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Gut inflammation provides a respiratory electron acceptor for *Salmonella*

Sebastian E. Winter,

Department of Medical Microbiology and Immunology, School of Medicine, University of California at Davis, One Shields Ave; Davis CA 95616, USA

Parameth Thiennimitr,

Department of Medical Microbiology and Immunology, School of Medicine, University of California at Davis, One Shields Ave; Davis CA 95616, USA

Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

Maria G. Winter,

Department of Medical Microbiology and Immunology, School of Medicine, University of California at Davis, One Shields Ave; Davis CA 95616, USA

Brian P. Butler,

Department of Medical Microbiology and Immunology, School of Medicine, University of California at Davis, One Shields Ave; Davis CA 95616, USA

Douglas L. Huseby,

Department of Microbiology, University of California at Davis, One Shields Ave; Davis CA 95616, USA

Robert W. Crawford,

Department of Medical Microbiology and Immunology, School of Medicine, University of California at Davis, One Shields Ave; Davis CA 95616, USA

Joseph M. Russell,

Department of Medical Microbiology and Immunology, School of Medicine, University of California at Davis, One Shields Ave; Davis CA 95616, USA

Charles L. Bevins,

Department of Medical Microbiology and Immunology, School of Medicine, University of California at Davis, One Shields Ave; Davis CA 95616, USA

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Correspondence to: Andreas J. Bäumlér (ajbaumlér@ucdavis.edu).

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L. Garry Adams,

Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843, USA

Renée M. Tsois,

Department of Medical Microbiology and Immunology, School of Medicine, University of California at Davis, One Shields Ave; Davis CA 95616, USA

John R. Roth, and

Department of Microbiology, University of California at Davis, One Shields Ave; Davis CA 95616, USA

Andreas J. Bäuml

Department of Medical Microbiology and Immunology, School of Medicine, University of California at Davis, One Shields Ave; Davis CA 95616, USA

Abstract

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 reacted with endogenous, luminal sulphur compounds (thiosulfate) to form a new respiratory electron acceptor, tetrathionate. The genes conferring the ability to utilize tetrathionate as an electron acceptor produced 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.

Introduction

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 employing 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 neither known sources of tetrathionate in the mammalian host, nor does a *S. Typhimurium* mutant deficient for tetrathionate respiration exhibit reduced virulence in a mouse model of typhoid fever 8(Figure S1). These observations suggested that tetrathionate respiration encoded by the *ttrSR ttrBCA* gene cluster (Figure S1A) might be most important when free-living bacteria grow in tetrathionate-containing environments such as soil or decomposing carcasses 9.

S₄O₆²⁻ availability in the gut

A fresh look at sulphur metabolism in the inflamed intestine suggested an alternative to this conventional wisdom (Figure 1). Colonic bacteria produce large quantities of hydrogen sulfide (H₂S), a highly toxic compound. The cecal mucosa protects itself from the injurious effects of H₂S by converting it to thiosulfate (S₂O₃²⁻) 10,11 (Figure 1A). While thiosulfate 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 thiosulfate, not tetrathionate (S₄O₆²⁻). Prior to use of the medium, thiosulfate is oxidized to tetrathionate by addition of the strong oxidant iodine (Figure 1A). We reasoned that oxidation of thiosulfate might occur during intestinal inflammation, a condition accompanied by neutrophil transmigration into the gut lumen (Figure 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 to mock-infected animals, infection of mice (C57BL/6) with *S. Typhimurium* resulted in acute cecal inflammation (Figure 1C, 1D, and Figure S2). Infection with a mutant deficient for tetrathionate respiration (*ttr* mutant) was accompanied by increased tetrathionate levels, which were detected in cecal contents by reverse phase high performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) (Figure 1E). *S. Typhimurium* causes intestinal inflammation by employing 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 (Figure 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.

