An Incomplete TCA Cycle Increases Survival of Salmonella Typhimurium during Infection of Resting and Activated Murine Macrophages

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Abstract

Background: In comparison to the comprehensive analyses performed on virulence gene expression, regulation and action, the intracellular metabolism of Salmonella during infection is a relatively under-studied area. We investigated the role of the tricarboxylic acid (TCA) cycle in the intracellular replication of Salmonella Typhimurium in resting and activated macrophages, epithelial cells, and during infection of mice.

Methodology/Principal Findings: We constructed deletion mutations of 5 TCA cycle genes in S. Typhimurium including gltA, mdh, sdhCDAB, sucAB, and sucCD. We found that the mutants exhibited increased net intracellular replication in resting and activated murine macrophages compared to the wild-type. In contrast, an epithelial cell infection model showed that the S. Typhimurium ΔsucAB and ΔsucCD strains had reduced net intracellular replication compared to the wild-type. The glyoxylate shunt was not responsible for the net increased replication of the TCA cycle mutants within resting macrophages. We also confirmed that, in a murine infection model, the S. Typhimurium ΔsucAB and ΔsucCD strains are attenuated for virulence.

Conclusions/Significance: Our results suggest that disruption of the TCA cycle increases the ability of S. Typhimurium to survive within resting and activated murine macrophages. In contrast, epithelial cells are non-phagocytic cells and unlike macrophages cannot mount an oxidative and nitrosative defence response against pathogens; our results show that in HeLa cells the S. Typhimurium TCA cycle mutant strains show reduced or no change in intracellular levels compared to the wild-type [1]. The attenuation of the S. Typhimurium ΔsucAB and ΔsucCD mutants in mice, compared to their increased net intracellular replication in resting and activated macrophages suggest that Salmonella may encounter environments within the host where a complete TCA cycle is advantageous.

Introduction

Salmonella enterica is one of the most common food-borne bacterial pathogens and the disease outcomes range from a self-limited gastroenteritis to typhoid fever in mammals. Typhoidal Salmonella serovars, such as Salmonella enterica serovars Typhi and Paratyphi, cause an estimated 20 million cases of typhoid and 200,000 human deaths worldwide per annum [2]. Typhoid infection involves transmission of Salmonella via the ingestion of contaminated food and water followed by bacterial penetration of the small intestinal barrier by invading gut epithelial cells causing bloody diarrhoea. Subsequently, Salmonella can enter the mesenteric lymph nodes and invade phagocytic cells such as macrophages [3,4]. Within macrophages, the Salmonella bacteria are compartmentalised into a modified intracellular phagosome termed the “Salmonella containing vacuole” (SCV). The SCV protects the Salmonella by preventing lysosomal fusion [5,6]. The antimicrobial defences deployed by macrophages include reactive oxygen and reactive nitrosative intermediates (ROI and RNI respectively), as well as antimicrobial peptides [7,8]. The ROI response is bactericidal and occurs approximately 1 h post-infection of macrophages whereas the RNI response is bacteriostatic and occurs approximately 8 h post-infection [9,10,11].

Recently, research is being directed towards establishing the role of central metabolic pathways in the virulence of pathogenic bacteria [12]. For example, it has been shown that fitness of Escherichia coli during urinary tract infection is reliant upon gluconeogenesis and the TCA cycle [13]. In Salmonella, we and others have demonstrated that glycolysis and glucose are required for the intracellular replication of S. Typhimurium in macrophages and mice [14,15,16]. Other work has shown that full virulence
In the current study, we investigated the effect of disrupting the TCA cycle on the ability of \textit{S}. Typhimurium to replicate within murine RAW macrophages and HeLa epithelial cells by deleting genes encoding specific TCA cycle enzymes. The HeLa epithelial cell line is a well-defined model for infection of mammalian cells with \textit{S}. Typhimurium, and has been used to characterize the biogenesis and evolution of the SCV [18,19,20]. We deleted the suc\textit{AB}, suc\textit{CD}, \textit{sdhCDAB}, \textit{mdh} and \textit{gltA} genes which encode 2-ketoglutarate dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase, malate dehydrogenase and citrate synthase respectively (Figure 1) [21]. Interestingly, we found that disruption of the TCA cycle can result in an increase in the levels of the \textit{S}. Typhimurium mutant strains within resting and activated macrophages compared to the wild-type. Mutants that showed increased net replication in macrophages did not exhibit the same phenotype in epithelial cells and, in agreement with previously published results, were severely attenuated in mouse infection assays compared to the wild-type [17].

