Role of the *Salmonella typhimurium* Fur regulon in mouse infection

by

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A DISSERTATION

Presented to the Department of Molecular

Microbiology and Immunology and the

Oregon Health Sciences University

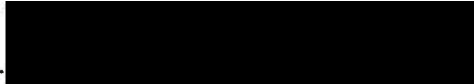
in partial fulfillment of

the requirements for the degree of

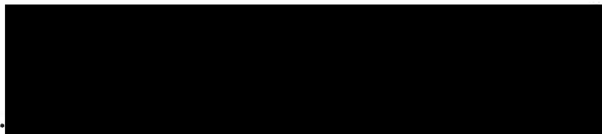
Doctor of Philosophy

December 1995

APPROVED



Fred Heffron, Ph.D., Thesis Advisor



Richard A. Maurer, Ph.D. Chairman, Graduate Council

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Acknowledgments:

My sincere thanks to all of the people who helped me to achieve this goal. First to Fred Heffron, who provided me with limitless opportunies during my studies. I would also like to thank my committee members, Maggie So, Jorge Crosa, Caroline Enns and Gary Thomas for their time and advice during the course of this work.

Studying here would not have been nearly as enjoyable without the support and friendship of past and present members of the Heffron and So labs. I am especially grateful to Andreas Bäumler, for his endless support, encouragement, and advice and to Igor Stojiljkovic for his support and enthusiasm. Suzana Anic provided me with several kb of DNA sequence, without which I might still be reading gels. Peter Valentine, Susanne Lindgren, Hans Kusters and Brian Ahmer labored over many versions of manuscripts, and Frances Bowe and Jeff Lipps helped me with animal experiments.

Abstract:

The pathogenesis of *Salmonella typhimurium* infection requires bacterial growth in the host. Although the bacteria can synthesize most compounds required for their growth, iron is a nutrient which must be acquired from the external environment. Mammalian hosts exploit this weakness by withholding iron to inhibit the growth of microorganisms. Bacteria, in turn, use this low iron concentration as a signal for the induction of virulence genes. These virulence genes may encode toxins, which release cellular iron compounds by lysis of host cells, specialized uptake systems for host iron proteins, or enzymes for withstanding oxidative stress. Expression of these virulence factors is regulated via a common pathway for iron-responsive gene regulation. This regulation occurs at the transcriptional level and is mediated by the Fur repressor. During characterization of a *S. typhimurium fur* mutant it was found the mutant strain was recovered in higher numbers from murine macrophages than the parent *Salmonella* strain. Since survival in macrophages is essential to the pathogenesis of mouse typhoid caused by *S. typhimurium*, we therefore examined whether Fur might regulate genes required for macrophage survival, and possibly other virulence genes of *S. typhimurium*. In order to address this question, we took two different approaches to identify Fur regulated genes involved in virulence. In the first approach, we screened the *S. typhimurium* genome for new genes regulated by Fur. This screen identified 14 new Fur-regulated genes in *Salmonella*. The second approach included cloning and inactivation of *S. typhimurium* Fur regulated genes known to exist in other enteric bacteria. These included the *sodA* gene, encoding Mn-cofactored superoxide dismutase (MnSOD), the *feoB* gene, encoding an Iron(II) uptake system, and the *tonB* gene, which encodes a protein required for several different mechanisms of Iron(III) uptake. Our results show that overexpression of MnSOD allows *S. typhimurium* to survive phagocytosis by macrophages better than the wild type, but that the enzyme is not absolutely required for infection of mice. Characterization of *tonB* and *feoB* mutants

showed that the iron uptake systems encoded by these genes function in distinct phases of infection. While the Iron(II) uptake system encoded by *feo* is utilized during growth in the mouse intestine, TonB-mediated Iron(III) uptake was required for growth in the mesenteric lymph node and the Peyer's patches. These data indicate that *S. typhimurium* utilizes different iron sources in the various compartments of its murine host.

Introduction and Literature Review:

Iron withholding response of the host

To cause disease, most microorganisms must be able to multiply in their hosts. Iron is essential for DNA synthesis and energy metabolism, and is therefore a major requirement for this growth. Thus, withholding iron from microbes limits their ability to multiply and cause disease. In fact, iron withholding is a nonspecific host immune defense mechanism for the prevention of disease. One strategy the host uses to limit the availability of iron is the synthesis of high-affinity iron binding proteins such as lactoferrin and transferrin. Transferrin is an iron-binding glycoprotein synthesized primarily by hepatocytes and found in plasma, interstitial fluids, lymph and pleural fluids. In the human adult, the proportion of transferrin molecules bound to iron ranges from 20-30% (38). Transferrin plays a dual role in the host--it not only serves to limit the availability of iron in body fluids, but also functions in iron transport to specific sites (e.g. bone marrow) where it is required. For maximal iron binding activity, transferrin requires a pH value above 6.5 (31). The idea that transferrin functions in bacteriostasis in vivo is borne out by two observations: (i) addition of apotransferrin to bacterial cultures in vitro has been shown to inhibit their growth, and (ii) patients with defects in transferrin synthesis are at greater risk for bacterial infections (38).

A second iron binding protein, lactoferrin, was first identified in human milk, where it accounts for up to 20% of milk protein. Infants that are breast-fed have a lower incidence of infection than infants fed cow's milk or milk formula, which contains one-tenth the amount of lactoferrin (6). From this observation, a role for lactoferrin in preventing infection of infants has been proposed. In addition, lactoferrin is present in secretions of other exocrine glands, including tears, saliva, bronchial, nasal and cervical mucus, seminal fluid, gastrointestinal fluid and bile (6). Furthermore, lactoferrin is a major component of the granules of polymorphonuclear neutrophils (PMN). Upon contact with bacteria, PMNs

release lactoferrin, which can scavenge iron at sites of infection. The lactoferrin-iron complex is phagocytosed by macrophages, thereby removing iron from the infected site. It has been found that patients deficient in lactoferrin-containing granules of PMN experience recurrent bacterial infections, which further emphasizes the importance of lactoferrin's antimicrobial activity (10). Lactoferrin and transferrin are structurally related, however lactoferrin is able to bind iron at a pH of <4.0. This property enables it to function in acidic environments. It is known that the pH is lowered at sites of inflammation and in the digestive tract , two sites where lactoferrin is present (38).

The withholding of iron by lactoferrin and transferrin is supported by additional iron withholding mechanisms during infection (38). As early as the 1930's, it was recognized that the plasma of patients with inflammatory diseases was hypoferremic. Further investigation revealed that these low plasma iron levels were caused by suppression of iron release from hepatic and splenic macrophages involved in recycling iron from senescent red blood cells. This reduced iron release was accompanied by increased synthesis of ferritin, an intracellular iron storage protein. Thus, during infection, the body's iron stores are shifted to the liver and spleen and the saturation of serum transferrin is reduced to 20% of its normal level. This response is known as hypoferremia of infection. Together with the hypoferremic response, both the assimilation of dietary iron and the iron saturation of transferrin are decreased during infection. Recent studies indicate that the iron withholding mechanisms induced during infection are elicited by cytokines, including IL-1, IL-6 and TNF-α (37).