S₄O₆²⁻ 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 (Figure S1A and B) were co-cultured in tetrathionate broth in the presence or absence of oxygen (Figure S1C). When thiosulfate 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 (Figure S1D) ($P < 0.01$). 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 (Figure 1F) ($P < 0.01$). Collectively, these data suggested that tetrathionate respiration might provide a benefit during the anaerobic growth conditions encountered *in vivo*, e.g. 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 (Figure 2). *S. Typhimurium* infection resulted in prominent intestinal inflammation (Figures 2A and B) and increased mRNA levels of *Kc*, encoding a neutrophil chemoattractant, and *Nos2*, encoding inducible nitric oxide synthase (iNOS) (Figure 2C). A marked enrichment for the *S. Typhimurium* wild-type strain was observed 4 days after infection in the colon contents (Figure 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 (Fig S1F), 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 (Figure 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, while equal numbers of both strains were recovered from the luminal fluid 8 hours after infection. These data suggested 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 (Figures 2A-C). Equal numbers of both strains were recovered from colon contents (Figure 2D). During the early stages of infection modelled in bovine ligated ileal loops, intestinal inflammation is largely dependent on T3SS-1 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 (Figure 3). Collectively, these data suggested that tetrathionate respiration provided no growth benefit in the absence of intestinal inflammation.

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

Induction of a respiratory burst in blood leukocytes resulted in oxidation of thiosulfate to tetrathionate (Figure S1G). To determine whether iNOS 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 (Figure 2A and B) and increased mRNA levels of *Kc* (Figure 2C). While 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 (Figure 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 (Figure 1E). Collectively, these data suggested 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 utilized. 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 (Figure 4). Mice were inoculated with the *S. Typhimurium* wild-type strain or a *ttrA* mutant and bacteria were recovered four 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) (Figure 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 *S. Typhimurium* wild-type strain, but not the *ttrA* mutant, was able to outcompete other bacteria inhabiting the cecum (Figure 4D and Figure S3). These results suggested that the ability of *S. Typhimurium* to outgrow the microbiota during inflammation depends on tetrathionate respiration.

Discussion

An important recent conceptual advance in bacterial pathogenesis is the demonstration that enteric pathogens can utilize 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 in the competitive environment of the gut by utilizing 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 fecal-oral route 6. The selective advantage conferred by tetrathionate respiration is likely an important reason why *S. Typhimurium* causes gastrointestinal disease, since this property places virulence factors (i.e. 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 is historically 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 tetrathionate

