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Gut inflammation provides a respiratory electron acceptor for *Salmonella*

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***Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) causes acute gut inflammation by using its virulence factors to invade the intestinal epithelium and survive in mucosal macrophages. The inflammatory response enhances the transmission success of *S.* Typhimurium by promoting its outgrowth in the gut lumen through unknown mechanisms. Here we show that reactive oxygen species generated during inflammation react with endogenous, luminal sulphur compounds (thiosulphate) to form a new respiratory electron acceptor, tetrathionate. The genes conferring the ability to use tetrathionate as an electron acceptor produce a growth advantage for *S.* Typhimurium over the competing microbiota in the lumen of the inflamed gut. We conclude that *S.* Typhimurium virulence factors induce host-driven production of a new electron acceptor that allows the pathogen to use respiration to compete with fermenting gut microbes. Thus the ability to trigger intestinal inflammation is crucial for the biology of this diarrhoeal pathogen.**

S. Typhimurium is an invasive enteric pathogen associated with diarrhoea, acute intestinal inflammation and the presence of neutrophils in stool samples[1]. The pathogen triggers intestinal inflammation by using two type III secretion systems (T3SS-1 and T3SS-2) that enable *S.* Typhimurium to invade the intestinal epithelium and survive in mucosal macrophages[2]. Recent studies suggest that acute intestinal inflammation enhances growth of *S.* Typhimurium in the intestinal lumen[3–5]. The resulting increase in numbers establishes the pathogen as a prominent species in the gut, thereby enhancing its transmission success[6]. However, the mechanisms by which *S.* Typhimurium can overgrow other microbes in the hostile environment of the inflamed gut remain uncharacterized.

The ability of *S.* Typhimurium to overgrow other microbes under certain *in vitro* growth conditions has been exploited for enrichment methods that facilitate its isolation from biological samples containing competing microbes. A commonly used approach, known as tetrathionate enrichment, was developed in 1923, and is based on the ability of *S.* Typhimurium to use tetrathionate as a terminal electron acceptor[7]. It is widely believed that tetrathionate respiration is not important during infection, because there are no known sources of tetrathionate in the mammalian host, nor does an *S.* Typhimurium mutant deficient for tetrathionate respiration exhibit reduced virulence in a mouse model of typhoid fever[8] (Supplementary Fig. 1). These observations suggest that tetrathionate respiration encoded by the *ttrSR ttrBCA* gene cluster (Supplementary Fig. 1a) might be most important when free-living bacteria grow in tetrathionate-containing environments such as soil or decomposing carcasses[9].

$S_4O_6^{2-}$ availability in the gut

A fresh look at sulphur metabolism in the inflamed intestine suggested an alternative to this conventional wisdom (Fig. 1). Colonic bacteria produce large quantities of hydrogen sulphide (H_2S), a highly toxic compound. The caecal mucosa protects itself from the injurious effects of H_2S by converting it to thiosulphate ($S_2O_3^{2-}$)[10,11] (Fig. 1a). Although thiosulphate is therefore likely to be present in the intestinal lumen, this compound cannot be used as an electron acceptor by the *ttrSR ttrBCA* gene cluster[12]. However, tetrathionate broth used for enrichment of *Salmonella* serotypes contains thiosulphate, not tetrathionate ($S_4O_6^{2-}$). Before use of the medium, thiosulphate is oxidized to tetrathionate by addition of the strong oxidant iodine (Fig. 1a). We reasoned that oxidation of thiosulphate might occur during intestinal inflammation, a condition accompanied by neutrophil transmigration into the gut lumen (Fig. 1b) and production of nitric oxide radicals (NO) and reactive oxygen species[13].

