from the start site) of two SPI-1 genes, invF and sic4, were transcriptionally fused upstream of a promoterless lacZ in pHG86. The lacZ transcriptional fusions were transformed into WT and ΔIgl strains. pFPV25.1-mCherry (Chakravortty et al., 2002) transformed S. Typhimurium was used for analysing the expression of intracellular SPI-2 effectors.

**Gentamicin protection assays.** RAW264.7, U937 and INT-407 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS. For Caco-2 cells, non-essential amino acids solution was added as a supplement. Bone-marrow-derived macrophages (BMDMs) were obtained as described previously (Das et al., 2010). Gentamicin protection assays were performed as described previously at m.o.i. 10 (Eswarappa et al., 2008). The intracellular growth was determined by dividing the c.f.u. at 16 h by that at 2 h post-infection (p.i.). For epithelial cell infections, overnight culture was subcultured at 1:33 and incubated for 3 h.

For the epithelial cell invasion assay, infected cells were lysed after 1 h of gentamicin (100 µg ml<sup>−1</sup>) treatment. The invasiveness was represented as: (no. of intracellular bacteria/no. of input bacteria) × 100.

To inhibit vacuolar acidification, bafilomycin A1 (BAF) was used at 50 nM. To enumerate the percentage of cytosolic S. Typhimurium, chloroquine was used at 50 µg ml<sup>−1</sup>. Cells were pretreated with BAF for 45 min followed by gentamicin protection assay. Chloroquine was added at the desired time points p.i. and an additional 1 h was given for the compound to kill the vacuolar bacteria before lysing with 0.1% Triton X-100. The number of cytosolic bacteria was determined as the percentage of bacteria recoverable after chloroquine treatment compared with the untreated total (vacuolar and cytosolic both) bacteria.

**In vitro secretion assays and immunoblotting.** For analyses of expression of SPI-1 and SPI-2 proteins, the procedure of Ehrbar et al. (2003) and Höfler & Hensel (2010) was adapted with slight modifications. Bacteria were diluted in either SPI-1-inducing medium (LB with 0.3 M NaCl) or SPI-2-inducing medium (F-medium), and incubated for 3 and 7 h, respectively. Bacteria from 1 ml culture were harvested and resuspended in SDS loading buffer according to the optical density (OD<sub>600</sub> of 1 ml of culture × 100 = µl sample buffer). This fraction represented the whole-cell lysate. Secreted or membrane-attached protein fractions were isolated from 10 µl cultures grown in appropriate media. For extraction of the secreted protein fraction (SpIC), culture supernatant was filtered (0.2 µm membrane filter) and proteins were precipitated overnight at 4 °C with 10% (w/v) TCA. For isolation of the surface-associated protein fraction...
(SseB and SseD), the bacterial pellet (from 10 ml culture volume) was mixed with ice-cold PBS (1 ml), vortexed vigorously for 2 min and centrifuged for 30 min at 8000 g. The rapid vortexing enabled the loosely attached surface proteins to come into the suspension. The TCA precipitates of both the supernatant fraction and the surface-attached fraction were washed with ice-cold acetone, air-dried and finally resuspended in SDS loading buffer; 50 mg total protein was loaded on 12 % SDS-PAGE gels. For immunoblot analysis, affinity-purified rabbit polyclonal antibodies against Salmonella SipC, SseB and SseD were used. Equal protein loading for cell-associated protein fractions was determined by levels of DnaK. For secreted and surface-attached proteins, either Coomassie-blue-stained gels or culture volumes with equivalent OD600 were used for equal loading.

Immunofluorescence microscopy. For intracellular SPI-2 expression and SCV maturation dynamics, RAW264.7 or INT-407 cells, seeded at a density of 3 6 10^4 cells per coverslip, were infected with ice-cold acetone, air-dried and finally resuspended in SDS loading buffer; 50 mg total protein was loaded on 12 % SDS-PAGE gels. For immunoblot analysis, affinity-purified rabbit polyclonal antibodies against Salmonella SipC, SseB and SseD were used. Equal protein loading for cell-associated protein fractions was determined by levels of DnaK. For secreted and surface-attached proteins, either Coomassie-blue-stained gels or culture volumes with equivalent OD600 were used for equal loading.

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Mouse experiment. BALB/c and C57BL/6 mice (6–8 weeks old) were infected either orally or intraperitoneally under aseptic conditions with 10^6 and 10^3 c.f.u. per mouse, respectively, of overnight bacterial culture. For organ infiltration studies, livers and spleens were aseptically removed, 4 days after infection. They were weighed and homogenized in 1 ml ice-cold PBS. Serial dilutions of
the homogenate were plated on LB agar with appropriate antibiotics and the c.f.u. was calculated per gram weight of organ for liver and per organ for spleen.

**Antimicrobial peptide (AMP) sensitivity assay.** Exponentially grown bacteria (obtained after 3 h of growth post 1:50 subculture) were freshly subcultured in 0.5% tryptone/0.5% sodium chloride and 10 mM potassium phosphate buffer for determining the sensitivity against protamine sulfate and human β-defensin (HBD 1), respectively, as described previously (Eswarappa et al., 2008). For flow-cytometric analysis, the samples (both treated and untreated) were treated with bis-(1,3-dibutylbarbituric acid)-trimethine oxonol [DiBAC$_{4(3)}$] at 1 µg ml$^{-1}$ for 10 min followed by FACs analysis. The weighted means for DiBAC$_{4(3)}$ fluorescence was calculated. For assessing the co-localization of HD5 with intracellular bacteria, INT-407 cells were infected with either mCherry-WT or mCherry-DΔgl. At the specified time points, the infected monolayers were stained with affinity-purified anti-HD5 (Alpha Diagnostic International).