Although these iron withholding mechanisms are effective against extracellular pathogens, they do not effectively remove iron from bacteria, fungi or protozoa that multiply within host cells. However, the host possesses additional mechanisms for intracellular depletion of iron (37). The signal for triggering these iron depletion mechanisms is delivered by interferon gamma (IFN-γ). Upon stimulation of host cells with IFN-γ, the activity of the enzyme nitric oxide synthase (NOS) increases markedly

(37). The reactive nitrogen intermediates generated by NOS then cause an efflux of nonheme iron from cells. In addition, IFN-γ stimulates down regulation of transferrin receptor expression on activated macrophages (14). IFN-γ treated macrophages have been shown to retard growth of *Salmonella typhimurium*, *Listeria monocytogenes*, *Legionella pneumophila*, and *Mycobacterium bovis* (13, 23, 37). For *L. pneumophila*, this growth inhibition was shown to be the result of iron deprivation. IFN-γ-mediated growth inhibition of *Legionella* could be reversed by added iron-saturated lactoferrin but not by apolactoferrin (13). Interestingly, it was also found that the alveolar macrophages of smokers are unable to kill *Listeria*, while macrophages obtained from non-smokers, which have a 75% lower iron content, are able to kill the bacteria (29).

Iron-responsive regulation of bacterial genes by Fur

Iron uptake genes

Iron is an essential component of nearly all known organisms. Enzymes and proteins containing iron function in DNA synthesis, oxygen transport and electron transport. Under aerobic conditions, however, iron is present as insoluble iron(III) hydroxide complexes, making its acquisition a difficult task. For this reason both bacteria and eukaryotes have developed highly efficient systems for assimilation of iron. In addition to its necessity for cellular processes, however, an excess of iron can be hazardous to the cell. Iron acts as a catalyst for the generation of toxic oxygen radicals. These radicals are highly reactive and cause damage to DNA and to membranes by peroxidation of lipids. For this reason, the synthesis of components of iron uptake systems are tightly regulated in response to intracellular iron concentration.

In bacteria, expression of iron uptake genes is controlled at the transcriptional level by the iron response regulator Fur (26). In *E. coli*, it has been proposed that an intracellular pool of free or loosely bound iron exists (39). When intracellular iron levels are high, the Fur-apoprotein binds ferrous ions. In this form, ferrated Fur acts as a

repressor of transcription of iron uptake genes (1). The isolation of dominant negative mutations in the *E. coli fur* gene indicated that Fur binds as a dimer or possibly a multimer to DNA (9). The DNA binding site for Fur is a 19 bp repeat known as the Fur-box, which is located in the promoter region of Fur-regulated genes (19). When the intracellular iron pool becomes depleted, Fur is present as the apoprotein and is unable to function as a repressor. Thus, when iron is limited, iron uptake genes are derepressed.

Bacterial genes for control of oxidative damage

In addition to iron uptake genes, two other groups of genes have been shown to be regulated by Fur in response to iron availability--genes involved in oxygen defense mechanisms and virulence genes (9). Prevention of oxygen-mediated cell damage is important during iron uptake, because of the potential for superoxide generation (21). Transition metals such as iron are able to catalyze the one-electron reduction of O_2 to superoxide (O_2^-). Superoxide causes cellular damage by attacking proteins containing iron-sulfur clusters. More importantly, superoxide can dismutate spontaneously to hydrogen peroxide (H_2O_2). This in turn can react with iron via the Fenton reaction to produce hydroxyl radicals:

$$H_2O_2 + Fe^{2+} \dashrightarrow OH\cdot + H_2O + Fe^{3+} \qquad (21)$$

Hydroxyl radicals are highly reactive and will oxidize virtually any biomolecule, including membrane lipids and DNA.

Bacteria synthesize two types of enzymes to protect them from damage by oxygen radicals--superoxide dismutases (SODs) and catalases. SODs, as their name indicates, catalyze the dismutation of O_2^- to H_2O_2. This H_2O_2 is then prevented from causing cellular damage by the action of catalases, which convert hydrogen peroxide to $H_2 + O_2$.

E. coli produces three different SODs--FeSOD, MnSOD, and a newly discovered Cu/ZnSOD (5). Whereas FeSOD is expressed constitutively, expression of Mn-cofactored

SOD is regulated by Fur (36). The coordinate regulation of the gene that encodes MnSOD (*sodA*) with those for iron uptake may thus be a security measure against iron-catalyzed oxidative damage by reactive oxygen species after derepression of iron uptake genes (16).

It has been suggested that in addition to detoxifying reactive oxygen species arising during metabolic processes such as iron uptake, SODs and catalases may contribute to the pathogenesis of certain bacteria. An important killing mechanism of macrophages and neutrophils is the oxidative burst. In the oxidative burst, glucose is oxidized through the hexose monophosphate shunt, producing NADPH, the substrate of NADPH oxidase. NADPH oxidase is a complex that forms an electron transfer chain able to convert molecular oxygen into superoxide (33). The ability to resist the oxidative burst might therefore enable some pathogens to survive within mononuclear phagocytes. For example, mutants of *Nocardia asteroides* lacking a SOD have been shown to be attenuated for virulence (4). There are also data suggesting that *S. typhimurium* must withstand the oxidative burst of macrophages to cause disease. The DNA repair enzymes RecA and RecBC, which repair oxygen-induced DNA damage, are necessary for survival of *Salmonella* within macrophages and for virulence in mice (12).

Virulence genes

Iron limitation can serve as an environmental signal for the expression of bacterial virulence genes inside a mammalian host. Bacterial toxins such as the Shiga-like toxin produced by enterohemorrhagic*E. coli*, the hemolysins of *Serratia marcescens*, *Vibrio cholerae*, and *E. coli*, and the Shiga toxin of *Shigella dysenteriae* are all regulated by Fur (9). It has been postulated that these toxins assist in bacterial iron uptake by lysing host cells and releasing heme or hemoglobin. In addition, genes involved in the adaptive acid tolerance response of *S. typhimurium* have been shown to be regulated by Fur (24).

