INTRODUCTION

*Salmonella* is a facultative intracellular pathogen that resides inside specialized compartments called *Salmonella*-containing vacuoles (SCV) of host phagocytic and non-phagocytic cells (Haraga et al., 2008). As a response to infection, macrophages produce reactive oxygen species (ROS) by the phagocyte NADPH oxidase (Phox) to kill engulfed bacteria (Vazquez-Torres et al., 2000). Patients with chronic granulomatous disease lack a functional Phox and cannot produce an oxidative burst. This defect is associated with an increased susceptibility to extraintestinal *Salmonella* infections (Winkelstein et al., 2000) implicating the importance of ROS in control of salmonellosis. Likewise, Phox-deficient murine macrophages are highly susceptible to *Salmonella* infections (Vazquez-Torres et al., 2000).

Bacteria detoxify ROS using superoxide dismutases, catalases and peroxiredoxin-type peroxidases (peroxiredoxins) (Aussel et al., 2011; Fang et al., 1999; Hébrard et al., 2009; Horst et al., 2010; Kim et al., 2010; Krishnakumar et al., 2004; Uzzau et al., 2002). Besides detoxification, repair of damage caused by ROS is crucial. The amino acid methionine is highly susceptible to oxidation due to its easily oxidizable sulfur residue (Moskovitz, 2005). Oxidation affects both free and protein-bound methionine, and generates two epimers; methionine-(S)-sulfoxide and methionine-(R)-sulfoxide (Weissbach et al., 2002). Most organisms possess methionine sulfoxide reductases (Msr), an evolutionally conserved specific repair system for the regeneration of oxidized methionine (Moskovitz, 2005). Recently we found that the Msr system of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) is composed of three proteins. MsrA is specific for the (S)-epimer, and repairs free and protein-bound methionine-(S)-sulfoxide. MsrB and MsrC are specific for the (R)-epimer. MsrB reduces protein-bound methionine-(R)-sulfoxide while MsrC is responsible for the repair of free methionine-(R)-sulfoxide (Denkel et al., 2011). MsrA and MsrC were shown to mediate resistance of *S. Typhimurium* exposed to H$_2$O$_2$ *in vitro* and were essential for replication in IFN-γ activated macrophages. MsrA alone and MsrC together with MsrB are essential for growth of *S. Typhimurium* in mice.

In *Escherichia coli*, besides MsrA a biotin sulfoxide reductase, encoded by *bisC*, reduces free methionine-(S)-sulfoxide (Ezraty et al., 2005). Initially, the *E. coli* and...
Rhodobacter sphaeroides: BisC was shown to reduce biotin sulfoxide to biotin (del Campillo-Campbell & Campbell, 1982; Pollock & Barber, 1997). Biotin is an essential cofactor for carboxylases involved in central metabolic pathways of eukaryotes and prokaryotes (Streit & Entcheva, 2003). It also plays a significant role in epigenetic gene regulation, cell signalling and chromatin structure (Zempleni, 2005). Oxidation of biotin preferentially generates biotin-sulfoxide, and to a lesser degree biotin-l-sulfoxide. Both forms are biologically inactive (Melville, 1954; Melville et al., 1954; Nielsen et al., 1942). Thus reduction of oxidized biotin is required to use biotin sulfoxide as a biotin source (Dykhuizen, 1973).

The de novo biotin biosynthesis pathway has been shown to be essential for pathogenesis of intracellular bacteria such as Mycobacterium tuberculosis, Francisella tularensis and S. Typhimurium (Dey et al., 2010; Napier et al., 2012; Shi et al., 2009; Woong Park et al., 2011). In this study, we examined two functions of this enzyme, the methionine sulfoxide reductase and biotin sulfoxide reductase activity. We tested the possible role of BisC in the oxidative stress response and virulence of S. Typhimurium. We dissected two functions of this enzyme, the methionine sulfoxide reductase and biotin sulfoxide reductase activity. We examined a AbisC mutant of S. Typhimurium exposed to H2O2 in vitro, in murine macrophages and during infection in mice. Our data showed that after exposure to H2O2 in vitro, the methionine sulfoxide reductase activity of BisC sustained the supply with free methionine for de novo protein biosynthesis, which was required for bacterial survival during oxidative stress. In IFN-γ activated macrophages and in mice, however, it was the biotin sulfoxide reductase activity that became essential to mediate bacterial replication. Thus BisC is essential for virulence in S. Typhimurium. The fact that BisC is not present in mammals (Nielsen et al., 1942) makes it a potential drug target for the treatment of Salmonella infections.