**β-Galactosidase reporter assays.** Promoter activity of two SPI-1 genes, _invF_ and _sick_ was assessed using transcriptional fusions to lacZ as described previously (Marathe et al., 2010). Reporter strains were grown in SPI-1-inducing media until exponential phase and β-galactosidase activity was checked by measuring the absorbance of the coloured product at the end of the assay at 495 nm. Data were expressed as Miller units normalized to bacterial growth (OD$_{600}$).

**Quantitative real-time (qRT)-PCR.** For analysing the transcript levels of _STM3117_ expressed from pQ660 and pTrc99c, total RNA was isolated from ΔΔgl-pQE::Δgl and ΔΔgl-pTrc::Δgl strains. The relative expression level of _STM3117_ was normalized to that from WT. For SPI-1 genes, total bacterial RNA was isolated from cultures grown under SPI-1-inducing medium for 3 h. For the expression levels of RNA repair genes, _katB_ and _spa_, and SPI-2 genes _savV_ and _savU_ in intramacrophage _S. Typhimurium_, RNA was isolated from intracellular bacteria within RAW264.7 cells (4 h p.i.) as described previously (Eriksson et al., 2003). Approximately, 2 × 10$^8$ bacteria ml$^{-1}$ were taken for resuspension in TRIzol (Sigma) followed by isolation of RNA as per the manufacturer’s protocol. Random hexamer primers were used to generate a cDNA pool from each RNA sample. Total DNase-treated RNA (1 µg) was reverse transcribed using BioScript Moloney murine leukemia virus reverse transcriptase (Biolime) according to the manufacturer’s protocol. qRT-PCRs were carried out using the Kapa SYBR Green RT-PCR kit (Kapa Biosystems). Specific primer pairs are listed in Table 2. They generated ~100–150 bp amplicons. Cycle threshold ($C_t$) was measured for each reaction and the expression fold change was measured by the 2$^{-ΔΔC_t}$ method. Relative expression levels were normalized to 16S rRNA levels.

**Eukaryotic cells and DNA transfection.** RAW264.7 and INT-407 cells were transiently transfected with pCMV::hTLR2-FLAG and pCMV::hTLR4 constructs (kind gift from Dr Markus Schnare, University of Marburg, Germany) using polyethyleneimine. Transfected cells were examined by confocal microscopy for expression of membrane FLAG tagged human TLR2 and 4. At 48 h post-transfection, the cells were infected with WT and ΔΔgl, and the monolayers were fixed at the indicated time points for analysis by confocal microscopy. Untransfected RAW264.7 and INT-407 cells were also tested for expression of TLR4 by immunofluorescence. RAW264.7 cells transiently transfected with a dominant-negative mutant construct of mouse TLR2 (kind gift from Professor K. N. Balaji, Indian Institute of Science, India) and 4 (kind gift from Dr Markus Schnare) were infected similarly for analysis by confocal microscopy.

**Measurement of phagosomal pH.** Analysis of the pH of _S. Typhimurium_ SCVs was performed according to a previous report (Wong et al., 2011). Bacteria were either dually labelled with a pH-sensitive probe (pHrodo, which emits red fluorescence in an acidic environment; Invitrogen) and a pH-insensitive probe Alexa Fluor 488 (green) or singly with pH-sensitive FITC as described previously (Bernardo et al., 2010). The calculated fluorescence ratios of pH-sensitive and pH-insensitive probes (in the dual-labeling process) and mean fluorescence intensity (in the single-labeling process) were used to determine phagosomal pH according to a calibration curve. Phagosomal pH was measured with a FACScanto II (BD Biosciences). pH calibration was performed by incubating infected RAW264.7 or INT-407 cells in 10 mM phosphate citrate buffer of predetermined pH (4.0–6.0) containing 20 µM nigericin for equilibration.

**RESULTS**

**STM3117-encoded lactoylglutathione lyase metabolizes endogenous methylglyoxal in _S. Typhimurium_**

In _S. Typhimurium_, the four coding regions from _STM3117_ to _STM3120_ constitute an operon, and encode lactoylglutathione lyase, monoamine oxidase, acetyl-CoA hydrolase and a LysR-type transcriptional regulator, respectively (Fig. 1a) (Haneda et al., 2009). Eriksson et al. (2003), based on the transcriptome analysis, had predicted that _STM3117_ promoted methylglyoxal neutralization during macrophage infection. Isogenic inactivation of _STM3117_ in _S. Typhimurium_ proved not to be lethal, alluding to the presence of either other detoxifying enzymes in the genome or sublethal concentrations of endogenous methylglyoxal under the conditions tested. Remarkably, ΔΔgl (deletion mutant of _STM3117_) was hypersensitive to growth inhibition by external methylglyoxal compared with WT (Fig. 1b). During growth in LB medium, ΔΔgl accumulated sublethal levels of endogenous methylglyoxal, 12 ± 0.3 nmol (g wet weight)$^{-1}$, compared with 0.9 ± 0.07 nmol (g wet weight)$^{-1}$ of methylglyoxal produced by WT, as quantified by the levels of 2-methylquinoxaline (derivatized product of methylglyoxal) in HPLC. The reduced ability to degrade methylglyoxal resulted in the inhibition of growth of the mutant in the exponential growth phase. Enzymic activity also demonstrated that _STM3117_ in the presence of the metal ion Co$^{2+}$ isomerized the methylglyoxal–GSH adduct hemithioacetal into S-D-lactoylglutathione (product) (Fig. 1c). The results validated the involvement of _STM3117_ in methylglyoxal detoxification.