Bacterial iron uptake mechanisms

Siderophore-mediated iron uptake

Pathogens are able to grow in diverse host environments including the acidic stomach, the anaerobic intestine, the serum, and intracellular vacuoles. In these various niches, the pathogens presented with different sources of iron. Thus, it is not surprising that pathogens have evolved a variety of strategies to acquire iron in the many environments they face during growth in their mammalian hosts. Of these, the best studied is the utilization of siderophores, which are low molecular weight iron binding compounds. Due to their high affinity for iron, siderophores are able to scavenge iron from transferrin. Bacteria that produce particular siderophores also possess high-affinity uptake systems for the assimilation of the bound iron (reviewed in (8, 18)). Some bacteria are also able to utilize siderophores produced by other bacterial species or by fungi. For example, *S. typhimurium*, which produces enterobactin and (in 4% of isolates) aerobactin, is also able to utilize iron bound to a wide range of other siderophores, including the clinically used chelator desferoxamine (Desferal) (31, 33)

Siderophores are taken up by Gram-negative bacteria in a two-step process. In the first step, the siderophore binds to an integral outer membrane receptor. Many siderophore receptors have been characterized and found to be similar in structure. They are thought to consist of membrane spanning β-strands that form a barrel in the outer membrane. These membrane-spanning segments are connected by "loops" at the cell surface and in the periplasm. One surface loop ("gating loop") closes the pore formed in the outer membrane by the β-barrel. These surface-exposed loops are also thought to bind the siderophore. Upon siderophore binding, a conformational change occurs in which the gating loop opens and the siderophore can pass through the pore. The conformational change in the receptor required for translocation of the siderophore through the outer membrane requires energy. Since the outer membrane has no energy potential, this transport step is energized by a cytoplasmic membrane protein, TonB, which acts as an "extension cord" to transmit the

potential of the cytoplasmic membrane to the outer membrane receptors (4, 7). Simultaneous binding of TonB and the siderophore to the outer membrane receptor triggers a conformational change in the receptor. This change results in translocation of the substrate from the surface to the periplasm. The periplasm contains binding proteins that target the substrate to an ABC (ATP binding cassette) transport system in the cytoplasmic membrane. Whereas the transport across the outer membrane is driven by proton motive force, the second transport step across the cytoplasmic membrane is ATP dependent. In addition, while the outer membrane receptor is highly specific for one siderophore, the cytoplasmic membrane permease is less selective and can transport several related substrates (8).

Utilization of host iron compounds

A second strategy used by pathogenic bacteria is the direct assimilation of host iron compounds. Transferrin and lactoferrin are accessible to bacteria on mucosal surfaces or in body fluids. Heme, another host iron compound, can be acquired from lysed or dead cells. In the human intestine, for example,the constant renewal of the mucosal epithelium (approximately every three days) releases dead epithelial cells into the lumen. Thus, a constant source of heme is available to intestinal bacteria. Heme can also be obtained directly from live cells by bacterial cytotoxins.

Organisms such as the pathogenic Neisseriae, *Haemophilus influenzae*, *Vibrio cholerae*, *Shigella* and *Yersinia* possess outer membrane receptors for heme, transferrin, lactoferrin and hemoglobin (17, 32). These receptors can either extract iron from the host compound at the cell surface or transport the entire compound into the bacterial cell (35).

Both siderophore-mediated iron uptake and assimilation of host proteins utilize ferric iron. Furthermore, these Iron(III) uptake mechanisms transport iron across the outer

membrane via integral outer membrane proteins belonging to the TonB-dependent family of receptors.

Iron(II) transport

Whereas the mechanisms mentioned above function in acquisition of Iron(III) from the environment, several organisms are also able to use iron(II). Iron(II) is present in the mammalian intestine, which is colonized by anaerobic and facultative anaerobic bacteria such as *E. coli*. In addition, Iron(II) is the form of iron that is incorporated into ferritin in mammalian cells. Thus, it may be a source of iron for intracellular bacteria. Yeasts and *Listeria monocytogenes* possess ferric reductases, which convert iron(III) present in the environment to Iron(II). In contrast to Iron(III), Iron(II) is soluble and able to diffuse across the bacterial outer membrane through porins. The only bacterial transport system for Iron(II) to be characterized at the molecular level is the Feo system of *E. coli* (28) The Feo operon contains two genes--*feoA* and *feoB*. Whereas the function of FeoA remains to be elucidated, FeoB appears to be a cytoplasmic membrane transport ATPase that functions as a pump for ferrous ions. Interestingly, this system has been found to function during growth of *E. coli* in the murine intestine (34).

Pathogenesis of mouse typhoid caused by *Salmonella typhimurium*

Penetration of the intestinal mucosa

S. typhimurium causes a disease in susceptible mice that is similar to typhoid fever caused by *S.typhi* in humans. In mice, only about 1% of the inoculum survive the acidic conditions of the stomach and go on to infect the intestine (15). *S. typhimurium* is enteroinvasive in mice and is able to attach to and invade enterocytes at the tips of the villi and M-cells in the Peyer's patches. It is believed that the ability of *S. typhimurium* to adhere to enterocytes and M-cells is encoded by distinct adhesins. The tropism of *Salmonella* for the Peyer's patches has been shown to be mediated by an adhesin associated

with the long polar fimbriae (3). The adhesins mediating association of *S. typhimurium* with enterocytes have not yet been found.

Although adhesion seems to require unique determinants for each cell type, the invasion apparatus mediates entry into both M-cells and enterocytes (2). This apparatus is encoded by the *inv* locus of the *Salmonella* chromosome and is a type III secretion system (25). The components of this system appear to form an injection apparatus for an unknown protein into the host cell. The signal transduction pathways triggered by the injection process cause rearrangements of the cell surface known as "ruffling," which result in internalization of *S. typhimurium*. Subsequent to invasion, enterocytes recover from the distortion of their apical membrane, but M-cells are destroyed (27). Following entry into M-cells and enterocytes, bacteria are detected in the Peyer's patches of the terminal ileum, from where they spread to the draining mesenteric lymph node (15).

Intracellular growth in the liver and spleen

From the mesenteric lymph node, *Salmonella* is thought to travel through the lymphatics into the bloodstream. Upon passage through the liver and spleen, bacteria are filtered out by the resident macrophages of these organs. There are several lines of evidence that indicate that *S. typhimurium* can grow in these cells. First, *S. typhimurium* mutants that are unable to survive within macrophages are avirulent (22). Second, experiments in which infected animals were treated with gentamicin, which is unable to penetrate into cells, demonstrated that *Salmonella* was still able to grow in the liver and the spleen, and must therefore be intracellular (20). Third, enrichment of infected splenic cell populations by FACS suggests that the majority of *Salmonella* recovered from the spleen are in macrophages (11). Finally, a mouse gene was identified which confers immunity to *S. typhimurium* infection. This gene, *Ity*, was found to be expressed in cells of the reticuloendothelial system. Resident macrophages from *Ity*^{s} mouse strains support growth

of *S. typhimurium,* while those isolated from *Ity*^{r} mice do not. In the liver and spleen of infected mice, bacteria undergo rapid multiplication--an inoculum of fewer than 10 bacteria given i.v. can reach levels of $>10^9$ within three to five days. In vitro, intracellular *S. typhimurium* have been shown to kill macrophages, possibly by induction of apoptosis (30). This may be the mechanism bacteria use to escape the macrophages. In the final stage of infection, a massive bacteremia precedes the death of infected animals.