respiration deficient mutants 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(CFU) /ml 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 the Texas A&M University (calf experiments). Ligated ileal loop surgery was performed as described previously 17. A *S. Typhimurium* mouse colitis model has been described 14. Groups of 10-12 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 10-fold dilutions of tissue homogenates on selective media. The competitive 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, Hematoxylin and Eosin (H&E) stained cecal sections were examined for signs of inflammation (Supplementary Figure 2). Tetrathionate concentration of cecal extracts was measured by RP-LC-MS. To measure relative expression levels of *Kc* and *Nos2* mRNA, total RNA was isolated from the cecum 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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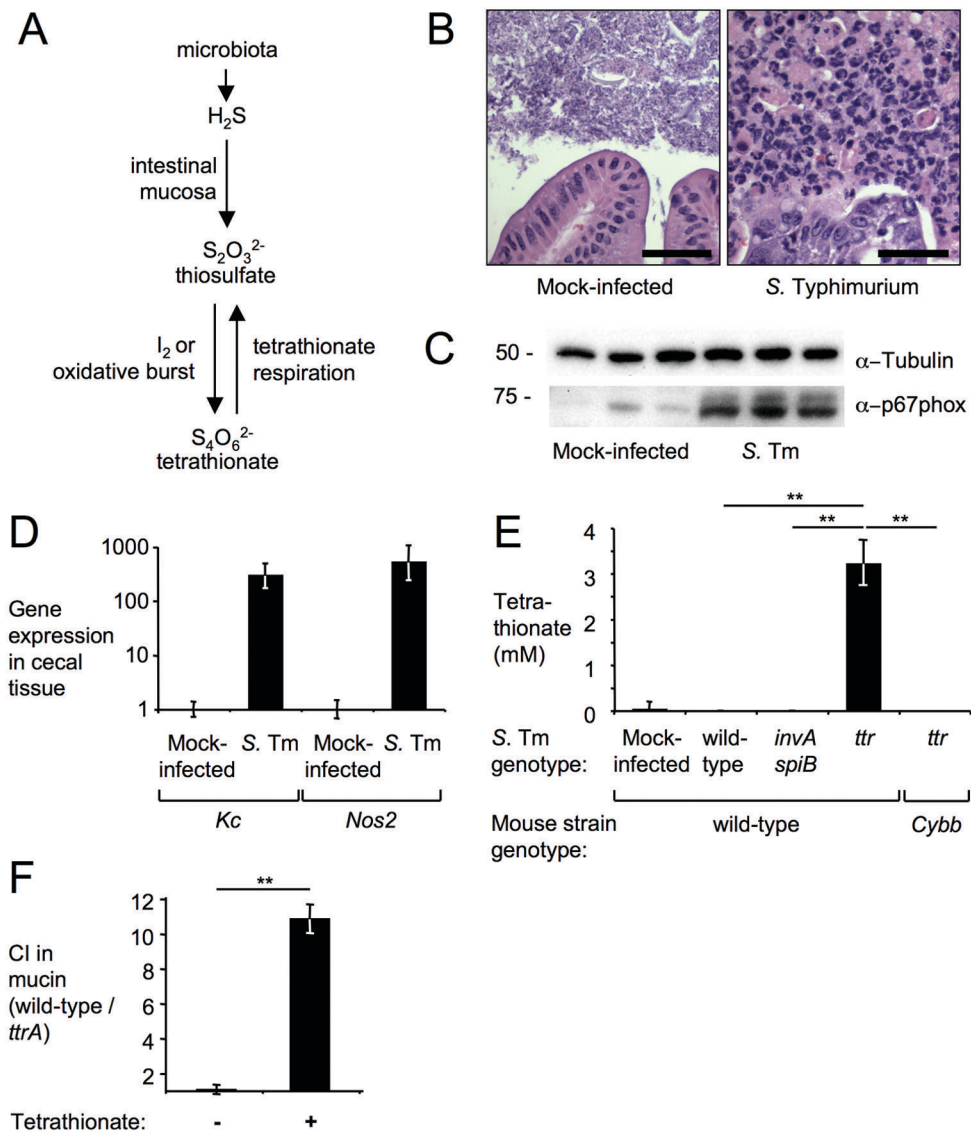


Figure 1. Tetrathionate becomes available during inflammation

(A) Schematic of intestinal sulfur metabolism. (B-E) Samples from a mouse colitis model four days after infection with *S. Typhimurium* (*S. Tm*) or mock-infection. (B) H&E stained cecal sections. Scale bar, 100 μ m. (C) Detection of NADPH oxidase (α -p67phox) or tubulin (α -tubulin) in cecal extracts (n=3). (D) Expression of *Kc* and *Nos2* in cecal RNA samples (n=3) using qRT-PCR (fold-increases over mock-infection). (E) Tetrathionate detected in cecal contents using LC-MS (n=3). (F) Competitive indices (CI) for anaerobic growth in mucin broth with (+) or without (-) tetrathionate (n=3). (D-F) Bars represent geometric means \pm standard error. **, $P < 0.01$.