**Expression of STM3117 results in differential replication of _S. Typhimurium_ in phagocytic versus non-phagocytic cells**

In order to investigate if _STM3117_ was essential for intracellular survival, we compared the proliferation of ΔΔgl with WT in macrophages and epithelial cells. In murine macrophage line RAW264.7, an increased proliferation of ~30-fold was observed for the mutant compared with ~15-fold for WT (Fig. 2a). Similar trends were observed in human macrophage line U937 and murine BMDMs (Fig. 2a). Whilst complementation (ΔΔgl-pQE::Δgl) restored the
WT phenotype, the overexpressing strain Δlgl-pTrc:lgl exhibited an extremely low proliferation compared with the other strains. qRT-PCR analysis of STM3117 expression confirmed that pTrc99c driven expression was ~15-fold higher than that from pQE60 (Fig. S1e, available in the online Supplementary Material). We hypothesized that the infection outcomes were affected by the critical level of STM3117 within bacteria, which when perturbed yielded differences in intracellular proliferation rates. Δlgl with empty pTrc99c control did not proliferate like Δlgl because LacI (Lac repressor) in pTrc99c reduces Salmonella virulence (Eswarappa et al., 2009). Therefore, the proliferation defect of the overexpression strain observed in our gentamicin protection assays was the consequence of both LacI and excess STM3117-mediated repression of proliferation.

In cultured epithelial cell lines such as INT-407 and HeLa, the mutant exhibited a defective proliferation (Fig. 2b) along with reduced numbers of intracellular bacteria at 2 h.p.i (Fig. S1a). The invasion assay on epithelial cells revealed limited internalization of Δlgl in INT-407 (~9%) and CaCo-2 cells (~24%) compared with WT, whose internalization capacity was considered 100% (Fig. 2c). As the mutant showed restricted entry, we sought to determine the SPI-1 status in Δlgl. SPI-1-encoded TTSS-1 spans the bacterial membrane and helps translocate effector molecules across the host plasma membrane, where they cause active actin rearrangement and invasion (Lara-Tejero & Galán, 2009; Lostroh & Lee, 2001). qRT-PCR analyses and β-galactosidase reporter assay did not reveal any appreciable difference in the expression of the SPI-1 genes between the mutant and the WT (Fig. S1b, c). However, there was a significant decrease in the level of secreted SipC in the culture supernatant of the mutant (Fig. 2d), which was also reflected in the amount of translocated SipC from Δlgl into the host cell during invasion (Fig. S1d). The results showed a reduced ability of invasion of the mutant bacteria due to an impaired translocation of SPI-1 effectors.

**Fig. 1.** (a) ORF map of the STM3117–STM3121 locus in S. Typhimurium. Arrows depict the direction of transcription. The rip operon of Yersinia pestis and the corresponding homologous region of SPI-6 of S. enteritidis are compared. Similar coloured arrows represent the corresponding sequence homologues across species. In Y. pestis the rip operon genes have been recently annotated as per their substrate specific enzymatic activities. (b) Growth rate of bacteria with/without methylglyoxal (0.2 mM). Growth rate was calculated by dividing the c.f.u. ml⁻¹ by the initial c.f.u. ml⁻¹ every hour. Columns represent mean ± SD of three independent experiments. ***P<0.001. (c) Activity of purified STM3117 enzyme of S. Typhimurium with (●) or without (●) divalent metal ions (Co²⁺). Initial rates were monitored by the increase in absorbance at 240 nm due to the conversion of hemithioacetal (HTA) to S-d-lactoylglutathione.
 Nonetheless, this indicated that the activity of STM3117 in intracellular Salmonella determines the extent of methylglyoxal detoxification, which additionally transpires to affect the proliferation rates within SCVs. The ability of the Δlgl mutant strain to replicate in the macrophages is in contrast to its replication defect in epithelial cells.

The capacity of Δlgl to colonize in vivo was also analysed. BALB/c and C57BL/6 mice were infected orally (Fig. 2e), and the former was also infected intraperitoneally (Fig. S1f). Compared with the WT-infected mice, which were moribund on day 4, the mutant-infected mice were active and showed minimal pathological symptoms of the disease. Both BALB/c and C57BL/6 mice showed ~75-fold minimized Δlgl burden in livers and spleens as compared with the WT. The mutant population manifested a systemic spread, albeit at a lower rate. Our results are in agreement with earlier reports on the inability of STM3117 (Santiviago et al., 2009) or STM3117–3120 (Haneda et al., 2009) mutants to survive in vivo. In fact, mice infected with such mutants show decreased morbidity and mortality (Haneda et al., 2009; Shah et al., 2005).

**Activity of kefB in response to the intracellular level of methylglyoxal–GSH adducts determines the proliferation rate of intramacrophagic S. Typhimurium**

Proliferation dynamics of Δlgl within macrophages was in disagreement with the reported attenuation of this mutant inside macrophages (Shi et al., 2006). We considered bacterial proliferation with respect to the SCV environment and SPI-2 activity, the two being key players in intracellular survival and growth, to resolve the ambiguity. First, we confirmed whether STM3117 detoxified methylglyoxal in intracellular S. Typhimurium.