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Manuscript #1

Role of *Salmonella typhimurium* Mn-Superoxide dismutase (SodA) in protection against early killing by J774 macrophages

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Published in Infection and Immunity, **63:** 1739-1744 (1995)

Summary

The *Salmonella typhimurium* gene for Mn-cofactored superoxide dismutase (*sodA*) was cloned by complementation of an *Escherichia coli sodA sodB* mutant for growth on minimal medium. Sequence analysis revealed an open reading frame of 618 base pairs, encoding a polypeptide with 97% identity to *E.coli* SodA. A *S. typhimurium sodA* mutant was created by allelic exchange and tested for the ability to survive in the murine macrophage-like cell line J774. Growth of bacteria under iron limiting conditions, inactivation of the Fur repressor, or expression of *sodA* from a plasmid resulted in increased resistance to early killing by J774 cells, which was abolished in the *sodA* mutant. These results suggest that resistance to the early oxygen-dependent microbicidal mechanisms of phagocytes involves the SodA gene product. The *S. typhimurium sodA* mutant was not significantly attenuated in mice, however, which suggests that resistance to early oxygen-dependent microbicidal mechanisms in vivo may play only a minor role in *Salmonella* pathogenesis.

Introduction

The killing of most extracellular organisms by mononuclear phagocytes depends upon this cell's capacity to convert oxygen to microbicidal metabolites, including reactive oxygen intermediates (ROI) such as superoxide anions and hydrogen peroxide. Aerobic bacteria contain several protective enzymes which detoxify active oxygen species: superoxide dismutases (SodA and SodB), catalases (KatG and KatE), glutathione synthetase (GshAB) and glutathione reductase (Gor) [10]. Several findings point to the importance of these enzymes in protection of bacteria from the oxidative killing mechanisms of host phagocytes. Exogenously added superoxide dismutase or catalase were shown to protect *E. coli* from phagocytic killing [1]. In addition, studies on initial survival within phagocytes of *katFG* and *sodB* mutants of *Shigella flexneri*, a close relative of *E. coli*, indicated that the most efficient protective mechanism against oxygen toxicity in this species is formed by superoxide dismutase, with catalase activity participating to a lesser extent [11]. Conflicting results were reported by a Papp-Szabò and coworkers, who showed that a *sodB* mutation in *E. coli* had no effect on killing by human PMN [26]. Similarly, *S. typhimurium* mutants in *katG* and *oxyR*, which are involved in defense against ROI were found to resist killing by human PMN as well as the wild type, and a *katE katG* double mutant was found to have equal sensitivity to murine macrophages as the wild type [5, 25]. Thus, the contribution of a particular ROI detoxifying enzyme in protection against phagocytic killing mechanisms may vary with the organism and the model system studied.

During studies on survival of *Nocardia* in polymorphonuclear (PMN) leukocytes it was demonstrated that bactericidal activity at early time points was primarily due to oxidative metabolism, whereas killing after three hours was by both oxidative- and non oxidative mechanisms [1]. Resistance to early killing in phagocytes is therefore most likely mediated by proteins which provide protection against oxygen-dependent microbicidal mechanisms.

Since *Salmonella typhimurium* is able to persist within macrophages in the liver and spleen of the mouse, it must also be able to circumvent these oxidative killing mechanisms. Since the manganese cofactored superoxide dismutase (MnSOD), SodA, is induced under conditions shown to exist intracellularly (low iron; [12]), it might play an important role in defense against macrophage-induced oxidative damage in *S. typhimurium*. In this study we investigated the role of MnSOD in survival of *S. typhimurium* within the macrophage-like cell line J774 and in mouse virulence.

Materials and Methods:

Bacterial strains, media and growth conditions. The strains used are listed in table 1. All bacteria were cultured aerobically at 37°C. Antibiotics, when required, were included in the culture medium or plates at the following concentrations: kanamycin 60 mg/l, carbenicillin, 100 mg/l, chloramphenicol 20 mg/l, tetracycline, 20 mg/l. Complementation of the growth defect of QC774 (*sodA sodB*) was performed on M9 agar plates [21]. A spontaneous nalidixic acid resistant derivative of *S. typhimurium* ATCC14028 was selected by plating 10^9 bacteria on LB plates containing 50 mg/l nalidixic acid [31].

Conjugation. Conjugation between bacterial strains was performed over night on LB agar plates. For selection on minimal plates, conjugation mixtures were resuspended and washed with 1x M9 salts (to remove residual nutrients from the LB agar) before plating on M9 agar. For allelic exchange using the suicide vector pEP185.2 [15], conjugation mixtures were plated on LB selective plates, and individual colonies screened for loss of the vector resistance marker on LB agar containing 20 mg/l chloramphenicol.

Recombinant DNA techniques. Plasmid DNA was isolated using ion exchange columns from Qiagen (Hilden, Germany). Standard methods were used for restriction endonuclease analyses, ligation and transformation of plasmid DNA and isolation of chromosomal DNA from bacteria. The construction of the gene bank from *S. typhimurium* ATCC14028 in the cosmid vector pLAFRII has been described elsewhere [20]. Sequencing was performed by the dideoxy chain termination method according to a protocol of Kraft using α-[^{35}S]-dATP (Amersham, Arlington Heights, IL) for labeling [17].

The coding sequence of *sodA* was cloned without its upstream regulatory sequence by polymerase chain reaction (PCR) amplification using Taq polymerase and the primers 1: 5'-GCTCGACAACCATGGAGATGATTATGAG-3' and 2: 5'-ACTCGCTTCTAGAGACGTGCAATGC-3'. The 695 bp PCR product was cloned behind the IPTG inducible Trc promoter in the vector pTrc99A (Pharmacia, Alameda, CA) using the enzymes *Xba*I and *Nco*I.

Southern hybridization. Southern transfer of DNA onto a nylon membrane was performed as previously described. Labeling of DNA probes, hybridization and immunological detection were performed using the DNA labeling and detection kit (non-radioactive) from Boehringer-Mannheim (Mannheim, Germany). The DNA was labeled by random primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate. Hybridization was performed at 65°C in solutions without formamide. For Southern hybridization with cosmids of a gene bank or with chromosomal DNA a nonstringent wash (10 minutes at room temperature in 2 x SSC, 0.1% SDS) and a stringent wash (30 minutes at 65°C in 0.2 x SSC, 0.1% SDS) were performed. Hybrids were detected by an enzyme linked immunoassay using an antibody conjugate (anti-digoxygenin alkaline phosphatase conjugate) and the substrate AMPPD (3-(2'-spiroademantane)-4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxethane, Boehringer-Mannheim). The light emitted by the dephosphorylated AMPPD was detected by X-ray film.

Computer analysis. The nucleotide sequences were compared to nonredundant updates of SWISS-PROT, PIR(R), and GenPept using the program blastX, and were compared to non redundant updates of GenBank and EMBL using the program blastN. Nucleotide sequences were further analyzed using the PC/GENE software package.

SOD activity assay. Bacterial lysates were prepared by a modification of the procedure described by Touati [32]. A volume of an overnight culture in LB equivalent to 5 x 10^9 bacteria (calculated by optical density measurements at 578 nm) was centrifuged briefly at 14,000 rpm to pellet cells. Cells were washed by resuspending in PBS and pelleting again. Bacteria were then resuspended in 1/20 volume of 10mM Potassium phosphate, 0.1mM EDTA buffer containing 0.3mg/ml lysozyme and subjected to 10 freeze-thaw cycles by dipping tubes alternately for 1 min. in an ethanol-dry ice bath and a 42°C water bath. Lysates were cleared by centrifugation at 14,000 rpm for 10 minutes. Total protein content of the lysates was determined by the Bradford assay [3]. Equivalent amounts of total protein were loaded onto a polyacrylamide gel run under nondenaturing conditions. SOD bands were visualized in gels by the activity staining method of Beauchamp and Fridovich [2].