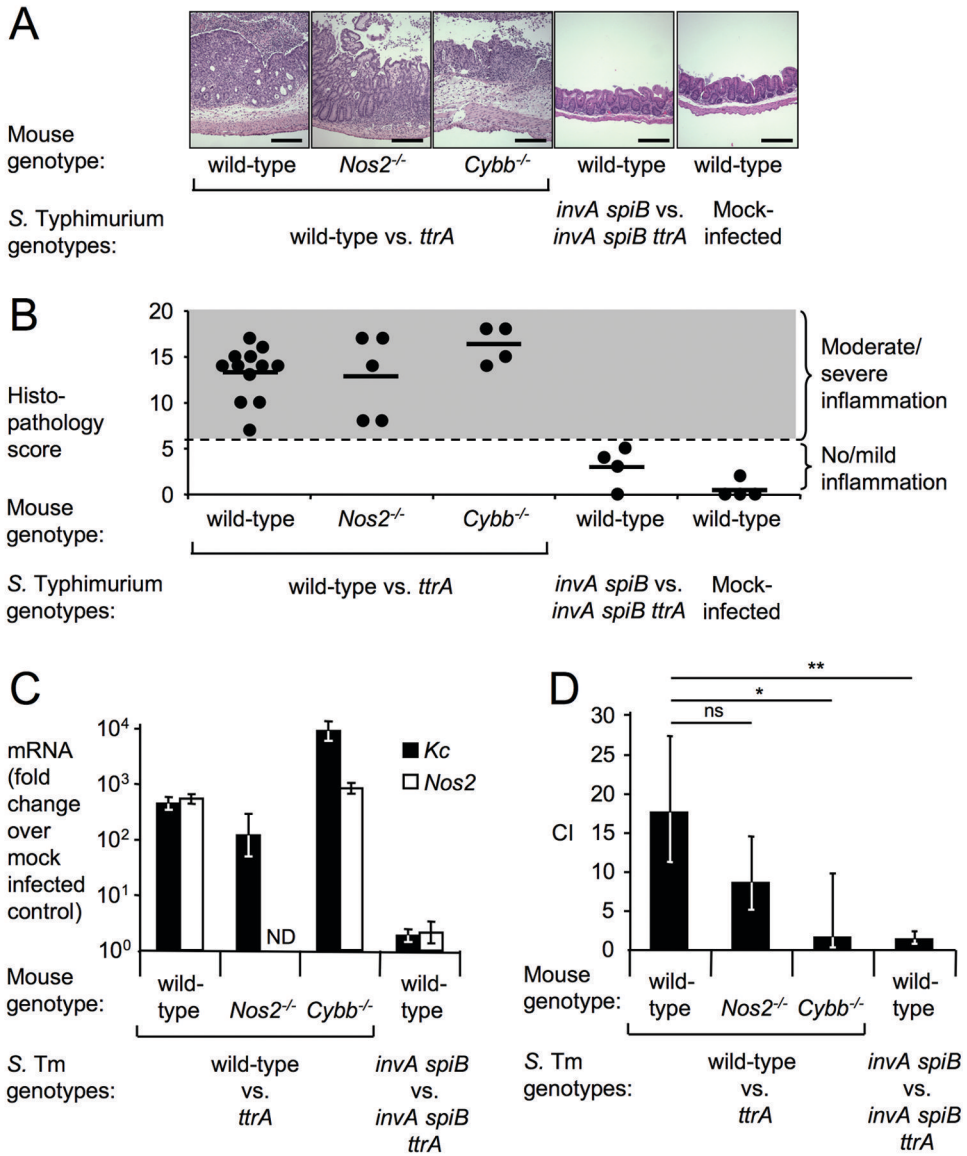


Figure 2. Tetrathionate respiration confers growth advantage
 (A-D) Samples from a mouse colitis model (n indicated in B) four days after infection with *S. Typhimurium* (*S. Tm*) or mock-infection. (A) H&E stained cecal sections. Scale bar, 400 μ m. (B) Blinded histopathology scoring showing averages (bars) and individual scores (circles). (C) *Kc* (closed bars) and *Nos2* (open bars) expression in cecal RNA samples using qRT-PCR (fold-increases over mock-infection). (D) Competitive indices (CI) of indicated *S. Typhimurium* strains determined by recovering bacteria from colon contents. (C-D) Bars represent geometric means \pm standard error. *, $P < 0.05$; **, $P < 0.01$; ns, not significant, ND, not determined.