METHODS

Ethics statement. The mouse experiments were approved by the animal welfare committee of Hannover Medical School and the Niedersächsische Landesamt für Verbraucherschutz und Lebensmittelsicherheit Oldenburg. All mouse experiments were performed in accordance with both institutional and national guidelines (ethical permit 12/0697).

Bacterial strains and plasmids. The bacterial strains used in this study are given in Table 1. S. Typhimurium ATCC 14028 was used as the wild-type strain and all mutants were generated in this genetic background. Bacteria were grown routinely overnight in Luria-Bertani (LB) broth with shaking or in a roller drum at 37°C. Antibiotics were added if necessary (ampicillin 100 μg ml⁻¹, chloramphenicol 10 μg ml⁻¹, kanamycin 25 μg ml⁻¹).

Construction of deletion mutants in S. Typhimurium 14028 and complementation. The database colibase (S. enterica subspecies enterica serovar Typhimurium strain LT2) served as reference database. Deletion mutants of bisC (STM3644), bioB (STM0794) and leuBCD (STM0112, STM0111, STM0110) were generated using the one-step inactivation via homologous recombination (Datsenko & Wanner, 2000) as described recently (Denkel et al., 2011). Primers and plasmids are listed in Tables 2 and 3.

If necessary, antibiotic resistance genes were eliminated by the helper plasmid pCP20 (Table 3) carrying a FLP recombinase (Datsenko & Wanner, 2000). pCP20 was transformed into mutants containing FRT-flanked antibiotic resistance cassettes. Ampicillin-resistant transformants were selected at 30°C and the FLP recombinase removed antibiotic resistance cassettes flanked by FRT sequences. The temperature sensitive pCP20 could possibly be disposed by cultivation of bacteria at 42°C.

All multiple deletion mutants (ΔametCΔmsrA, ΔmetCΔbisC, ΔametCΔmsrAΔbisC, ΔbioBAΔmsrA, ΔbioBAΔbisC, ΔbioBAΔbisCΔmsrA) were generated by transduction with the bacteriophage P22int (Schmieg, 1972) that transfers mutations between different strains.

To construct the complemented strain of the ΔbisC mutant the ORFs of wild-type bisC from S. Typhimurium 14028 including upstream regions between 250 bp and 400 bp were amplified by PCR using Phusion™ High Fidelity DNA Polymerase (NEB). Primers (Table 2) included restriction sites for subcloning the PCR product into the vector pBAD30 (Guzman et al., 1995). All genes were under control of their own promoter. The complementation plasmid pBAD30 carrying the bisC gene was subsequently transformed into the ΔbisC mutant.

Culture of Salmonella on methionine and methionine sulfoxide. Overnight cultures of S. Typhimurium in LB were harvested by centrifugation, washed three times in M9 minimal medium, and inoculated in M9 minimal medium at OD600 0.05. Defined concentrations (5, 10 or 20 μg ml⁻¹) of methionine, methionine-(S)-sulfoxide or methionine-(R)-sulfoxide were added as the methionine source. Purity of methionine-(S)-sulfoxide (85% ± 5%) and methionine-(R)-sulfoxide (92.5 ± 2.5%) was determined by 1H-NMR analysis according to the declaration of the manufacturer. Bacteria were incubated aerobically with shaking (37°C, 130 r.p.m.). Growth was measured by OD600 after 24 h of cultivation. Optical density was detected using the cell density meter (WPA Biowave CO 8000) and cuvettes of 1 cm path length. Samples were diluted 1:10 in 1 ml M9 minimal medium. Experiments were performed three times, each in duplicate.