Cell culture techniques and macrophage survival assay. The macrophage cell line J774 was cultivated in Dulbecco's modified Eagle's medium (DMEM; GibcoBRL, Gaithersburg, MD) supplemented with 10% heat-inactivated equine serum (Gibco), 1% non-essential amino acids, and 1 mM glutamine (DMEMsup). J774 cells were tested periodically for production of oxygen radicals via the hexose monophosphate shunt (oxidative burst) after induction with phorbol myristate acetate (PMA, Consolidated Midland Corp., Brewster, NY) as described elsewhere [9].

For macrophage survival assays, the bacteria were grown over night, washed in phosphate buffered saline (PBS) and opsonized in fresh mouse serum for 20 min. 24-well microtiter plates were seeded with macrophages at a concentration of $5x10^5$ cells/well in 0.5 ml of DMEMsup and incubated over night at 37°C in 5% CO_2. The bacterial cultures were then diluted and about 5 x 10^6 bacteria in 0.25 ml of DMEMsup were added to each well of macrophages. To create iron limitation the overnight cultures were grown in LB plus 0.2 mM 2,2' dipyridyl, harvested and grown for two hours in DMEMsup plus 2

mg/ml apotransferrin (Boehringer Mannheim) and 10 mM sodium bicarbonate buffer. After opsonization the bacteria were diluted in DMEMsup plus 2 mg/ml apotransferrin and 10 mM sodium bicarbonate buffer.

Microtiter plates were centrifuged at 250 x g for 5 min. at room temperature in order to synchronize infection. Cells were incubated for 15 min. at 37°C in 5% CO_2, free bacteria were removed by three washes with PBS, and the zero time point was taken as described below. The washing solutions were collected and extracellular bacteria were quantified by dilution in sterile PBS and plating on LB agar. DMEMsup plus 6μg gentamicin per ml was added to the wells, and the cells were incubated at 37°C in 5% CO_2. Wells were sampled at appropriate time points after infection by aspirating the medium, lysing the macrophages with 0.5 ml of 1% deoxycholate and rinsing each well with 0.5 ml of PBS. Viable bacteria were quantified by dilution in sterile PBS and plating on LB agar. All experiments were performed independently at least three times and the standard error for each time point calculated.

Infection of mice. Virulence of the *sodA* mutant was tested by infecting 6 to 8 week old female BALB/c ByJ mice obtained from Jackson Laboratories (Bar Harbor, ME). Serial tenfold dilutions ranging from 9.2×10^7 to 9.2×10^4 were made in LB and 0.2 ml of these dilutions administered intragastrically (i.g.) to groups of four mice. Mortality was recorded at 28 days post infection and the 50% lethal dose (LD_{50}) values calculated by the method of Reed and Muench [28]. Stability of the *sodA* mutation in vivo was assessed by plating the liver and spleen of a moribund infected mouse. Liver and spleen were each homogenized in 1 ml of PBS using a Stomacher (Tekmar, Cincinnati, OH) and 0.1 ml of diluted homogenate plated on both LB+Nal and LB+Kan. Viable counts were compared on each plate to determine stability of the *sodA::kan* mutation. From LB+Nal plates, ten colonies were picked and grown for SOD activity assays as described above.

Table I: Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Source
S. typhimurium		
ATCC 14028	wild type strain	ATCC
IR 715	nalidixic acid resistant derivative of ATCC 14028	I. Stojiljkovic (31)
SA1	IR715 *sodA::Km*	this study
E. coli		
LE392	F- *e14-(mcrA-)hsdR*514($r_k^- m_k^+$)*supE44 supF58lacY1 or Δ(lacIZY)6 galK2galT22 metB1trpR55*	lab collection
S17-1 *λpir*	*Prp thi recA hsdR*: chromosomal RP4-2 (Tn*1*::ISR*1 tet*::Mu Km::Tn7); *λpir*	lab collection
QC772	F- Δ*lac*4169 *rpsL* Φ (*sodA-lacZ*)49 Cm^r	D. Touati (7)
QC773	F- Δ*lac*4169 *rpsL* Φ (*sodB-kan*)1-Δ2 Km^r	D. Touati (7)
QC774	F- Δ*lac*4169 *rpsL* Φ (*sodA-lacZ*)49 Cm^r Φ (*sodB-kan*)1-Δ2 Km^r	D. Touati (7)
DH5α	*endA1hsdR17(rk-mk-) supE44t hi-1 recA1 gyrA relA1 Δ(lacZYA-argF) U169 deoR (φ80 dlac Δ (lacZ)M15)*	lab collection
pBluescript KS	colE1, *bla* Stratagene	
PTrc99A	P_{trc}, $lacI^q$, *bla* Pharmacia	
pMH152	pACYC184, fur-	K. Hantke (4)
pEP185.2	pGP704, pBluescript MCS, *cat*	J. Pepe (15)

Results

Cloning and sequencing of the *S. typhimurium sodA* gene

In order to clone the *S. typhimurium sodA* gene, we used a strategy which has been described previously (for example [6]; [13]. The *E. coli* strain QC774 (*sodA sodB)* exhibits a growth defect when grown aerobically on minimal medium. Growth can be restored by introduction of either *sodA* or *sodB* on a plasmid. We therefore attempted to clone *S. typhimurium sodA* by complementation of QC774 for growth on minimal medium. A cosmid library of *S. typhimurium* ATCC 14028 constructed in pLAFRII was introduced by conjugation into QC774 on LB agar. Twelve exconjugants were picked and assayed for SOD activity [2]. One of these clones, designated pSA8.0, showed SOD activity corresponding to *E. coli sodA* on an SOD activity gel. This cosmid was digested with *Eco*RV and the fragments cloned into pBluescriptKS. A pool of subclones was used to transform QC774 and the transformants were tested for complementation of the growth defect on minimal medium. A clone able to grow on M9 plates was found to contain a plasmid (pSA8.1) carrying a 2 kb *Eco*RV fragment. Plasmid pSA8.1 was shown to encode *sodA* by activity staining (data not shown). For sequencing, further subclones of pSA8.1 were constructed using the enzymes *Sal*I, *Pst*I and *Eco*RI.

Sequence analysis revealed an open reading frame of 618 bp (Fig. 1). A potential Fur box located upstream of the open reading frame was identical to the Fur box located in the promoter region of *E. coli sodA* [23](Fig. 1). The deduced amino acid sequence of the open reading frame shared 97% identity with *E. coli* SodA (Fig. 2). These data show that the cloned DNA fragment encoded *sodA* and therefore confirmed the data obtained by complementation of QC774 and SOD activity staining.