To test this idea, we measured the formation of tetrathionate *in vivo* using a mouse colitis model[14]. Compared with mock-infected animals, infection of mice (C57BL/6) with *S.* Typhimurium resulted in acute caecal inflammation (Fig. 1c, d and Supplementary Fig. 2). Infection with a mutant deficient for tetrathionate respiration (*ttr* mutant) was accompanied by increased tetrathionate levels, which were detected in caecal contents by reverse phase high-performance liquid chromatography coupled with mass spectrometry (Fig. 1e). *S.* Typhimurium causes intestinal inflammation by using two type III secretion systems, T3SS-1 and T3SS-2, which mediate epithelial invasion and macrophage survival, respectively[15]. Inactivation of T3SS-1 (through a mutation in *invA*) and T3SS-2 (through a mutation in *spiB*) renders *S.* Typhimurium unable to trigger intestinal inflammation in the mouse colitis model[16] (Fig. 2). Tetrathionate was not detected in mice infected with an *invA spiB* mutant ($P < 0.01$), suggesting that inflammation is required for generating tetrathionate in the intestine. Furthermore, tetrathionate did not accumulate during infection with the *S.* Typhimurium wild-type strain ($P < 0.01$), which raised the possibility that the *ttr* genes might promote consumption of this electron acceptor during infection.

$S_4O_6^{2-}$ promotes growth in the gut

To investigate the growth benefit conferred by tetrathionate respiration *in vitro*, the *S.* Typhimurium wild-type strain and a *ttrA* mutant

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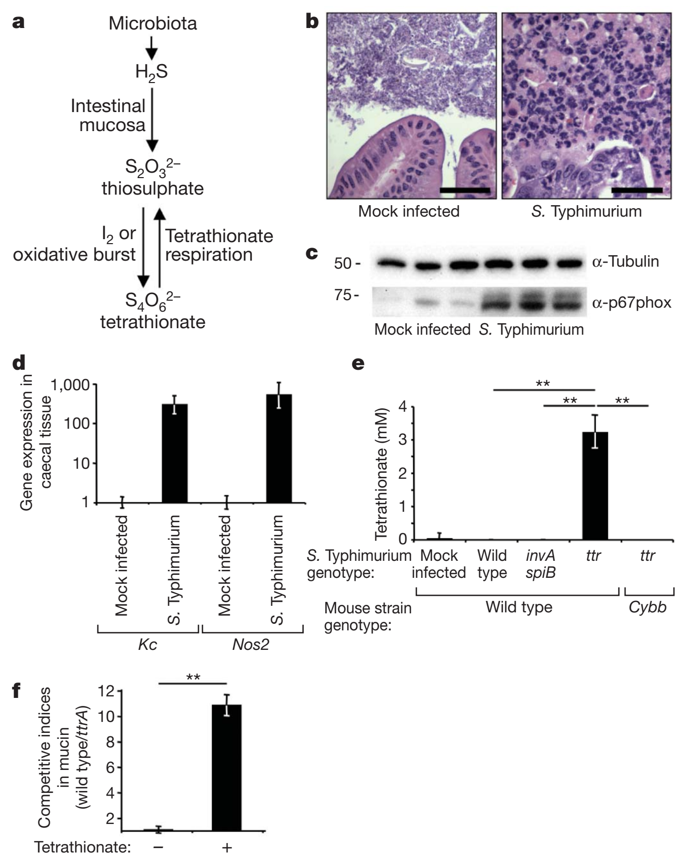

Figure 1 | Tetrathionate becomes available during inflammation. **a**, Schematic of intestinal sulphur metabolism. **b–e**, Samples from a mouse colitis model 4 days after infection with *S.* Typhimurium or mock-infection. **b**, Haematoxylin and eosin-stained caecal sections. Scale bar, 100 μm. **c**, Detection of NADPH oxidase (α-p67phox) or tubulin (α-tubulin) in caecal extracts ($n = 3$). **d**, Expression of *Kc* and *Nos2* in caecal RNA samples ($n \geq 3$) using quantitative real-time PCR (fold-increases over mock-infection). **e**, Tetrathionate detected in caecal contents using liquid chromatography–mass spectrometry ($n \geq 3$). **f**, Competitive indices for anaerobic growth in mucin broth with (+) or without (−) tetrathionate ($n = 3$). **d–f**, Bars, geometric means ± s.e.m. $^{**}P < 0.01$.