Culture of Salmonella on biotin and biotin sulfoxide. S. Typhimurium strains were grown in LB overnight. Bacteria were harvested by centrifugation, washed three times in M9 minimal medium and inoculated in M9 minimal medium at OD600 0.05.
Table 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>bisC_For</td>
<td>CTC CCT GCA AAC CGT TGT GCA TGA CCA GTG TCA GAC GCG GTA GGC TGG AGC TGC TTC</td>
<td>Deletion of bisC</td>
</tr>
<tr>
<td>bisC_Rev</td>
<td>CGC CTG CGGTAG GTT CAG CGG GCC ATG CGC CTT CGT GAA TAC</td>
<td>Deletion of bisC</td>
</tr>
<tr>
<td>bioB_For</td>
<td>GCC TGC CCG GAG GAT TGT AAA TAC TGC CCG CAG AGC TCG GGC TAT</td>
<td>Deletion of bioB</td>
</tr>
<tr>
<td>bioB_Rev</td>
<td>GTT CGC CCC GGC CAT AAA GCA CAT CGC CTG GTT CTC GTT CAT</td>
<td>Deletion of bioB</td>
</tr>
<tr>
<td>leuBCD_For</td>
<td>GCC CTG AAA GTT ATG GAT GCG GTA CGC AGC GCT TTT GAT AGT CGT</td>
<td>Deletion of leuBCD</td>
</tr>
<tr>
<td>leuBCD_Rev</td>
<td>GCG GCG GAA GTC GTC GTC GAT TTT AAA GCT GTA GGT TTT ATC GCC TGC</td>
<td>Deletion of leuBCD</td>
</tr>
<tr>
<td>bisC1_HindIII</td>
<td>ATATAT AAG CTT GCA GTT TCG CTT CCT GTA CG</td>
<td>Complementation with bisC</td>
</tr>
<tr>
<td>bisC2_HindIII</td>
<td>ATATAT AAG CTT GCC TGG ATG AGA TCC CTA CC</td>
<td>Complementation with bisC</td>
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Defined concentrations (10^{-3} \text{ g ml}^{-1}) of biotin (Sigma) or biotin sulfoxide (custom made by HWI ANALYTIK) were added as the biotin source. Purity of biotin sulfoxide was determined by liquid chromatography and equates to 95.21% biotin-d-sulfoxide and 3.37% biotin-l-sulfoxide according to the declaration of the manufacturer. Bacteria were incubated aerobically with shaking (37 °C, 130 r.p.m.). Growth was measured by OD_{600} after 24 h of cultivation. Samples were diluted 1:10 in 1 ml M9 minimal medium. Experiments were performed three times, each in duplicate.

Susceptibility of S. Typhimurium to oxidative stress in vitro. S. Typhimurium from overnight LB agar plate culture was suspended in starvation medium (56.51 mM Na_{2}HPO_{4}, 22.0 mM KH_{2}PO_{4}, 8.56 mM NaCl, 18.70 mM NH_{4}Cl, 2 mM MgSO_{4} and 0.1 mM CaCl_{2}) that resembles M9 medium without any carbon, amino acid or biotin source. Bacteria were cultured overnight at 37 °C with shaking. These cultures of S. Typhimurium were diluted with M9 starvation medium to an OD_{600} of 0.02, and 100 µl of the bacterial suspension was mixed with 100 µl 4 mM H_{2}O_{2} solution (Sigma). Therefore the final concentration of H_{2}O_{2} was 2 mM in a bacterial suspension of OD_{600} 0.01. For the non-stress control, bacterial cultures were treated with 100 µl M9 starvation medium instead of H_{2}O_{2}. Chloramphenicol was used in the designated experiment (Fig. 3d) at a concentration of 10 µg ml^{-1}. The assay was carried out in 96-well plates at 37 °C without shaking. Survival of bacteria was examined by plating for colony counts on LB agar after 3, 6 and 9 h of exposure. Experiments were done in duplicate and repeated at least twice.

Infection of the macrophage cell line RAW 264.7. RAW 264.7 macrophages (ECACC) were grown in RPMI (Gibco) supplemented with 10% heat inactivated FCS (Sigma), 4 mM glutamine (Gibco) and 20 mM HEPES (Gibco). A gentamicin protection assay was performed as previously described (Chakravortty et al., 2002). Briefly, cells were seeded at a density of 5 × 10^4 cells per well in 24-well tissue culture plates. S. Typhimurium cultures were grown overnight in M9 minimal medium and diluted to OD_{600} 0.2. 1.5 ml of the culture was harvested by centrifugation, washed twice with PBS and resuspended in 1 ml RPMI without any supplements. The bacterial suspension was further diluted 1:100 in RPMI and 100 µl was used for infection of RAW 264.7 cells in 900 µl (m.o.i. of 10:1) RPMI medium without any supplements. Plates were centrifuged at 220 g for 5 min and uptake of Salmonella was allowed during incubation at 37 °C and 5% CO_{2} for 30 min. Extracellular bacteria were removed by washing three times with PBS, followed by a 60 min incubation period with RPMI containing 100 µg ml^{-1} gentamicin. Subsequently medium was replaced by RPMI with 10 µg ml^{-1} gentamicin for the rest of the experiment. The bacterial load in the macrophages was assessed 2 h and 16 h post-infection (p.i.). Cells were again washed three times with PBS and lysed with 1 ml sterile bidistilled water per well. Bacteria were plated on LB agar for colony counts. To calculate bacterial growth, colony counts 16 h post-infection was divided by colony counts 2 h post-infection. In the case of activated RAW 264.7 macrophages, cells were stimulated with murine IFN-γ (Sigma) 18–24 h prior to infection. All experiments were performed in triplicate.