Figure 1: DNA sequence and deduced amino acid sequence of *S. typhimurium sodA*. Potential -10 and -35 sequences and a potential Fur binding site (identified by homology to the *E. coli* sequence) are shown. *Eco*RI sites used to generate the *sodA* mutation are indicated. Annealing sites of primers 1 and 2 used to amplify the *sodA* coding sequence by PCR are shown with arrows. This sequence has been assigned GenBank accession number U20645.

```
   1 CGCCGCC CGT TTT CTC TTC CAC TTG CTG CCC CAG GAA GTG CAA CGC ATC
  50 TTT TAC CGG CTG GCT ATT TTC AGC GTA GGC CAG GCG CAA AAT GGT TTC
  98 GCC GTG GGA GAA GAA AGA GAG TGT GGA GAG TAA GGC CAA CGT AGC CAG
 146 ACG TAT AAA ACC AGG TTG CTT CAT GAT TCC CTC GCA ATT GTA ATA ATT
 194 TAC CGG GAA TGT AAT TGC AGT GTG AAA TTA TAA CCT TCT TGA TTT TGC
 242 CAC CGC TGA CAA AAT TTG GCA CCA AAA GAA AGA TTT TGA AAC CCT GTT
                                   EcoRI
 290 TCA CAT TTC ATG ATT TCC AGG AAT TCA TCA ACA GGC GGT TTC GGT TGG
 338 AGG CGT AAA AAA CGG TTT TTT CAG CGG ATG CCG TAA CGT TTA TAA CCC
                                                -35
                                     Fur box
 386 TGG AAA AAG TAC GGC ATT GAT AAT CAT TTT CAA TAT CAT TTA ATT AAC
       -10                                                 >
 434 TAT AAT GAA CCA ACT GCT TAC GCG GCG TTA ACA CTG TGC CGC TCG ACA
          Primer1               >
 482 ATA ATG GAG ATG ATT ATG AGT TAT ACA CTG CCA TCC CTG CCG TAC GCT
                          Met Ser Tyr Thr Leu Pro Ser Leu Pro Tyr Ala

 530 TAT GAT GCA CTG GAA CCG CAC TTC GAT AAG CAG ACG ATG GAG ATT CAC
     Tyr Asp Ala Leu Glu Pro His Phe Asp Lys Gln Thr Met Glu Ile His

 578 CAC ACC AAA CAC CAT CAA ACC TAT GTC AAC AAC GCT AAC GCG GCG CTG
     His Thr Lys His His Gln Thr Tyr Val Asn Asn Ala Asn Ala Ala Leu

 626 GAA AAC CTG CCT GAG TTT GCC AGC CTG CCG GTT GAA GAA CTG ATT ACT
     Glu Asn Leu Pro Glu Phe Ala Ser Leu Pro Val Glu Glu Leu Ile Thr

 674 AAA CTG GAC CAG GTG CCA GCG GAC AAA AAA ACT GTG CTG CGT AAC AAC
     Lys Leu Asp Gln Val Pro Ala Asp Lys Lys Thr Val Leu Arg Asn Asn

 722 GCG GGC GGC CAT GCT AAC CAC AGC CTG TTC TGG AAA GGG CTG AAA ACA
     Ala Gly Gly His Ala Asn His Ser Leu Phe Trp Lys Gly Leu Lys Thr

 770 GGC ACC ACT CTG CAG GGT GAT CTG AAA GCG GCT ATC GAG CGT GAC TTC
     Gly Thr Thr Leu Gln Gly Asp Leu Lys Ala Ala Ile Glu Arg Asp Phe
                                        EcoRI
 818 GGT TCC GTT GAC AAC TTC AAA GCT GAA TTC GAA AAA GCA GCA GCA ACC
     Gly Ser Val Asp Asn Phe Lys Ala Glu Phe Glu Lys Ala Ala Ala Thr

 866 CGT TTC GGC TCC GGC TGG GCG TGG CTG GTG CTG AAA GGC GAC AAA CTG
     Arg Phe Gly Ser Gly Trp Ala Trp Leu Val Leu Lys Gly Asp Lys Leu

 914 GCT GTG GTT TCT ACC GCA AAC CAG GAT TCC CCG CTG ATG GGT GAA GCC
     Ala Val Val Ser Thr Ala Asn Gln Asp Ser Pro Leu Met Gly Glu Ala

 962 ATT TCC GGC GCT TCC GGC TTC CCG ATC CTG GGC CTG GAC GTG TGG GAA
     Ile Ser Gly Ala Ser Gly Phe Pro Ile Leu Gly Leu Asp Val Trp Glu

1010 CAC GCT TAC TAC CTG AAA TTC CAG AAC CGC CGC CCG GAC TAC ATC AAA
     His Ala Tyr Tyr Leu Lys Phe Gln Asn Arg Arg Pro Asp Tyr Ile Lys

1058 GAG TTC TGG AAC GTG GTG AAC TGG GAC GAA GCA GCA GCG CGT TTC GCG
     Glu Phe Trp Asn Val Val Asn Trp Asp Glu Ala Ala Ala Arg Phe Ala
                 <              Primer 2                 <
1106 CTA AAA TAA TTT GCA TTG CAC GTC TGT AGA AGC GAG TCT GAT GAC TCG
     Leu Lys
1154 CTT TTT TTG TAT CCG CGT AAG GAG CAG CAG ATG CAT TAT CCG GTT GAC
1202 GTG TTT ATT GG
```

Construction of an *S. typhimurium sodA* mutant

A chromosomal *sodA* mutant of *S. typhimurium* IR715 was created by marker exchange. A pBluescriptKS derivative lacking the *Eco*RI polylinker site was created by digestion with *Eco*RV and *Sma*I and subsequent religation. The 2 kb *Eco*RV insert in pSA8.1 was cloned into this pBluescriptKS derivative to create plasmid pSA8.5. The kanamycin resistance cassette KIXX (Pharmacia) was introduced into the *Eco*RI sites indicated at nucleotides 310 and 842 of Fig. 1. The resulting insert was cloned into the suicide vector pEP185.2 using enzymes *Xba*I and *Kpn*I and the host strain S17-1λ *pir* [30] for propagation of the suicide vector. This construct (pSA8.8) was mated into IR715 and exconjugants selected on plates containing kanamycin and nalidixic acid. Exconjugants were restreaked on plates containing chloramphenicol to test for loss of the suicide vector. Exconjugants sensitive to chloramphenicol but resistant to kanamycin originate from allelic exchange between the chromosomal *sodA* and the mutated copy on pSA8.8.