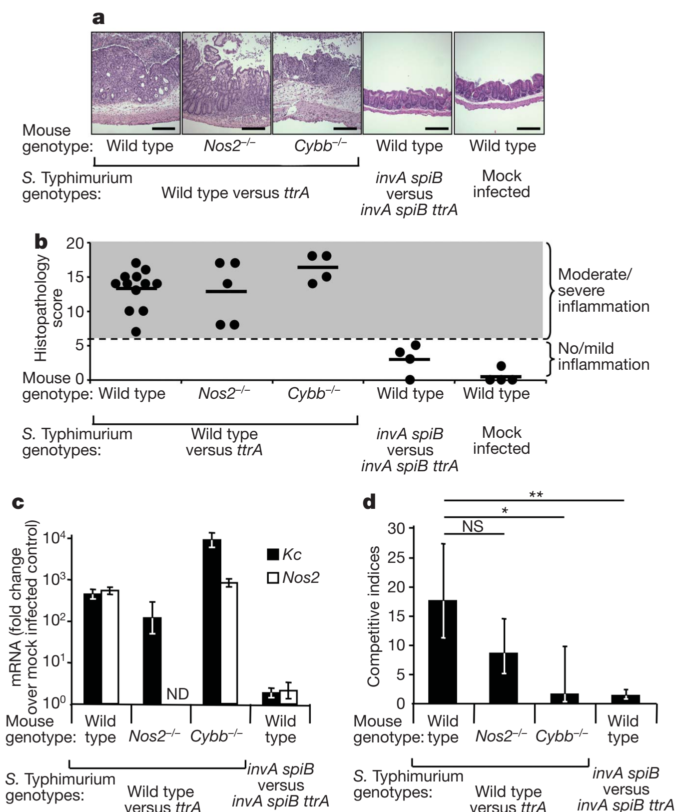

Figure 2 | Tetrathionate respiration confers growth advantage. **a–d**, Samples from a mouse colitis model (number indicated in **b**) 4 days after infection with *S.* Typhimurium or mock-infection. **a**, Haematoxylin and eosin-stained caecal sections. Scale bar, 400 μm. **b**, Blinded histopathology scoring showing averages (bars) and individual scores (circles). **c**, *Kc* (closed bars) and *Nos2* (open bars) expression in caecal RNA samples using quantitative real-time PCR (fold-increases over mock-infection). **d**, Competitive indices of indicated *S.* Typhimurium strains determined by recovering bacteria from colon contents. **c**, **d**, Bars, geometric means ± s.e.m. $^{*}P < 0.05$; $^{**}P < 0.01$; NS, not significant; ND, not determined.

(Supplementary Fig. 1a, b) were co-cultured in tetrathionate broth in the presence or absence of oxygen (Supplementary Fig. 1c). When thiosulphate was not oxidized to tetrathionate by the addition of iodine, the wild-type strain and the *ttrA* mutant grew equally well. However, in the presence of iodine, tetrathionate respiration promoted outgrowth of the *S.* Typhimurium wild-type strain under anaerobic and microaerobic, but not under aerobic, growth conditions. A tetrathionate concentration of 2.5 mM was sufficient to promote outgrowth of the wild-type strain (Supplementary Fig. 1d) ($P < 0.01$). Co-culture of the *S.* Typhimurium wild-type strain and the *ttrA* mutant in mucin broth resulted in enrichment for the wild type only in the presence of tetrathionate (Fig. 1f) ($P < 0.01$). Collectively, these data suggest that tetrathionate respiration might provide a benefit during the anaerobic growth conditions encountered *in vivo,* for example, in the intestinal mucus layer.