In E. coli, the GSH conjugates of methylglyoxal, i.e. hemithioacetal and lactoylglutathione, induce potassium efflux channels (kefB and kefC) (Ferguson et al., 1995). The channels work by effluxing K⁺ ions, with a simultaneous influx of H⁺ into the bacterial cytosol, whenever bacteria accumulate methylglyoxal–GSH adducts. Enhanced expression of kefB in the intramacrophagic mutant population indicated that kefB was elicited due to accumulated methylglyoxal–GSH adducts. Enhanced toxicity of Δlgl in the intramacrophagic mutant population was due to the efflux of H⁺ ions (Fig. 3a). The accumulation of toxic adducts in intracellular Δlgl was further confirmed by the increased induction of DNA N-glycosylases (mutM, mutT and mutY repair enzymes) (Fig. 3b). DNA repair enzymes had been previously speculated to become activated in response to low cytosolic pH (due to H⁺ influx) to rescue the bacteria from methylglyoxal-mediated DNA damage (Ferguson et al., 1998). The question now was whether KefB antiported H⁺ ions from SCVs during the efflux of K⁺ ions. FACS analysis of the pH of MCVs revealed a significant increase in the mean fluorescence intensity of FITC (pH-sensitive probe) corresponding to pH 5.8 compared with WT-containing phagosomes (corresponding to pH 4.8) (Fig. 3c). FITC increases fluorescence upon an increase in pH. Dual labelling of bacteria with a pH-sensitive dye (pHrodo with fluorescence maxima at acidic pH) and a pH-insensitive dye (Alexa Fluor 488) followed by infection yielded an increase in the mean pHrodo fluorescence intensity for WT SCVs as seen in the overlaid FACS histogram in Fig. 3(d). This again indicated that the pH of MCVs was less acidic than the WT SCVs. Mutant bacteria rapidly antiported phagosomal H⁺ by KefB to activate the repair enzymes.

The pH of epithelial cell SCVs, however, did not differ much between the WT and the mutant bacteria (Fig. S2), which indicated two possibilities – either KefB did not actively antiport H⁺ from MCVs or insufficient mutant bacteria resided within the endosomal vacuoles to give a net pH downshift. Moreover, epithelial cell SCVs are less acidic than macrophage SCVs (Hautefort et al., 2008), which could also be a limiting factor for efficient KefB activity in the case of the mutant. To further validate that the increased proliferation of Δlgl in macrophages was mediated by KefB activity, we employed ΔkefB and Δlgl/ΔkefB mutant strains. The increased proliferation of Δlgl should be negated in a ΔkefB mutant background. In accordance, ΔkefB and the Δlgl/ΔkefB double mutant had the same proliferative index in macrophages as in the WT (Fig. 3e). On the whole, the results showed that methylglyoxal detoxification was vital to survival within macrophages, which when impaired perturbs the dynamics of SCV pH.

**Rate of SPI-2 translocon secretion but not changes in expression level, accounts for the increased proliferation of Δlgl in macrophages**

Within host cells, a low pH (4–5) of the phagosomal compartment is considered to be an important cue for SPI-2 induction (Rathman et al., 1996; Yu et al., 2010). We have shown previously that MCVs of macrophages exhibited a higher pH than those of the WT SCVs due to the import of H⁺ from MCVs. We then hypothesized that a low pH of the bacterial cytosol (pH$_{bact}$) could enhance SPI-2 activity over and above the activity occurring due to phagosomal acidification.

When V-ATPase was inhibited with BAF to abolish vacuolar acidification in both RAW264.7 and INT-407 cells, a significant reduction in the net growth of Δlgl was observed in macrophages (Fig. 4a). It indicated that an acidic phagosomal niche is a prerequisite for the mutant to proliferate.

We quantified SPI-2 translocon proteins SseB and SseD, which are filament and pore-forming components of TTSS-2, respectively, and are secreted across the host phagosomal membrane in response to acidification (Hölzer & Hensel, 2010). Expression of ssaP and ssaV (SPI-2 genes) did not increase appreciably in intracellular Δlgl, indicating that the mutant’s increased proliferation was not due to enhanced transcription of SPI-2 (Fig. 4b). Therefore, translocation of SPI-2 proteins was analysed to account for the enhanced
Fig. 2. Lactoylglutathione lyase contributes to intracellular proliferation and virulence. (a, b) Net intracellular replication of WT, Δlgl, complement strain (Δlgl-pQE::lgl) and overexpression strain (Δlgl-pTrc::lgl) from 2 to 16 h in RAW264.7 macrophages, U937 cells, murine BMDMs (a), and INT-407 and HeLa cells (b). In RAW264.7 cells the mutant with empty pTrc99c vector was kept as control to account for the virulence-lowering effect of the LacI repressor present in the vector. ΔssaV mutant was used as negative control. Each experiment was performed three times with each strain infecting the host cells in triplicate. The columns represent the mean ± SEM from three different experiments. One-way ANOVA with Dunnett’s post hoc analyses was used to compare the means relative to WT in intracellular proliferation assays. *P<0.05; **P<0.01; ***P<0.001; NS, non-significant. (c) Per cent internalization of the indicated strains relative to the initial inocula in CaCo-2 and INT-407 cells. Values shown are the mean ± SEM of three independent experiments. Student’s t-test was used to compare the means. (d)
Replication of Δlgl. Bacteria grown in F-medium (SCV-like medium) (Eswarappa et al., 2008) were processed for three different protein fractions: surface-attached, whole-cell lysate and whole-cell lysate without the surface-attached fraction. Δlgl showed an increased level of both SseB and SseD in the surface-attached protein fraction and whole-cell lysate (Figs 4c and S3), whereas the overexpression strain displayed a reduced amount, corresponding well with the earlier data on intracellular proliferation (Fig. 4c). However, in the whole-cell lysate fraction where the surface-attached proteins were removed prior to analysis, we found similar levels of the protein among the strains. This indicated that STM3117 did not regulate the expression but negatively influenced transport across the TTSS-2 needle apparatus. Immunofluorescent detection of SseB and SseD in intramacrophage Δlgl and WT (Fig. 4d) further confirmed that there was no impairment whatsoever in expression of SPI-2 proteins in the mutant. The increased pH of Δlgl-containing phagosomes in macrophages in response to the metabolic status of the methylglyoxal–GSH conjugate presumably caused a lowering of the bacterial pH, which enhanced SPI-2 translocon secretion in Δlgl. However, within epithelial cells, the mutant failed to show detectable levels of SseD p.i. (Fig. 4d) as was expected due to poor proliferation. Based on these observations we believe that, beyond a threshold, STM3117 concentrations negatively regulated translocation of SPI-2 proteins by some unknown mechanism, thereby disfavouring vacuolar replication of the overexpression strain. There is evidence of a positive regulatory effect of virulence factors on infection outcomes; nonetheless, there are cases where a pathogen needs to negatively regulate certain virulence factors to achieve steady-state dynamics within the host cells (Coombes et al., 2005).