### Materials and Methods

#### Bacterial strains, growth conditions and reagents

\textit{S}. Typhimurium strains and plasmids used in this work are listed in Table S1. All mutants were constructed in the wild-type strain 4/74 which is the prototrophic parent of the well-characterized SL1344 strain [22]. Strains were maintained in Luria-Bertani (LB) broth or on plates with appropriate antibiotics at the following concentrations;ampicillin (Ap, Sigma Aldrich), 50 \textmu{g}.ml\textsuperscript{-1}, chloramphenicol (Cm, Sigma Aldrich), 12.5 \textmu{g}.ml\textsuperscript{-1} and kanamycin (Kn, Sigma Aldrich), 50 \textmu{g}.ml\textsuperscript{-1}. Oligonucleotide primers were purchased from Sigma Genosys or Illumina, (California).

#### Mutant construction

\textit{S}. Typhimurium mutant strains were constructed according to published procedures (Datsenko and Wanner 2000) and as briefly described in [14]. Primers used to construct the \textit{S}. Typhimurium deletion mutant strains were purchased from Sigma-Genosys (Table 1). P22-derived transductants were screened on green agar plates to obtain lysogen-free colonies and all deletion mutants were transduced into a clean wild-type background [24]. The complete absence of the structural genes was verified by DNA sequencing of the deleted regions of the chromosome.

#### Plasmid construction

The suc\textit{CD} genes were PCR amplified from \textit{S}. Typhimurium 4/74 genomic DNA using primers suc\textit{F} (5'- TTTTAAGCTTAT-GAACCTGATCATATGACATATCA) and suc\textit{R} (5'- TTTTGGA-TCCTCAGCGAGAAGACATCTACATATGAATATCCTCCTTAG). The PCR product was digested with BamHI and HindIII, ligated into the low-copy-number vector pWKS30 [25], and transformed into \textit{E}. coli strain DH5\textalpha\, by electroporation [26]. The resulting plasmid was designated pWKS30::suc\textit{CD} and was confirmed by PCR using

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### Table 1. Primers used to construct \textit{S}. Typhimurium gene deletion mutants.

<table>
<thead>
<tr>
<th>Primer Name</th>
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</tr>
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<td>ace\textit{Aredf}</td>
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<tr>
<td>ace\textit{Aredr}</td>
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Figure 1. TCA cycle and glyoxylate shunt showing intermediate products and genes encoding enzymes within the pathway. Deleted genes are shown in bold. 

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Table 1. Primers used to construct \textit{S}. Typhimurium gene deletion mutants.
primers sucF and sucR. Plasmids pWKS30 and pWKS30::sucCD were then transformed into 4/74 and AT3449 by electroporation.

**Macrophage infection assays**

Infection assays in murine RAW 264.7 macrophages (obtained from American Type Culture Collection; Rockville, MD; ATCC# TIB-71) were performed essentially as previously described [27]. Where indicated, macrophages were activated 24 h prior to infection by seeding the macrophages in MEM culture medium supplemented with 20 units.ml⁻¹ (2 ng.ml⁻¹) of IFN-γ (Sigma) as previously described [28]. The multiplicity of infection (MOI) for all experiments was 10:1. The infection assays were allowed to proceed for 2 h and 18 h post infection. To estimate the amount of intracellular bacteria at each time point, cells were lysed using 1% Triton X-100 (Sigma), and samples were taken for viable counts [10]. Statistical significance was assessed by using Student’s unpaired t test, and a P value of 0.05 was considered significant.

**Cytotoxicity assays**

Macrophages were infected as described above with the following modifications: DMEM without Phenol Red was used to prevent interference with the assay and the PBS content of the complete tissue culture medium was reduced to 5% due to associated lactate dehydrogenase (LDH) activity. LDH activity released from the mammalian cells was measured in the tissue culture medium using the Cytotox 96 non-radioactive cytotoxicity assay kit (Cat. G1780, Promega) and was considered to reflect cytotoxicity of S. Typhimurium. Statistical significance was assessed by using Student’s unpaired t test, and a P value of 0.05 was considered significant.

**HeLa cell infection assays**

Infection assays in human HeLa epithelial cells (obtained from American Type Culture Collection; Rockville, MD; ATCC#c CCL-2) were performed according to [1]. Approximately, 1×10⁵ HeLa cells were seeded into each well of a 6-well cell culture plates and infected with S. Typhimurium 4/74 and mutant strains at an MOI of 10:1. Prior to infection the S. Typhimurium strains had been grown aerobically to an OD₅₆₀ of ~2.0 in LB broth at 250 rpm at 37°C to allow expression of the SPI-1 Type 3 secretion system.