To test this idea, mice were infected with an equal mixture of the *S.* Typhimurium wild-type strain and a *ttrA* mutant (Fig. 2). *S.* Typhimurium infection resulted in prominent intestinal inflammation (Fig. 2a, b) and increased messenger RNA (mRNA) levels of *Kc*, encoding a neutrophil chemoattractant, and *Nos2*, encoding inducible nitric oxide synthase (Fig. 2c). A marked enrichment for the *S.* Typhimurium wild-type strain was observed 4 days after infection in the colon contents (Fig. 2d), suggesting that tetrathionate respiration provided an advantage during growth in the lumen of the inflamed gut. In contrast, both strains were recovered in similar numbers from the spleen in a mouse model of typhoid fever (Supplementary Fig. 1f), suggesting that tetrathionate was not available for growth at systemic sites. We next validated our results using a bovine ligated small-intestinal (ileal) loop model in which *S.* Typhimurium causes acute mucosal inflammation (Fig. 3)[17]. Upon infection with an equal mixture of the *S.* Typhimurium wild type and a *ttrA* mutant, higher numbers of the wild-type strain were associated with the mucus fraction and with the intestinal mucosa, whereas equal numbers of both strains were recovered from the luminal fluid 8 h after infection. These data suggest that the selective advantage conferred by tetrathionate respiration was greatest in close proximity to the inflamed mucosal surface.

To determine whether tetrathionate respiration provides a colonization advantage in the absence of inflammation, mice were infected with an equal mixture of an *invA spiB* mutant and an *invA spiB ttrA* mutant. Mice infected with this mixture neither developed intestinal pathology nor exhibited elevated levels of *Nos2* or *Kc* mRNA (Fig. 2a–c). Equal numbers of both strains were recovered from colon contents (Fig. 2d). During the early stages of infection modelled in bovine ligated ileal loops, intestinal inflammation is largely dependent on T3SS-1 (ref. 17). Infection with an equal mixture of an *invA* mutant and an *invA ttrA* mutant resulted in equal recovery of both strains from bovine ligated ileal loops (Fig. 3). Collectively, these data suggest that tetrathionate respiration provided no growth benefit in the absence of intestinal inflammation.

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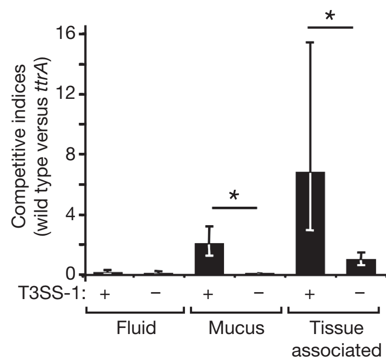

Figure 3 | Tetrathionate respiration promotes growth of *S.* Typhimurium in close proximity to the mucosal surface. Bovine ligated ileal loops ($n = 3$ animals) were infected with a mixture of *S.* Typhimurium T3SS-1 proficient (+) strains (wild type (AJB715) versus *ttrA* mutant(SW661)) or T3SS-1-deficient (−) strains (*invA* mutant (SW737) versus *invA ttrA* mutant (SW736)) and samples collected 8 h after infection from the luminal fluid, mucus scrapings and tissue punches (tissue-associated bacteria). Bars, geometric means ± s.e.m. $*P \leq 0.05$.

Oxygen radicals generate $S_4O_6^{2-}$ *in vivo*

Induction of a respiratory burst in blood leukocytes resulted in oxidation of thiosulphate to tetrathionate (Supplementary Fig. 1g). To determine whether inducible nitric oxide synthase or NADPH oxidase are required for tetrathionate respiration *in vivo*, *Nos2*-deficient mice and *Cybb* (gp91phox)-deficient mice were infected with an equal mixture of the *S.* Typhimurium wild-type strain and the *ttrA* mutant. *S.* Typhimurium infection resulted in marked intestinal inflammation (Fig. 2a, b) and increased mRNA levels of *Kc* (Fig. 2c). Although enrichment for wild-type bacteria was detectable in *Nos2*-deficient mice, no enrichment for the *S.* Typhimurium wild-type strain was observed in *Cybb*-deficient mice (Fig. 2d) ($P < 0.05$). Thus, oxygen radicals produced by NADPH oxidase may be more important than nitric oxide radicals in promoting tetrathionate respiration *in vivo*. Infection of *Cybb*-deficient mice with a *ttr* mutant was not accompanied by production of tetrathionate (Fig. 1e). Collectively, these data suggest that the respiratory burst of phagocytes recruited during inflammation stimulates growth of *S.* Typhimurium in the gut by providing a terminal electron acceptor.