Competitive infection of mice with S. Typhimurium. The fitness of mutant strains was calculated by competition experiments in mice (BALB/c or 129S2/SvPasCrl, purchased from Charles River) comparing in vivo replication of the tested mutant strain and the wild-type. As described recently (Denkel et al., 2011) Salmonella were taken

Table 3. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Selection marker</th>
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<tr>
<td>pKD3</td>
<td>Chloramphenicol, ampicillin</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>pKD4</td>
<td>Kanamycin, ampicillin</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>pKD46</td>
<td>Ampicillin</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>pCP20</td>
<td>Chloramphenicol, ampicillin</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>pBAD30</td>
<td>Ampicillin</td>
<td>Guzman et al. (1995)</td>
</tr>
<tr>
<td>pBAD30::msrA</td>
<td>Ampicillin</td>
<td>Denkel et al. (2011)</td>
</tr>
<tr>
<td>pBAD30::bisC</td>
<td>Ampicillin</td>
<td>This study</td>
</tr>
</tbody>
</table>
from an overnight LB agar plate, resuspended in PBS and the number of cells was adjusted to 5 × 10^3 bacteria for each strain. Groups of five female BALB/c or 129S2/SvPasCrl mice (age 6–8 weeks) were infected intraperitoneally with a 1:1 mixture of two strains containing a total of 1 × 10^8 bacteria. Mice were killed on day 1 and day 3 p.i., livers and spleens were removed and homogenized in PBS. Bacteria were plated for colony counts on LB agar and on LB agar containing the appropriate antibiotic (chloramphenicol for ΔbisC, kanamycin for ΔbioB, chloramphenicol and kanamycin for ΔbioBΔbisC) to distinguish between wild-type and mutant. Competitive indexes (CIs) were calculated as described earlier (Beuzón & Holden, 2001). Briefly, the CI measures the attenuation of the tested mutant strain and is calculated as the ratio of the bacterial number between the mutant and the wild-type at day 1 or 3 divided by the ratio of the bacterial number between mutant and wild-type at day 0.

**Statistical analysis.** The t-test was applied for statistical analyses and P-values <0.05 were considered to be significant. Significance of the CIs was calculated using the Wilcoxon signed rank test.

## RESULTS

### MsrA and BisC of *S. Typhimurium* reduce free methionine-(S)-sulfoxide

In *E. coli*, BisC encodes a biotin sulfoxide reductase that reduces free methionine-(S)-sulfoxide (Ezraty *et al.*, 2005). On the chromosome of *S. Typhimurium, STM3644* was annotated as bisC, with 85% identity (amino acids) to BisC (AAC76575) of *E. coli* (coliBASE) (McClelland *et al.*, 2001). To examine the methionine sulfoxide activity of BisC in *Salmonella*, we deleted bisC in a methionine auxotrophic (*ΔmetC*) background. The *ΔmetC* mutant required the methionine sulfoxide reductase activity to reduce methionine sulfoxide back to methionine, and thus made it accessible as a methionine source (Denkel *et al.*, 2011). This step is essential since acylate tRNA<sub>met</sub> cannot use methionine sulfoxide as a substrate for protein biosynthesis (Ejiri *et al.*, 1979). The *ΔmetCΔbisC* mutant did not show a growth defect in minimal medium supplemented with methionine-(S)-sulfoxide (Fig. 1a). The ability to reduce methionine-(S)-sulfoxide in the *ΔmetCΔbisC* was presumably mediated by the methionine sulfoxide reductase MsrA. We also deleted msrA in a methionine auxotrophic (*ΔmetC*) background. The *ΔmetCΔmsrA* mutant still grew in minimal medium supplemented with methionine-(S)-sulfoxide (Fig. 1b). Only the deletion of both methionine sulfoxide reductases, BisC and MsrA, in the methionine auxotrophic background (*ΔmetCΔmsrAΔbisC*) led to a loss of the ability to utilize methionine-(S)-sulfoxide (Fig. 1c) in this strain. Thus, we conclude that MsrA and BisC of *S. Typhimurium* reduce free methionine-(S)-sulfoxide. None of the mutants showed a growth defect on methionine-(R)-sulfoxide (Fig. 1a–c) indicating that MsrA and BisC are specific for reduction of methionine-(S)-sulfoxide.