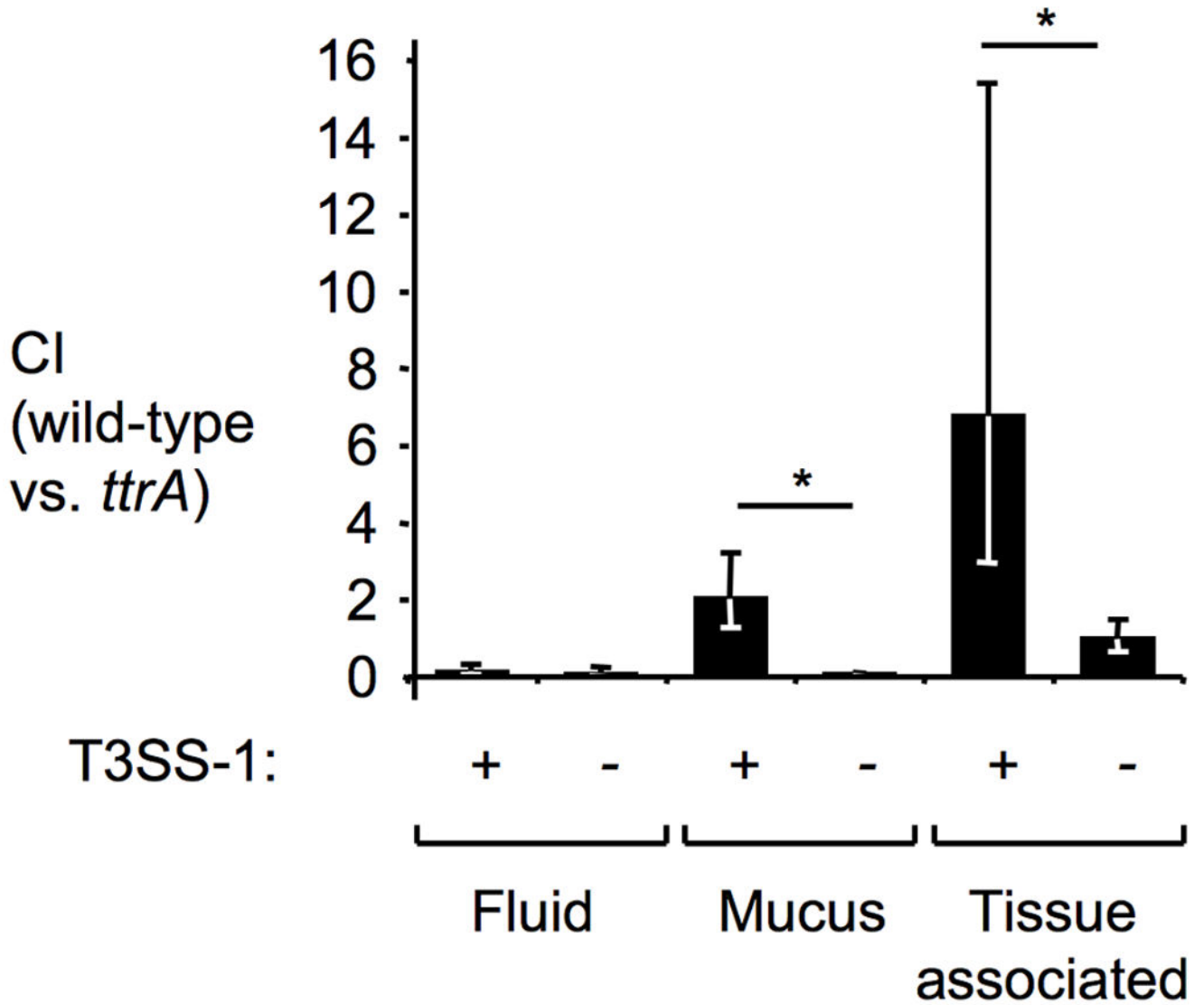


Figure 3. Tetrathionate respiration promotes growth of *S. Typhimurium* in close proximity to the mucosal surface

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 hours after infection from the luminal fluid, mucus scrapings and tissue punches (tissue-associated bacteria). Bars represent geometric means \pm standard error. *, $P < 0.05$.