**STM3117 promotes maturation of SCVs**

The proliferation defect of Δlgl in non-phagocytic cells cannot be due to poor SPI-2 activity alone since the impairment was observed at the downstream translocation stage and not at the expression stage (Fig. 4b, c). Also, in our studies with SPI-2 we observed a notable decrease in colocalisation of MCVs with the classical endocytic marker LAMP1 (Fig. 4d), which prompted us to believe that compromised vacuole maturation together with poor SPI-2 activity must be the cause of non-proliferative MCVs. As both EEA1 and LAMP1 are recruited sequentially onto the SCV membrane, and the latter is retained throughout the course of infection (Steele-Mortimer, 2008), we considered bacterial co-localization with EEA1 and LAMP1 to be representative of SCV maturation.

In RAW264.7 macrophages, both Δlgl and WT showed similar co-localization with LAMP1 at 2 and 6 h (~70% co-localization) (Fig. 5a) and ~80% of the mutant bacteria co-localized with LAMP1+ SCVs, similar to the WT (Fig. 5b). Co-localization of the non-LAMP1+ bacteria with ubiquitinated aggregates early in infection (1 h p.i) confirmed their cytosolic nature (Fig. S4a). The cytosolic population of *S. Typhimurium* is targeted by the ubiquitinated proteins which are further degraded by proteasome (Perrin et al., 2004).

In INT-407 cells, however, Δlgl exhibited significantly decreased co-localization of 19, 30 and 42% with LAMP1 at 2, 6 and 12 h p.i., respectively (Fig. 5c). The amount of LAMP1 recruitment did not seem to increase even at late time points p.i. (12 h). Enumeration of the percentage of SCV-localized bacteria (LAMP1+) revealed 40–50% Δlgl in vacuoles with the rest in the cytosol (Fig. 5d). Co-localization of EEA1 was 20% with Δlgl compared with 80% with WT (Fig. 5e). Even at 1.5 h p.i., only a limited number of mutant bacteria had co-localized with EEA1.

To further determine if some of the mutant population escaped/quit MCVs to enter the cytosol and were cleared thereafter, we incorporated chloroquine (50 μg ml⁻¹) in the gentamicin protection assay. The protonated form of chloroquine has the potential to kill vacuolar bacteria without any apparent effect on the cytosolic population (Marathe et al., 2012). As shown in Fig. S4(b, c), the mutant population was predominantly vacuolar throughout infection in macrophages, whilst it was largely cytosolic at late time points within epithelial cells. This further indicated the poor maturation of MCVs within epithelial cells.