Outgrowth by $S_4O_6^{2-}$ respiration

Under anaerobic conditions, microbes compete for high-energy resources that are available for fermentation, but fermentation end products cannot be further used. By reducing tetrathionate, *S.* Typhimurium is able to use fermentation end products that can only be respired, providing a substantial selective advantage. To test the magnitude of this growth advantage, we measured the effect of tetrathionate respiration on the abundance of *S.* Typhimurium in intestinal contents (Fig. 4). Mice were inoculated with the *S.* Typhimurium wild-type strain or a *ttrA* mutant, and bacteria were recovered 4 days after infection. The *S.* Typhimurium wild-type strain was recovered in approximately 80-fold higher numbers ($P < 0.01$) than the *ttrA* mutant (no tetrathionate respiration) (Fig. 4a–c). Restoration of tetrathionate respiration in the *ttrA* mutant by homologous recombination re-established growth at the level of the wild-type strain. Analysis of the microbiota composition indicated that the Typhimurium wild-type strain, but not the *ttrA* mutant, was able to outcompete other bacteria inhabiting the caecum (Fig. 4d and Supplementary Fig. 3). These results suggest that the ability of *S.* Typhimurium to outgrow the microbiota during inflammation depends on tetrathionate respiration.

An important recent conceptual advance in bacterial pathogenesis is the demonstration that enteric pathogens can use host responses to outgrow the intestinal microbiota, but the mechanisms were not clear[3,4,18]. Here we show that *S.* Typhimurium gains a growth advantage

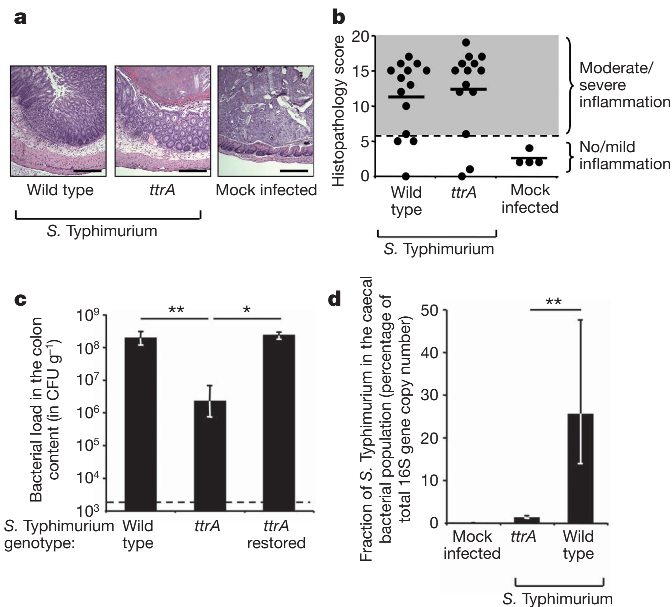

Figure 4 | Tetrathionate respiration increases the abundance of *S.* Typhimurium in the intestinal lumen. **a**–**c**, Samples from a mouse colitis model (number indicated in **b**) 4 days after infection with *S.* Typhimurium or mock-infection. **a**, Haematoxylin and eosin-stained caecal sections. Scale bar, 400 μm. **b**, Blinded histopathology scoring showing averages (bars) and individual scores (circles). **c**, Recovery of *S.* Typhimurium from colon contents. **d**, Fraction of *S.* Typhimurium as a percentage of the caecal bacterial population using 16S rRNA gene quantitative real-time (wild type $n = 6$, *ttrA* mutant $n = 6$, mock-infected $n = 4$). **c**, **d**, Bars, geometric means ± s.e.m. $*P < 0.05$; $**P < 0.01$.