To confirm the marker exchange, one of these exconjugants, designated SA1, was characterized further by Southern hybridization (Fig. 3). Using the insert of plasmid pSA8.1 as a probe, a 2 kb and a 3.2 kb fragment were detected in *Eco*RV digested chromosomal DNA of IR715 and SA1, respectively. The change in size of 1.2 kb is that which would be expected as a result of the allelic exchange. In addition, SA1 was found to show no detectable SodA activity by SOD activity staining (Fig. 4A). Introduction of pSA8.1 into SA1 resulted in SodA activity, as detected by activity staining (Fig. 4A). These results confirmed the inactivation of *sodA* by marker exchange in SA1.

```
E. c.          MSYTLPSLPYAYDALEPHFDKQTMEIHHTKHHQTYVNNANAALESLPEFA
S. t.          --------------------------------------------N-----

E. c.          NLPVEELITKLDQLPADKKTVLRNNAGGHANHSLFWKGLKKGTTLQGDLK
S. t.          S------------V--------------------------T---------

E. c.          AAIERDFGSVDNFKAEFEKAAASRFGSGWAWLVLKGDKLAVVSTANQDSP
S. t.          ----------------------T---------------------------

E. c.          LMGEAISGASGFPIMGLDVWEHAYYLKFQNRRPDYIKEFWNVVNWDEAAA
S. t.          --------------L-----------------------------------

E. c.          RFAAKK
S. t.          ---L-
```

Figure 2: Comparison of the deduced amino acid sequence of *E. coli* (E.c.) and *S. typhimurium* (S.t.) SodA. Identical amino acids are indicated by asterisks. The sequence alignment was prepared using the CLUSTALV program.

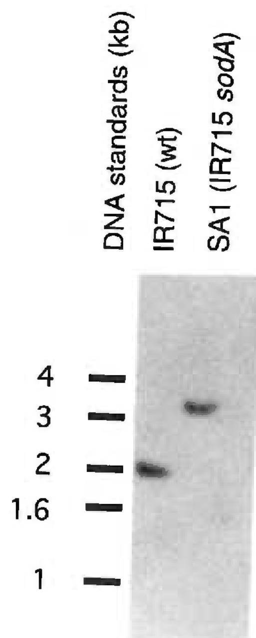

Figure 3: Southern hybridization of EcoRV digested chromosomal DNA prepared from IR715 and SA1 (IR715 *sodA*) with a probe containing *sodA*. Sizes and positions of DNA standards are given on the left.

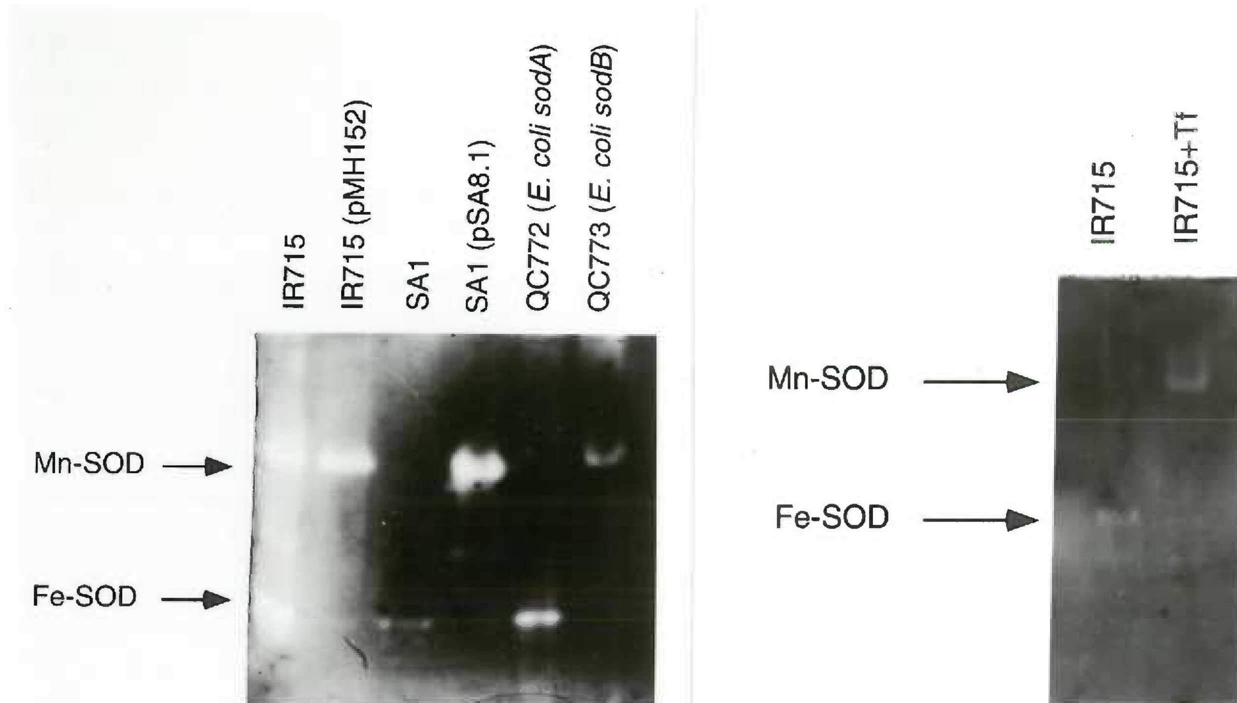

Figure 4 A: SOD activity gel of *S. typhimurium* IR715 and derivatives. Arrows indicate bands of SOD activity corresponding to *E. coli* SodA (MnSOD) and SodB (FeSOD). About 30mg of total protein (20ml of lysate) was loaded per lane. **B:** SOD Activity of IR715 grown under iron replete (LB) and iron limiting (DMEM+2 mg/ml apotransferrin; Tf) conditions. About 10mg of total protein was loaded per lane. Lysates were prepared and activity staining performed as described in Materials and Methods.

Survival of SA1 in J774 macrophages

In order to determine whether MnSOD is involved in bacterial defense against oxygen dependent microbicidal mechanisms of macrophages, strains IR715 and SA1 were tested for their ability to survive in the cell line J774. Since the oxidative burst is thought to occur immediately upon contact of macrophages with microbes [1], we investigated the ability of *S. typhimurium* to survive the initial contact with J774 cells (Fig. 5). The *sodA* mutant strain survived in macrophages at a rate similar to the wild type (Fig. 5). These data thus indicated that under the assay conditions used MnSOD activity does not contribute significantly to macrophage survival of *S. typhimurium.*

In *E. coli*, *sodA* expression is subject to regulation by six regulatory proteins [8]. Therefore, in order to ensure expression of *sodA* under the assay conditions used, using PCR we cloned a promoterless *S. typhimurium sodA* gene into plasmid pTrc99A (Pharmacia), yielding pSA8.9. The annealing sites for the primers used to amplify *sodA* are indicated in Fig. 1. In pSA8.9 *sodA* expression is under the control of the *trc* promoter. In this construct, we found the trc promoter to be “leaky”, allowing high levels of expression of MnSOD even without IPTG induction (data not shown). Addition of IPTG increased MnSOD expression even further. Plasmid pSA8.9 was introduced into SA1 and the resulting strain (pregrown without IPTG) tested for survival within J774 cells. The number of bacteria recovered from macrophages one hour after infection of J774 cells increased three to nine-fold as compared to SA1 or IR715 (Fig. 5). SOD activity gels showed high levels of MnSOD activity in SA1(pSA8.9) (data not shown). Thus, the elevated level of MnSOD present in SA1(pSA8.9) seemed to confer protection against early killing in J774 cells. Introduction of the empty vector, pTrc99A, into SA1 had no effect on survival within J774 cells (data not shown).