### BisC of *S. Typhimurium* reduces biotin sulfoxide

The ability of the *S. Typhimurium* BisC to reduce biotin sulfoxide was examined by a similar genetic approach.
First, we generated a biotin auxotrophic (ΔbioB) mutant that required biotin or biotin sulfoxide for full growth. Next, we disrupted bisC, msrA or both genes in the biotin auxotrophic background of S. Typhimurium. The ΔbioBΔbisC mutant was unable to use biotin sulfoxide for growth (Fig. 2a). Growth on biotin sulfoxide was impaired to a similar extent in the ΔbioBΔmsrAΔbisC mutant (Fig. 2c). In contrast, deletion of msrA did not impair growth of the biotin auxotrophic mutant with biotin sulfoxide as biotin source (Fig. 2b). These experiments suggest that BisC but not MsrA reduces biotin sulfoxide in S. Typhimurium.

BisC facilitates survival of Salmonella during exogenous H₂O₂ treatment

In vitro, H₂O₂ oxidizes the sulfur residue of biotin to biotin sulfoxide (Melville, 1954). Thus, we hypothesized that the ability to repair biotin sulfoxide by BisC might play a role in H₂O₂ tolerance of S. Typhimurium. To evaluate this idea, we generated a ΔbisC mutant in the S. Typhimurium wild-type background, and tested its sensitivity to oxidative stress in vitro in a starvation medium without biotin or amino acids. The ΔbisC mutant was more susceptible to exogenous H₂O₂ after 9 h of treatment than the wild-type (Fig. 3a). Without H₂O₂, the ΔbisC mutant was not impaired in its survival (Fig. 3b). The wild-type phenotype could be fully restored in the mutant by expressing bisC on a plasmid. Thus BisC contributed to H₂O₂ stress tolerance in S. Typhimurium. Interestingly, a plasmid carrying msrA also restored the wild-type phenotype.

The results above also implied that the methionine-(S)-sulfoxide reductase activity rather than the biotin sulfoxide reductase activity of BisC mediated survival of oxidative stress. This led us hypothesize that bisC was not essential for survival of H₂O₂-exposed S. Typhimurium in vitro. To test for this possibility we treated a biotin auxotrophic mutant (ΔbioB), which lacks endogenous biotin production, with exogenous H₂O₂. The ΔbioB mutant was not impaired for survival after exposure to H₂O₂. In contrast, a methionine auxotrophic mutant (ΔmetC) was highly sensitive to oxidative stress (Fig. 3c). Without H₂O₂ neither the ΔmetC mutant, nor the ΔbisC mutant were impaired in their survival (Fig. 3b).

Next, we wanted to know whether auxotrophy for another amino acid also caused a high sensitivity to exogenous...
**Fig. 3.** Survival of *S. Typhimurium* wild-type and mutant strains. Wild-type and mutant strains were cultivated overnight in starvation medium. OD\textsubscript{600} was adjusted to 0.01 and bacteria were treated with or without (control) H\textsubscript{2}O\textsubscript{2}. Survival was monitored by plating for colony counts at the indicated time points. The data shown are representative and were performed at least three times with identical results. Experiments were done in duplicate and means are shown ± SD. (a) Susceptibility of *S. Typhimurium* wild-type \(\Delta\text{bisC}, \Delta\text{bisC}+\text{pBAD30::bisC}\) and \(\Delta\text{bisC}+\text{pBAD30::msrA}\) to exogenous H\textsubscript{2}O\textsubscript{2}. (b) Survival of *S. Typhimurium* wild-type and mutant strains \(\Delta\text{metC}, \Delta\text{bisC}, \Delta\text{bioB}\) and \(\Delta\text{leuBCD}\) without exogenous H\textsubscript{2}O\textsubscript{2}. (c) Susceptibility of *S. Typhimurium* wild-type, \(\Delta\text{metC}, \Delta\text{bisC}, \Delta\text{bioB}\) and \(\Delta\text{leuBCD}\) to exogenous H\textsubscript{2}O\textsubscript{2}. (d) Susceptibility of *S. Typhimurium* wild-type to chloramphenicol, H\textsubscript{2}O\textsubscript{2}, and combined chloramphenicol and H\textsubscript{2}O\textsubscript{2} treatment. *P*-values <0.05 were considered to be significant as indicated by asterisks.
H₂O₂. We generated a leucin auxotrophic (ΔleuBCD) mutant, lacking three central genes of the leucin biosynthesis. The ΔleuBCD mutant did not show a survival defect without oxidative stress (Fig. 3b). Yet, it was highly sensitive to exogenous H₂O₂ (Fig. 3c), pointing to the importance of de novo protein biosynthesis of S. Typhimurium under these conditions. This conclusion was strongly supported by the observation that the protein biosynthesis inhibitor chloramphenicol dramatically increased sensitivity of wild-type S. Typhimurium to H₂O₂ (Fig. 3d).