in the competitive environment of the gut by using a virulence-factor-induced electron acceptor generated by the host respiratory burst. These data suggest that tetrathionate respiration provides a significant selective advantage, because enrichment for *S.* Typhimurium during growth in the inflamed gut leads to increased transmission by the faecal–oral route[6]. The selective advantage conferred by tetrathionate respiration is likely an important reason why *S.* Typhimurium causes gastrointestinal disease, because this property places virulence factors (that is, T3SS-1 and T3SS-2) that are required for inducing the inflammatory host response needed for the formation of tetrathionate *in vivo*, under selection. This may also explain why the ability to reduce tetrathionate is among a constellation of functions found in most *Salmonella* isolates and has historically been used as a criterion for identification of *Salmonellae*. It is noteworthy that the *ttr* gene cluster is also present in the enteric pathogen *Yersinia enterocolitica*, but is absent from a close relative, *Y. pestis*, which does not colonize the intestine[19].

METHODS SUMMARY

Bacterial strains and plasmids used are listed in Supplementary Table 1. *S.* Typhimurium was routinely cultured in LB broth or on LB agar plates. Construction of mutants deficient in tetrathionate respiration is described in the Supplementary Methods. Tetrathionate broth (BD Biosciences) or mucin broth (0.05% hog mucin (Sigma-Aldrich) in minimal media supplemented with 40 mM sodium tetrathionate as indicated) was inoculated with 100 colony forming units per millilitre of each strain and incubated at 37 °C for 16 h either with aeration, statically or anaerobically as indicated. All animal experiments were approved by the Institutional Animal Care and Use Committees at the University of California, Davis (mouse experiments) or Texas A&M University (calf experiments). Ligated ileal loop surgery was performed as described previously[17]. An *S.* Typhimurium mouse colitis model has been described[14]. Groups of 10- to 12-week-old, female mice (C57BL/6, B6.129S-$Cybb^{tm1Din}$/J, B6.129P2-$Nos2^{tm1Lau}$/J; the Jackson Laboratory) were orally infected with *S.* Typhimurium and tissue samples collected 4 days later. Bacterial numbers were determined by spreading serial tenfold dilutions of tissue homogenates on selective media. The competitive

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index was calculated by dividing the number of wild-type cells by the number of mutant cells and normalized by the input ratio. Formalin-fixed, haematoxylin and eosin-stained caecal sections were examined for signs of inflammation (Supplementary Fig. 2). The tetrathionate concentration of caecal extracts was measured by reverse-phase liquid chromatography–mass spectrometry. To measure relative expression levels of *Kc* and *Nos2* mRNA, total RNA was isolated from the caecum using TRI Reagent (Molecular Research Center), reverse transcribed (TaqMan reverse transcription reagents; Applied Biosystems) and SYBR-Green- (Applied Biosystems) based real-time PCR performed using the primers listed in Supplementary Table 2. Fold changes in mRNA levels measured by real-time PCR, tetrathionate concentrations and bacterial numbers underwent logarithmic transformation before ANOVA analysis followed by Student's *t*-test.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions S.E.W. contributed to the experimental design, constructed bacterial strains and contributed to Figs 1c–e, 2c, d, 3d, e and Supplementary Figs 1a, b, e, f and 3. P.T. contributed to Fig. 1f and Supplementary Fig. 1c, d and assisted with mouse experiments. M.G.W. assisted with mouse experiments and performed cloning experiments. B.P.B. contributed to Figs 1b, 2a, b, 3b, c and Supplementary Fig. 2. D.L.H. constructed bacterial strains. R.W.C. and J.M.R. contributed to Fig. 3a. L.G.A. performed the ligated loop surgery. C.L.B., L.G.A., R.M.T., J.R.R. and A.J.B. provided financial support for the study and contributed to the experimental design. S.E.W. and A.J.B. were responsible for the overall study design and for writing the manuscript.

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