As epithelial cell cytosol is more permissive to bacterial replication than the macrophage, the former provides an ideal niche for *S. Typhimurium* to divide efficiently (Brumell et al., 2002). However, the proliferation defect of the mutant within epithelial cells could not be due solely to immature MCVs, but due to targeting of these MCVs by the host defence system. Epithelial cells of various origin are known to express defensins such as HBD1, HBD3, HBD4 and HD5, which are potent AMPs and are known to kill pathogens by permeabilizing their cell membrane (Eswarappa et al., 2008; Ganz, 2003). Immunofluorescence results showed a uniform distribution of HD5 in *S. Typhimurium*-infected INT-407 cells, and there was a visible increment in the co-localization of HD5 with the...
Fig. 3. Impaired intracellular methylglyoxal detoxification in macrophages increases the pH of Δgl-containing SCVs more than that of WT SCVs. (a) qRT-PCR analysis of kefB in the indicated strains isolated from RAW264.7 macrophages at 4 h p.i. (b) Expression levels of mutM, mutT and mutY in intracellular Δgl relative to that in WT (isolated from RAW264.7) cells at 5 h p.i. The plot is representative of three independent experiments performed in duplicates. Bar, ±SD. (c) Representative histograms of FITC fluorescence as per the phagosomal pH either with infection (FITC labelled WT and Δgl) alone or infection followed by equilibration with known pH buffers (4, 4.6, 5, 6) and nigericin. FITC (pH sensitive dye) labelled bacteria were allowed to infect RAW264.7 cells for 5 hr before harvesting samples for flow-cytometry. Equilibration was done for 30 min just prior to flowcytometric analysis. WT maintained a phagosomal pH ~4.8, whereas phagosomes of the Δgl strain were less acidified and reached pH ~5.7. (d) Overlaid FACS histograms of pHrodo fluorescence intensity of the WT and Δgl. pHrodo fluoresces at a maximum at acidified pH. Phagosomes containing WT show an increased fluorescence, indicating a relatively lower phagosomal pH than those of the Δgl. Results of one independent experiment out of three is shown. (e) Net intracellular replication of WT, Δgl, ΔkefB and Δgl/ΔkefB from 2 to 16 h in RAW264.7 macrophages. The columns represent the mean ±SEM from three different experiments. One-way ANOVA with Dunnett's post hoc analyses was used to compare the means relative to WT in intracellular proliferation assays. ***P<0.001; NS, non-significant.
Fig. 4. SPI-2 translocon secretion increases in intramacrophage Δgl. (a) RAW264.7 and INT-407 cells pretreated with BAF (50 nM) were infected with the indicated strain and the fold proliferation from 2 to 16 h was calculated. Graphs are representative of three independent experiments with similar results. Student’s t-test was used to compare the means. **P<0.01; ***P<0.001. (b) qRT-PCR analysis of SPI-2 genes ssaP and ssaV in the indicated strains isolated from RAW264.7 macrophages at 4 h p.i. (c) Immunoblot analysis of SPI-2 translocon SseB from WT, Δgl, complement strain (Comp; Δgl-pQE::lgI) and overexpression strain (Ov-ex; Δgl-pTrc::lgI) in whole-cell lysate (W), surface-attached fraction (SA) and cell lysate without the surface-attached fraction (W-SA). Strains were grown for 7 h in SPI-2-inducing medium (F-medium). (d) Single confocal sections of WT- and Δgl (mCherry, red)-infected RAW264.7 and INT-407 cells, immunolabelled for SseB (green, arrowheads) and LAMP1 (blue, false coloured) at the indicated time points p.i. Δgl inside INT-407 cells showed an absence of detectable levels of SseB and were frequently found not to co-localize with LAMP1 at both time points checked (diamond arrowheads). Bars, 5 μm.
Δgl strain at 3 and 8 h p.i. (Fig. 5f). There was a significant increase in the sensitivity of the mutant to AMPs such as protamine and HBD1 during *in vitro* growth (Fig. S4d). AMP-treated Δgl was hypersensitive towards the treatment compared with the similarly treated WT, indicated by the increased incorporation of the membrane-potential-sensitive...
Fig. 5. The Δgl population within epithelial cells exhibits defective SCV maturation and becomes susceptible to AMPs. (a–d) Single confocal sections of WT- and Δgl-infected RAW264.7 (a) and INT-407 (c) cells at the indicated time points p.i. Bacteria were immunolabelled against Salmonella O-antigen and endosome marker LAMP1. Values represent the mean ± SEM of per cent co-localization from three independent experiments each involving the analysis of 20 microscopic fields with 10–12 infected cells for each time point. Bar, 5 µm. Percentage of bacteria (WT, Δgl and complement) (n = 200) showing either >20 (vacuolar) or <20 % (cytosolic) association with LAMP1 inside RAW264.7. ***P<0.001. (b) and INT-407 (d) cells at 2 and 8 h p.i. Data are represented as mean ± SEM of percentage of bacteria. NS, non-significant. (e) Single confocal sections of WT- and Δgl-infected INT-407 cells at the indicated time points post-uptake, immunolabelled for EEA1 (red). Bars, 5 µm (30 min); 5 µm (90 min). Quantification of the per cent co-localization of bacteria with EEA1 is shown alongside the images, with values representing the mean ± SEM of three independent experiments each involving the analysis of 50 infected cells for each time point. ***P<0.001. (f) Co-localization of the indicated strains with HD5 in INT-407 cells at two time points p.i. Mutant bacteria exhibit increased susceptibility to HD5. Bars, 5 µm.
dye DiBAC$_4$(3) (Fig. S4e). The anionic dye is known to readily cross permeabilized depolarized membranes and fluoresce. Thus, the amount of the membrane-potential-sensitive dye taken up by the AMP-treated cells would correlate directly with the loss of membrane integrity and depolarization (Nuding et al., 2006). Altogether, the results show that within epithelial cells the mutant escapes from MCVs very early in infection and the fraction of Δlgl which manages to stay inside MCVs is subsequently targeted by AMPs, contributing to their decreased net growth.

**Expression of TLR2 and 4 partially restores the growth of the STM3117 mutant inside epithelial cells**

Recently, Denise Monack’s group has revealed the importance of TLR signalling in recognition of intracellular S. Typhimurium (Arpaia et al., 2011). They have shown that the presence of TLR2, 4 and 9 in mouse BMDCs is necessary for the establishment of an acidic phagosomal compartment and induction of SPI-2 genes in Salmonella. Moreover, epithelial SCVs are less acidic than macrophage SCVs. As primary intestinal epithelial cells are known to negligibly express TLR2 and 4 on their membranes, but constitutively express TLR3 and 5 (Cario & Podolsky, 2000; Hornef et al., 2002), we used TLR-mediated signalling in epithelial cells to mimic the phagosomal environment of macrophages. We showed a lower expression of TLR4 in INT-407 cells compared with that in macrophages (Fig. 6a), and hence used FLAG-tagged human TLRs (TLR2 4) for transient transfections of INT-407 cells (Fig. S5a). Expression of either TLR did not suffice in restoring vacuolar localization of the mutant (Fig. S5b). However, INT-407 cells expressing both human TLR2 and 4 exhibited a significant increase in SCV localization of the mutant by 8 h p.i (Fig. 6b, c). Also, the proliferation of Δlgl increased significantly in transfected cells relative to that in the vector control (Fig. 6d). Transfected epithelial cells pretreated with BAF showed an intermediate proliferation rate in between that of inhibitor alone and transfection alone, further validating the role of TLR signalling in enhancing endosomal acidification in otherwise lower acidic endosomes of epithelial cells and proliferation thereof.