**BisC is important for Salmonella to replicate inside activated macrophages**

S. Typhimurium lacking methionine sulfoxide reductases have a growth defect in IFN-γ activated macrophages (Denkel et al., 2011). To address the question whether the biotin sulfoxide reductase BisC is important for intracellular replication of S. Typhimurium, the murine macrophage cell line RAW 264.7 was infected with the wild-type S. Typhimurium and the ΔbisC mutant. The ΔbisC mutant showed a growth defect in IFN-γ activated but not in non-stimulated cells (Fig. 4a and b). The wild-type phenotype was restored by a plasmid carrying bisC but not by a plasmid carrying mstrA (Fig. 4a), suggesting that it was the biotin sulfoxide reductase activity of BisC that mediated robust growth of S. Typhimurium in IFN-γ activated macrophages.

It has been reported that S. Typhimurium relies on biotin biosynthesis for intracellular replication (Shi et al., 2009). Hence, we generated a biotin auxotrophic mutant (ΔbioB), and showed that replication was impaired both in non-stimulated and in IFN-γ activated macrophages (Fig. 4b and c). These data demonstrated that during intracellular replication S. Typhimurium relied on endogenous biotin biosynthesis. However, in contrast to non-activated macrophages, in IFN-γ activated macrophages endogenous biotin synthesis was not sufficient to support growth but also the biotin sulfoxide reducing activity of BisC was also required. This conclusion was supported by the finding that mutants lacking either the biotin sulfoxide repair (ΔbisC) or the endogenous biotin biosynthesis (ΔbioB), or lacking both (ΔbisCΔbioB), had a similar growth defect in IFN-γ activated macrophages (Fig. 4c).

**BisC contributes to successful replication of Salmonella in vivo**

To examine the role of BisC in replication of S. Typhimurium in vivo, groups of five female BALB/c mice were infected intraperitoneally with a mixture of wild-type and ΔbisC mutant in a 1:1 ratio. Competition experiments allow a direct comparison of the fitness of the tested strains (Beuzón & Holden, 2001). CIs were calculated as described elsewhere (Beuzón & Holden, 2001). The ΔbisC mutant was significantly attenuated in liver and spleen 1 and 3 days p.i. (Fig. 5a) indicating that BisC contributed to virulence of S. Typhimurium. RAW 264.7 macrophages as well as BALB/c mice are deficient in the NRAMP1 protein that evidently affects mounting an efficient oxidative burst (Fritsche et al., 2003, 2012; Vidal et al., 1995). We therefore repeated the infection competition experiment in NRAMP1-proficient 129S2/SvPasCrl mice. In these mice, we noted a more pronounced proportional decrease in the growth of the ΔbisC mutant, both in the spleen (P<0.05) and liver (P<0.05) 3 days p.i. (Fig. 5b). Thus, the increased attenuation of the ΔbisC mutant in 129S2/SvPasCrl mice indicated that BisC strongly contributed to the replication of S. Typhimurium in vivo especially under harsh oxidative stress conditions.

Next, we raised the question whether the mutant lacking endogenous biotin biosynthesis (ΔbioB) would also show a growth defect in vivo. BALB/c mice were infected intraperitoneally with a mixture of wild-type and ΔbioB mutant in a 1:1 ratio. Replication of ΔbioB mutant was slightly affected (Fig. 5c). Similar results could be observed in the 129S2/SvPasCrl mice (Fig. 5d). Thus, in contrast to the ΔbisC mutant, the growth defect of the ΔbioB mutant appeared to be independent of the level of oxidative stress in vivo.