To increase the uptake of Salmonella, the 6-well plates were centrifuged at 1000 g for 5 min, and this was defined as time 0 h. After 1 h of infection, extracellular bacteria were killed with centrifuged at 1000 g for 5 min, and this was defined as time 0 h. After 1 h of infection, extracellular bacteria were killed with centrifuged at 1000 g for 5 min, and this was defined as time 0 h.

**Results and Discussion**

**Effect of disrupting the TCA cycle on intracellular replication of S. Typhimurium in resting macrophages**

An incomplete TCA cycle has been found in a surprisingly large number of bacterial pathogens including Helicobacter pylori, Haemophilus influenzae and Staphylococcus mutans [31,32,33]. The primary role of the TCA cycle is to provide NADH which is used by bacterial cells for ATP synthesis via the electron transport chain (ETC). However, the TCA cycle also plays a key role in the synthesis of intermediates for anabolic pathways; specifically 2-ketoglutarate, oxaloacetate and succinyl-CoA are starting points for the synthesis of glutamate, aspartate and porphyrin respectively (Figure 1). Bacteria that harbor an incomplete TCA cycle retain the ability to generate 2-ketoglutarate, oxaloacetate and succinyl-CoA from pyruvate. To determine the role of the complete TCA cycle for the intracellular replication of S. Typhimurium within macrophages we used a genetic approach to interrupt the TCA cycle. We tested S. Typhimurium strains carrying deletions of the gldC, mdh, sdcCDAB, sucAB and sucCD genes for their ability to replicate within resting macrophages compared to the wild-type (Figure 2A). Surprisingly, we recovered up to 40% higher levels of the S. Typhimurium Δmdh, ΔsdcCDAB, and ΔsucCD strains from infected resting macrophages than the wild-type; the level of the S. Typhimurium ΔsucAB strain was 17% higher than the wild-type (Figure 2B). The apparent ‘over-replication’ phenotypes of the S. Typhimurium Δmdh, ΔsucAB, ΔsucCD, and ΔsdcCDAB strains relative to the wild-type suggested that a complete TCA cycle is not necessary for growth within resting macrophages. The observed reduced intracellular replication of the S. Typhimurium ΔgldC strain compared to the wild-type may suggest that 2-ketoglutarate and therefore glutamate is limiting within the SCV (Figure 2A). This result supports a previous finding that simultaneous prevention of glutamine synthesis and high-affinity transport attenuates S. Typhimurium virulence in BALB/c mice and murine macrophages, suggesting a host environment with low levels of glutamine [34].

We confirmed that the apparent ‘over-replication’ phenotype of the S. Typhimurium ΔsucCD strain was due to deletion of the sucCD gene. We inserted the sucCD gene into the low-copy number vector pWKS30 [25], transformed the construct into the S. Typhimurium 4/74 and S. Typhimurium strains, and performed infection assays with macrophages. As shown in Figure 3A, the cloned copy of the sucCD gene fully complemented the sucCD deletion in clonal deletions of macrophages and restored intracellular replication of the S. Typhimurium ΔsucCD strain containing pWKS30::sucCD to wild-type levels. Figure 3B shows that the level of cytotoxicity of the S. Typhimurium ΔsucCD strain and the complemented strain are indistinguishable from that of the wild-type strain demonstrating that the intracellular replication phenotype of the ΔsucCD strain is not a result of increased cell death.

**Increased net replication of the S. Typhimurium ΔsucCD mutant in resting macrophages is due to increased flux through the glyoxylate shunt**