Conversely, RAW264.7 cells transfected with the dominant-negative mutant forms of mouse TLR2 and 4 resulted in ~40% of both WT and Δlgl in the cytosol (Fig. 5e, f). Dominant-negative forms of mouse TLRs impaired the optimal phagosomal acidification which would have served as a H$^+$ source for the mutant during methylglyoxal detoxification. These results demonstrated the interplay between SPI-2 translocon secretion in intracellular S. Typhimurium, induced by H$^+$ quenching from the surrounding acidified phagosomes.

**DISCUSSION**

Methylglyoxal, a toxic byproduct of many physiological reactions (Chauhan & Madhubala, 2009; Kim et al., 2012; Korithoski et al., 2007; MacLean et al., 1998; Yadav et al., 2005), arises primarily through various metabolic pathways, e.g. increased glycolytic flux of triose phosphates, threonine/glycine catabolism and acetone breakdown (Ferguson et al., 1998). However, regardless of the pathway of methylglyoxal formation, the detoxification is exclusively dependent on cellular glyoxalases (Ferguson et al., 1995; Korithoski et al., 2007; Ozymak et al., 2010). The presence of a lactoylglutathione lyase (a putative glyoxalase I) gene in S. enterica serovar Typhimurium, Enteritidis, Gallinarum and other non-typhoidal serovars, but absence in typhoidal serovars might be indicative of host-specific acquisition of virulence determinants and metabolic requirements (Charles et al., 2009; Pujol et al., 2005; Rathman et al., 1996). The non-phylogenetic distribution of the homologues of coding regions downstream of STM3117, i.e. STM3118–3121, in other species such as Pseudomonas aeruginosa, Coxiella burnetti, Mycoplasma synoviae and Yersinia pestis, indicate their acquisition through horizontal transfer. For instance, in Y. pestis, the rip operon genes (ripA, putative CoA transferase; ripB, putative monoamine oxidase; Y2383, putative citrate lyase β chain; Y2382, transcriptional regulator), which are homologues of the STM3118–3121 cluster, are involved in reducing nitric oxide levels within macrophages in order to persist in activated macrophages (Pujol et al., 2005). Recently, the rip operon genes, (Y2383–2385; Y2383 being annotated as ripC recently) have been shown to be involved in a step-by-step degradation of itaconate – an antimicrobial macropage factor (Saskaran et al., 2014). However, the corresponding homologue sequence of STM3117 does not exist in these pathogens.

Why an additional methylglyoxal degradation gene is present in non-typhoidal Salmonella serovars is something which is difficult to analyse at present based on the available literature and our own findings. However, we do see a decrease in intracellular proliferation of S. Typhi expressing STM3117 compared with WT S. Typhi in human macrophages and epithelial cells (data not shown), revealing that STM3117 apparently does not provide any proliferative advantage to S. Typhi. In S. Typhimurium, however, STM3117 had a profound effect on the intracellular proliferation that was mediated by the activity of KefB and SPI-2. SPI-2-encoded TTSS-2 predominantly modulates intracellular proliferation in S. Typhimurium (Brown et al., 2005; Dandekar et al., 2012; Hölzer & Hensel, 2010; Jantsch et al., 2011). The apparatus translocates proteins that mediate SCV maturation by positioning the SCV at the juxtanuclear site, making it a proliferation-productive niche (Steele-Mortimer, 2008). Whilst secretion of SPI-2 translocon components such as SseB and SseD was greatly enhanced in Δlgl, there was no difference in the expression of SPI-2 needle apparatus genes such as ssaV and ssaP (Fig. 3b) between WT and the mutant. SPI-2 activity was demonstrated to be modulated by the intracellular level of STM3117 by using low (Δlgl-pQE60: lgl) and high (Δlgl-pTrc: lgl) expression strains of STM3117 (Fig. 3).
The exact cues for SPI-2 translocon secretion are still debated, and there exists a complex coordination between bacterial virulence factors and the host microenvironment which together decide the fate of SPI-2 function and proliferation (Brown et al., 2005; Coombes et al., 2005; Dieye et al., 2009). Bacteria grown in acidic media show...
effective secretion of SPI-2 effectors and translocon components in a SsrB-dependent manner (Coombes et al., 2004). We predicted that an acidified bacterial cytosol could boost the SPI-2 translocon secretion further than the extent achieved by sensing of acidic phagosomes (Fig. 7). The hypothesis was based on the fact that cytoplasmic acidification in E. coli (Ferguson & Booth, 1998; Ferguson et al., 1995) happens in response to metabolite detoxification. The \( \text{H}^+ \)-mediated pH drop essentially gives bacteria a short time to adapt and activate the repair system in response to methylglyoxal toxicity (Ferguson et al., 1998), which was validated in our case by the induction of DNA repair enzymes (\( \text{mutM, mutT, and mutY} \)) and the \( \text{K}^+ \) efflux pump \( \text{kefB} \) in intracellular \( \Delta\text{lgl} \) (Figs 3a, b and 7). Therefore, the enhanced secretion of SPI-2 translocon components in the mutant could be explained by considering \( \text{kefB} \) mediated lowering of \( \text{pH}_{\text{Bact}} \) (Fig. 4). This fact was further substantiated by the presence of mildly acidic MCVs in macrophages (~pH 5.7) (Fig. 3c). The mutant bacteria of MCVs actively import \( \text{H}^+ \) ions (through \( \text{kefB} \) activity) from the phagosomes in response to the toxicity of the methylglyoxal-GSH adducts. The role of \( \text{kefB} \) in mediating this event was also confirmed by using \( \Delta\text{kefB} \) and \( \Delta\text{lgl}\Delta\text{kefB} \) double-knockout strains, both of which behaved similar to the WT (Fig. 2e).