**DISCUSSION**

In the present study, we characterized the biotin sulfoxide reductase BisC of an intracellular pathogen, and demonstrated its significance in the oxidative stress tolerance of S. Typhimurium. Biochemical properties of BisC have been investigated in detail for E. coli and R. sphaeroides (del Campillo-Campbell & Campbell, 1982, 1996; Dykhuizen, 1973; Ezraty et al., 2005; Garton et al., 2000; Johnson & Rajagopalan, 2001; Nelson & Rajagopalan, 2004; Pierson & Campbell, 1990; Pollock et al., 2002, 2003; Pollock & Barber, 1995, 1997, 2000, 2001). In E. coli, methionine sulfoxide and biotin sulfoxide were described as substrates of BisC (Ezraty et al., 2005). Accordingly, by using a genetic approach we demonstrated here that BisC of S. Typhimurium reduced these substrates. As a result, BisC allowed S. Typhimurium to regenerate biotin and methionine from their oxidized forms. This could be advantageous, especially for an intracellular lifestyle when energy sources and nutrients are scarce (Buchmeier & Libby, 1997; Chakravortty et al., 2002; Fields et al., 1986; Gallois et al., 2001; García-del Portillo et al., 2008; Hoiseth & Stocker, 1981; Shi et al., 2009).

In S. Typhimurium, biotin sulfoxide appeared to be exclusively reduced by BisC. However a second biotin sulfoxide reductase (BisZ) has been described for E. coli (del Campillo-Campbell & Campbell, 1996). This BisZ has recently been renamed to TorZ based on its higher affinity for trimethylamine-N-oxide when compared to biotin sulfoxide (Gon et al., 2001). BisC has already been proposed to act as a general protector against oxidative stress (Pollock & Barber, 2000). Specifically, in E. coli it has been presumed that BisC might protect from oxidative...
stress and makes biotin sulfoxide an accessible biotin source (Pierson & Campbell, 1990). However, an experimental link between BisC, oxidative stress response and bacterial virulence has not been reported so far.

To establish this link, we tested a ΔbisC mutant of S. Typhimurium for its sensitivity against oxidative stress in vitro, and for its virulence in macrophages and in mice. The in vitro \( \text{H}_2\text{O}_2 \) susceptibility assay clearly verified the importance of BisC for S. Typhimurium to survive oxidative stress facilitated by its methionine sulfoxide reductase activity. The methionine sulfoxide reductase of S. Typhimurium, MsrA, repairs methionine-(S)-sulfoxide, whereas MsrB and MsrC specifically reduce methionine-(R)-sulfoxide. (Denkel

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**Fig. 4.** Intracellular proliferation of S. Typhimurium strains in RAW 264.7 cells. Intracellular proliferation was determined in a gentamicin protection assay. Number of bacteria 16 h post-infection was divided by the number of bacteria 2 h post-infection. Results shown are the means ± SEM for three independent experiments, each in triplicate. *P*-values <0.05 were considered to be significant as indicated by asterisks. ns means non significant. (a) Proliferation of S. Typhimurium wild-type, ΔbisC, ΔbisC+pBAD30::bisC and ΔbisC+pBAD30::msrA in IFN-γ activated RAW 264.7 cells. (b) Proliferation of wild-type, ΔbisC and ΔbioB in non-stimulated macrophages. (c) Proliferation of S. Typhimurium wild-type, ΔbisC, ΔbioB, ΔbisCΔbioB in IFN-γ activated RAW 264.7 cells.
This study shows that besides MsrA, S. Typhimurium has a second methionine sulfoxide reductase that is specific for the (S)-epimer of methionine sulfoxide. The ΔbisC mutant was more susceptible to H₂O₂ despite the presence of an intact MsrA. This might be explained by the high concentrations of H₂O₂ that we used for the survival assays in vitro. Other studies addressing the role of bacterial methionine sulfoxide reductases in the oxidative stress response use similar or even higher concentrations of H₂O₂, which are presumably required because of the redundancy of methionine sulfoxide repairing enzymes (Denkel et al., 2011; Douglas et al., 2004; Lee et al., 2009; Singh & Singh, 2012; Zhao et al., 2010).