The observed increased net replication of the S. Typhimurium Δmdh, ΔsdcCDAB, ΔsucAB and ΔsucCD strains could potentially be...
due to an increased flux through the glyoxylate shunt that conferred a replicative advantage compared to the wild-type within resting macrophages (Figure 2AB). The glyoxylate shunt bypasses reactions of the TCA cycle in which CO2 is released, and conserves 4 carbon compounds for biosynthesis (Figure 1) [21]. The pathway is active during growth on 2 carbon compounds such as acetate from fatty acids, when 4-carbon TCA cycle intermediates need to be conserved [21]. The conversion of isocitrate to glyoxylate is catalysed by isocitrate lyase, which has been shown to be required for the persistence of *Mycobacterium tuberculosis* in infected macrophages [21,35]. Isocitrate lyase is encoded by the aceA gene of *S. Typhimurium* [36]. To test whether the glyoxylate shunt plays a role in the increased intracellular replication phenotype of the *S. Typhimurium ΔaceA* strain, we constructed *S. Typhimurium ΔaceA* and ΔsucCD mutants and tested them for their ability to replicate within resting macrophages. Figure 4A shows that the net intracellular replication of the *S. Typhimurium ΔaceA* strain is only slightly increased compared to the wild-type within resting macrophages. Furthermore, combining the ΔaceA and ΔsucCD mutations does not reduce the increased intracellular replication of the *S. Typhimurium ΔsucCDΔaceA* strain to wild-type levels. The data suggest that the glyoxylate shunt does not contribute to the increased net intracellular replication phenotype of the *S. Typhimurium ΔsucCD* strain in infected resting macrophages.

**Effect of disrupting the TCA cycle on intracellular replication of *S. Typhimurium* in activated macrophages**

In the light of the above results, one possibility is that the net increase in intracellular replication of the *S. Typhimurium Δmdh, ΔsucAB, ΔsucCD*, and ΔsdhCDAB strains may be due to their enhanced ability to survive the antimicrobial defense mechanisms...
of macrophages, which include the ROI and RNI response [37,38]. Production of ROI and RNI species are stimulated during activation of macrophages by lipopolysaccharide and IFN-γ [39]. We therefore tested the ability of S. Typhimurium ΔsucAB, ΔsucCD, Δmdh, ΔsucCD and ΔgltA mutants to replicate within activated macrophages compared to the wild-type (Figure 4B). In this experiment, the wild-type 4/74 strain showed no net replication within activated macrophages; however the ΔsucCD, Δmdh, ΔsucCD and ΔgltA strains all showed increased net replication (Figure 4B). The observation that the S. Typhimurium ΔsucAB shows no net replication within activated macrophages may reflect limited availability of succinyl-CoA within the SCV of activated macrophages. Succinyl-CoA is required for the biosynthesis of lysine, methionine and diaminopimelate. Diaminopimelate is essential for the synthesis of peptidoglycan and hence the antimicrobial ROI and RNI response in macrophages compared to the wild-type strain prompted us to test an alternate cell line (HeLa epithelial cells) in which S. Typhimurium is still within a vacuole, but not subject to the ROI and RNI responses [1].

Effect of disrupting the TCA cycle on intracellular replication of S. Typhimurium in epithelial cells

Figure 5 shows that the S. Typhimurium ΔsucCD and ΔgltA strains show 30% less intracellular replication than the wild-type strain between 2 h and 6 h post infection. The S. Typhimurium Δmdh strain showed the same level as the wild-type.

These observations suggest that, in contrast to resting and activated macrophages, an intact TCA cycle is required for optimal intracellular replication within epithelial cells. Since HeLa cells lack the antimicrobial ROI and RNI responses deployed by macrophages, the reduced intracellular replication of the S. Typhimurium ΔsucCD and ΔgltA strains supports the possibility that a disrupted TCA cycle favors the ability of S. Typhimurium to survive macrophage defense mechanisms [1]. Disruption of the TCA cycle may result in decreased flux through the ETC which could enhance the ability of the ΔsucCD, Δmdh, ΔsucCD and ΔgltA mutants to survive the antimicrobial response mounted by activated macrophages by reducing Fenton reaction-based oxidative damage [38]; this hypothesis is the subject of further investigation, although we cannot rule out the possibility that other differences between the mutant and wild-type strains contribute to their infection phenotypes at this stage. For example, disruption of the TCA cycle may alter the redox balance in Salmonella to reduce endogenous NADH levels and therefore ETC activity. This would reduce endogenous Fenton-based oxidative damage and may enhance intracellular survival. Interestingly it has been postulated that bactericidal antibiotics may act by stimulating the TCA cycle which would increase NADH flux through the ETC thus increasing hydroxyl radical formation via the Fenton reaction and causing cell death [42,43].

Effect of disrupting the TCA cycle on infection of mice by S. Typhimurium

The increased net replication of the S. Typhimurium ΔsucCD, Δmdh, ΔsucCD and ΔgltA strains in macrophages was surprising in the light of previous findings which showed that S. Typhimurium ΔsucAB, ΔsucCD and ΔgltA mutants were attenuated in orally infected mice [17]. We therefore tested our S. Typhimurium ΔsucCD and ΔsucCD strains for their ability to infect mice.