TTSS-2 is located in the bacterial membrane in such a way that the regulatory complex of SsaL/SsaM/SpiC interacts with the basal body of the needle apparatus and responds to subtle changes in the host cytosolic pH (pH$_{\text{Cyt}}$) through the action of an unknown pH sensor (Yu et al., 2010).
low pH$_{\text{Cyt}}$ (pH-5) has been suggested to strongly induce translocon secretion but suppress effector delivery while a near neutral pH is shown to trigger effector delivery. Yu et al. (2010) reported that a certain bacterial pH sensor could be sensing these changes and regulating the activity of SsaL/SsaM/SpiC complex, where in, dissociation of the complex happens at a near neutral pH$_{\text{Cyt}}$ followed by effector delivery. What has not been discussed is whether this unknown bacterial pH sensor also senses host phagosomal pH (pH$_{\text{Phag}}$) and bacterial cytosolic pH (pH$_{\text{Bact}}$). This question arises based on the fact that the TTSS-2 regulatory complex at the cytoplasmic side of the needle apparatus is directly under the influence of bacterial cytosolic pH. It is plausible therefore, that the intramacrophagic mutant bacteria with more acidic pH$_{\text{Bact}}$ first strongly induces translocon secretion and as the pH$_{\text{Phag}}$ increases (at 5 hr p.i. pH 5.7 vs pH 4.8 for WT) the regulatory complex triggers robust effector secretion by dissociating from the TTSS-2. Thus the interplay of pH$_{\text{Bact}}$, pH$_{\text{Phag}}$ and pH$_{\text{Cyt}}$ together determines the fate of SPI-2 activity. 

Epithelial SCVs require additional TLR-mediated signalling to achieve macrophage SCV-like characteristics. It is known that macrophage SCVs are more acidic in nature than epithelial SCVs (Hautefort et al., 2008). For instance, TLR2-, 4- and 9-mediated acidification of phagosomes is required for SPI-2 induction in S. Typhimurium (Arpaia et al., 2011). Interestingly, epithelial cells transfected with both human TLR2 and 4 partially rescued the intracellular proliferation of the mutant (Fig. 5b, d) by preventing their escape from the acidified SCVs. Insufficiently acidified SCVs of naive epithelial cells limited the extent of SPI-2 translocon secretion in ΔalgI, which translated to a defective proliferation. However, post-transfection with TLRs, the proportion of vacuolar ΔalgI and their proliferation increased. Conversely, RAW264.7 cells expressing dominant-inhibitory forms of mouse TLR2 and 4 resulted in ~30% of both WT and ΔalgI outside phagosomes, reinforcing the importance of phagosomal acidification in the initial stages of infection (Fig. 5e, f). These findings further strengthen the fact that pH$_{\text{Cyt}}$, pH$_{\text{Phag}}$ and pH$_{\text{Bact}}$ together influence the intracellular Salmonella proliferation and infection outcome.

Acid resistance genes become upregulated up to 10-fold in S. flexneri in response to low cytosolic pH of U937 cells compared with only twofold in HeLa cells (Lucchini et al., 2005). Such observations further reiterate the influence of the immediate environment (vacuole or cytosol) of a particular pathogen on the expression of virulence genes. Similarly, in S. Typhimurium, the genes from STM3117 to STM3120 are also highly expressed in macrophages (Eriksson et al., 2003), but are downregulated in HeLa cells (Hautefort et al., 2008), which certainly does not exclude their requirement in intraepithelial proliferation. Contrary to an existing report suggesting STM3117’s role in peptidoglycan synthesis, we found that the enzyme has glyoxalase I activity and is involved in methylglyoxal detoxification. Moreover, the STM3117 mutant did not reveal any defect in cell wall integrity or sensitivity to certain detergents (data not shown), further supporting the notion that the encoded enzyme is not involved in peptidoglycan synthesis pathway.

One interesting observation from this study was the apparent difference in the mutant’s behaviour in vitro and in vivo (Fig. 1a, e). Although our observations on mouse infection were in full agreement with previous work showing the inability of either STM3117 or STM3117–3120 mutants to cause infection or survive in vivo, the results on macrophage infection differed widely. As the STM3117 mutant maintained a non-proliferative phenotype in epithelial cells as well as had low internalization efficiency (Fig. 1b, c), this could account for one of the reasons behind failure of the mutant to gain entry through the intestinal epithelia. However, due to the mutant’s ability to replicate successfully within macrophages, we observed a systemic dissemination of the mutant in the livers and spleens of mice because CD18$^+$ phagocytes are the major carriers of Salmonella in vivo (Cano et al., 2001). The net outcome of the infection, however, was not detrimental to the host as the initial number of mutants translocating the gut epithelia itself was low. Further investigation is required to understand the intramacrophage mutant population in vivo and its contribution to the observed systemic spread.

Our results unraveled an important role of lactoylglutathione lyase in S. Typhimurium infection, and established the impact of metabolite detoxification on vacuole maturation dynamics. Our findings showed how the events of metabolite detoxification influence the acidification status of SCVs and vice versa, which ultimately translates to the infection outcome. Determining the subtleties of acidification and pH regulation in phagosomes and cytosol will promote studies on other intracellular pathogens which are known to modulate their virulence factors to combat the pH-based differences in their micro-environment.

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