Fig. 5. In vivo proliferation of S. Typhimurium mutant strains in competition with S. Typhimurium wild-type. P-values were calculated using the Wilcoxon Signed Rank Test to evaluate whether the actual mean differs significantly from 1 (theoretical mean). CIs are shown with means ± SEM. The data shown are representative; experiments were performed at least twice with identical results. (a) CIs of ΔbisC versus wild-type in BALB/c on day 1 and day 3 post-infection. (b) CIs of ΔbisC versus wild-type in 129S2/SvPasCrl on day 1 and day 3 post-infection. (c) CIs of ΔbioB versus wild-type in BALB/c on day 1 and day 3 post-infection. (d) CIs of ΔbioB versus wild-type in 129S2/SvPasCrl on day 1 and day 3 post-infection. ns, non significant.
Methionine is susceptible to oxidation in its free and protein-bound form (Weissbach et al., 2002). Oxidation of protein-bound methionine influences the biological activity of calmodulin, the ribosomal protein L12, α-1-proteinase inhibitor and a voltage-gated K⁺-channel (Brot & Weissbach, 2000). Other studies revealed that oxidation of the N-terminal protein-bound methionine disables its essential removal by methionine aminopeptidase and affects cell viability (Chang et al., 1989; Miller et al., 1987; Solbiati et al., 1999). The physiological role of reducing free methionine sulfoxide, however, has received little attention. Both protein-bound and free methionine have been described as scavengers of oxidative stress (Bagchi et al., 1997; Levine et al., 1996). Our data suggested another reason why oxidized methionine needed to be repaired during the oxidative stress response of S. Typhimurium. Besides the ΔbisC mutant, the methionine auxotrophic ΔmetC mutant, the leucin auxotrophic ΔleuCBD mutant and S. Typhimurium wild-type treated with chloramphenicol were highly sensitive to H₂O₂. These observations indicated that de novo protein biosynthesis and thus access to free amino acids was critical for S. Typhimurium to survive oxidative stress.

The growth attenuation of the ΔbisC mutant in IFN-γ activated macrophages could only be rescued by bisC, not by the methionine sulfoxide reductase msrA. Additionally, the ΔbioB mutant was attenuated in macrophages and in mice, but not for its survival after exposure to H₂O₂. These data suggested that in S. Typhimurium biotin appeared to be essential in replicating bacteria, whereas it was dispensable under non-replicating conditions. At present we have no mechanistic explanation for these observations. Interestingly, the acetyl-CoA carboxylase has been described to be essential for bacterial growth of E. coli. It catalyses the first step in fatty acid synthesis that is required to generate phospholipids for the construction of membranes (Cronan & Rock, 1996; Freiberg et al., 2004). Biotin serves as cofactor for this essential carboxylase (Streit & Entcheva, 2003). Accordingly we suggest that biotin restriction as a result of biotin auxotrophy (ΔbioB) or lacking reduction of biotin sulfoxide (ΔbisC) might lead to the observed growth defects due to a disturbed functionality of biotin-dependent proteins like the acetyl-CoA carboxylase.

In contrast to the ΔbioB mutant, the growth deficiency of the ΔbisC mutant only emerged in IFN-γ-activated RAW 264.7 macrophages. This supports the idea that the phenotype of the ΔbisC mutant depended on the host response following the infection with S. Typhimurium. This assumption was further strengthened by the strong attenuation of the ΔbisC mutant in the 129S2/SvPasCrI mice producing a more profound oxidative burst compared to BALB/c mice. BALB/c mice are homozygous for the NRAMP sensitive allele (NRAMP⁵) with a defective resistance-associated macrophage protein 1 (NRAMP) (Ho & Cheers, 1982). NRAMP⁵ is linked to an enhanced susceptibility to S. Typhimurium infection (Fritsche et al., 2012; Vidal et al., 1995). Weiss and coworkers (Fritsche et al., 2003) assigned NRAMP1, beside the role as transporter for divergent cations, as having the ability to upregulate pro-inflammatory immune pathways and antimicrobial effector mechanisms (e.g. production of ROS). Thus the 129S2/SvPasCrI mice, homozygous for the NRAMP⁸ allele, should provide a suitable model to study the oxidative stress response of S. Typhimurium by producing a stronger oxidative burst compared to the NRAMP⁵ BALB/c mice.

In summary, our work showed that the biotin sulfoxide reductase BisC significantly contributed to defence of Salmonella against host-derived stress. Recent studies highlighted the importance of de novo biotin biosynthesis especially for intracellular pathogens like M. tuberculosis, F. tularensis and S. Typhimurium (Dey et al., 2010; Napier et al., 2012; Shi et al., 2009; Woong Park et al., 2011). Here the in vivo attenuation of both the biotin auxotrophic ΔbioB mutant and the ΔbisC mutant emphasized the importance of biotin homeostasis during infection and demonstrated that BisC was crucial for full virulence of S. Typhimurium.

ACKNOWLEDGEMENTS

We thank S. Suerbaum for support. We thank Oliver Böhm for the purification of methionine sulfoxide to its S- and R-forms. We thank Mathias W. Hornef and Oliver Pabst for helpful discussions. Thanks especially to the group of M. W. Hornef for the warm welcome in their laboratory and support with the animal experiments. This work was supported by the International Research Training Group 1273 funded by the German Research Foundation to L.A.D.

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Edited by: T. Abee