Following infection of mice, S. Typhimurium disseminates systemically from the Peyer’s patches to the liver and spleen where it continues to grow within macrophages [44,45,46]. We inoculated mice with the S. Typhimurium ΔsucAB, ΔsucCD and wild-type strains via the intraperitoneal route. Salmonella bacteria were recovered and enumerated after 72 h of infection. The results demonstrate that the S. Typhimurium ΔsucAB mutant is severely attenuated (by ~10^3-fold) compared to the wild-type in the spleens and livers of infected mice (Figure 6A). The S. Typhimurium ΔsucCD strain is less attenuated than the S. Typhimurium ΔsucCD strain in livers and spleens of infected mice, but is still reduced by ~10-fold compared to the wild-type (Figure 6B). These results are consistent with the finding of Tchawa Yimga et al., [17], who found that S. Typhimurium SR11 ΔsucAB was avirulent and the ΔsucCD mutant was attenuated in orally infected BALB/c mice. The contrast between the attenuation of the S. Typhimurium ΔsucAB and ΔsucCD mutants in mice, and their increased intracellular replication in resting and activated macrophages suggest that Salmonella may encounter...
Figure 4. Increased intracellular replication of the *S. Typhimurium* ΔsucCD, ΔsdhCDAB, ΔgltA and Δmdh strains during infection of activated RAW macrophages. (A) Intracellular replication assays of *S. Typhimurium* 4/74, ΔsucCD (AT3449), ΔaceA (AT3385) and ΔaceAΔsucCD (AT3496) strains during infection of resting RAW macrophages (B) Intracellular replication assay of *S. Typhimurium* 4/74, ΔsucAB (AT3448), ΔsucCD, (AT3449), ΔsdhCDAB (AT3475), ΔgltA (AT3505) and Δmdh (AT3508) strains during infection of activated RAW macrophages. The data show the number of viable bacteria (expressed as percentages of the initial inocula) within activated macrophages at 2 h and 18 h post-infection. Each bar represent the statistical mean from three independent biological replicates and the error bars represent the standard deviation. (The significant differences between the parental 4/74 strain and the mutant strains are shown by asterisks p<0.05, * p<0.05, ** p<0.01, and *** p<0.001).

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Figure 5. Decreased intracellular replication of *S. Typhimurium* ΔsucCD and ΔgltA strains during infection of HeLa epithelial cells. Intracellular replication assays of *S. Typhimurium* 4/74, ΔsucCD (AT3449), ΔgltA (AT3505) and Δmdh (AT3508) strains during infection of HeLa cells. The data show the number of viable bacteria (expressed as percentages of the initial inocula) within macrophages at 2 h and 6 h post-infection. Since *S. Typhimurium* initiates intracellular replication much earlier in HeLa cells (3–4 h) compared to macrophages (~8 h post-infection) replication was assessed at 6 h post-infection (Hautefort *et al.*, 2008). Each bar represent the statistical mean from three independent biological replicates and the error bars represent the standard deviation. (The significant differences between the parental 4/74 strain and the mutant strains are shown by asterisks p<0.05, * p<0.05, ** p<0.01, and *** p<0.001).

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environments within the host where a complete TCA cycle is advantageous.

Summary

We investigated the ability of S. Typhimurium TCA cycle mutants to replicate within macrophages, HeLa epithelial cells, and to infect BALB/c mice. The S. Typhimurium Δndh, ΔSucCD, ΔldtCΔAB strains replicated to higher levels than the wild-type within resting and activated macrophages, suggesting an enhanced ability to survive antimicrobial mechanisms. This is supported by the observed decreased levels of the S. Typhimurium ΔSucCD and ΔldtC strains within infected epithelial cells, which lack an ROI and RNI response, although we cannot rule out other mechanisms at this stage. The increased level of the S. Typhimurium ΔSucCD in resting macrophages was not due to increased flux through the glyoxylate shunt. In agreement with previous work, we found that the S. Typhimurium ΔSucAB and ΔSucCD strains are attenuated in mice, suggesting an intact TCA cycle is required for successful infection within specific host environments.

References


Supporting Information

Table S1 Strains and plasmids used in this study. Found at: doi:10.1371/journal.pone.0013871.s001 (0.03 MB PDF)

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Author Contributions

Conceived and designed the experiments: SDB AT. Performed the experiments: SDB VKR GMK. Analyzed the data: SDB. Wrote the paper: JH AT.