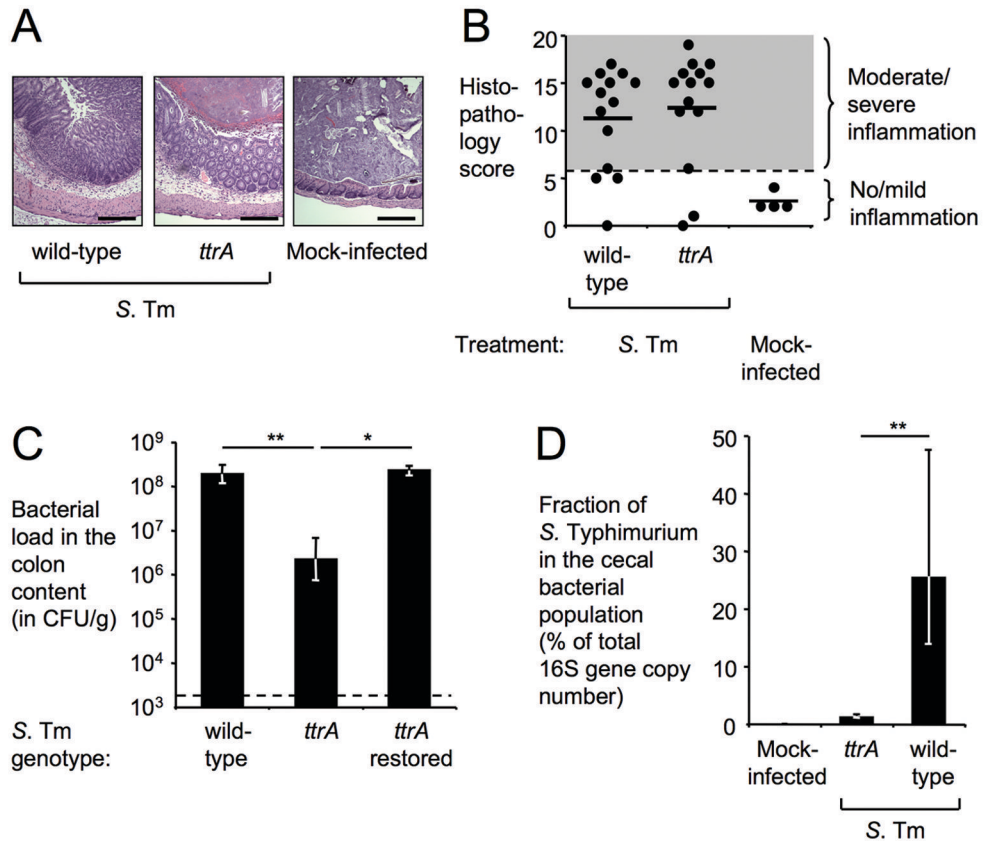


Figure 4. Tetrathionate respiration increases the abundance of *S. Typhimurium* in the intestinal lumen

(A-C) Samples from a mouse colitis model (n indicated in B) four days after infection with *S. Typhimurium* (*S. Tm*) or mock-infection. (A) H&E stained cecal sections. Scale bar, 400 μ m. (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 percentage of the cecal bacterial population using 16S rRNA gene qRT-PCR (wild-type n=6, *ttrA* mutant n=6, mock-infected n=4). (C-D) Bars represent geometric means \pm standard error. *, $P < 0.05$; **, $P < 0.01$.