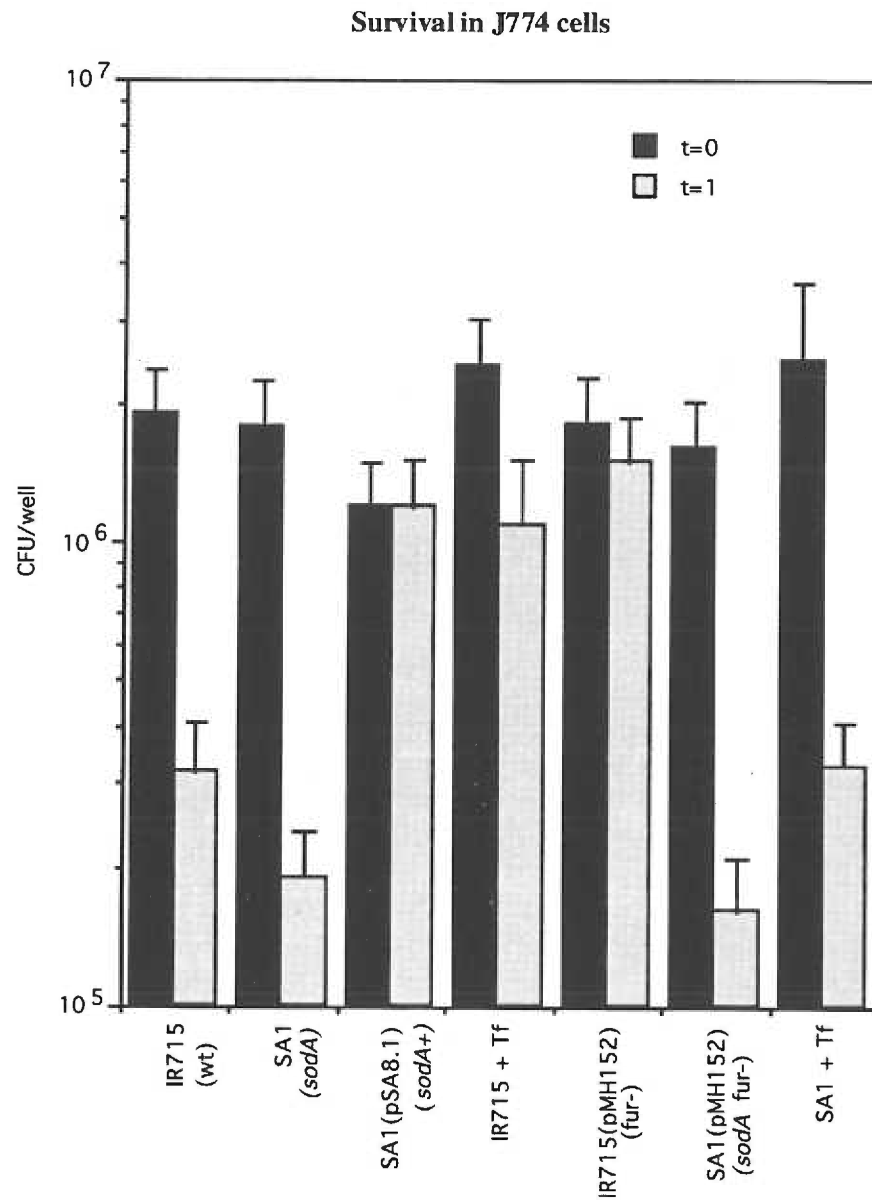

Figure 5: Survival of *S. typhimurium* strains in J774 cells. Strains were assayed for survival at 0 and 1h after infection of cells as described in Materials and Methods. Bars indicate averages of at least three experiments +/- SEM. Tf indicates growth of bacterial inoculum with 2 mg/ml apotransferrin to create iron starvation.

In order to determine whether elevated amounts of MnSOD are also expressed under conditions which more closely resemble the environment in the host, we performed macrophage survival assays using bacterial inocula pre-grown under iron limiting conditions. In *E. coli*, *sodA* is repressed by the iron response regulator Fur. Fur has been shown to strongly repress *sodA* expression if *E. coli* is grown in iron rich medium [8, 23]. If the iron concentration decreases, Fur dissociates from the *sodA* promoter thereby allowing elevated expression of *sodA*. The availability of iron for microbes has been shown to be low in serum as well as in an intracellular habitat [12] and thus iron limitation may more closely resemble in vivo growth conditions. IR715 did indeed survive better in macrophages if the bacteria were iron starved prior to infection (Fig. 5). We next investigated whether this increased bacterial survival could also be observed in the absence of Fur. Negative complementation was used to create a fur^- phenotype in IR715. By introducing a mutated *fur* gene carrying a point mutation in the DNA binding domain on a low copy number plasmid (pMH152), inactive heterodimers are formed which are unable to bind DNA. As a result, the merodiploid strain behaves as a *fur* mutant with respect to expression of *fur*-regulated genes[4]. Like expression of *sodA* in *Salmonella* from a plasmid or iron starvation of IR715, negative complementation resulted in increased resistance to early killing in J774 cells (Fig. 5). An increased amount of MnSOD activity was detected in IR715(pMH152) by SOD activity staining (Fig. 4A). This was accompanied by a decrease in FeSOD (SodB) activity. This finding is in agreement with the results of Niederhoffer et al., who found that *sodB* expression was strongly reduced in a *fur* mutant [23]. The relative decrease in FeSOD was also observed when IR715 was grown under iron-limiting conditions (Fig.4B). In order to ensure that the increased resistance to macrophage killing was due to derepression of *sodA*, plasmid pMH152 was introduced into the *sodA* mutant SA1 and the resulting strain tested for survival in J774 cells. The *sodA fur* merodiploid strain survived at rates similar to the parent SA1. In

addition, no increase in survival was observed when SA1 was iron starved prior to infection of macrophages (Fig. 5). Thus, the increase in macrophage survival caused by inactivation of Fur or pregrowth of bacteria under iron limiting conditions can be abolished by a mutation in *sodA*. These data show that under conditions of iron starvation elevated levels of MnSOD contribute to survival of *S. typhimurium* in J774 cells.

Virulence of the *S. typhimurium sodA* mutant in mice

In order to determine whether the increased resistance to macrophage killing mediated by elevated levels of MnSOD is important in vivo, the virulence of SA1 was compared with that of its parent, IR715, in the murine typhoid model of infection. The *sodA* mutant was found to be only slightly attenuated in mice. After intragastric infection, the LD_{50} of SA1 was 1.6×10^6 bacteria, while IR715 had a LD_{50} of 6×10^5 bacteria. The *sodA* mutation did not revert in vivo, as equal numbers of bacteria were enumerated on plates containing nalidixic acid (resistance of the parent strain) and kanamycin from the liver and spleen of an infected mouse. In addition, ten colonies isolated from the spleen and picked at random showed no SodA activity when examined by SOD activity staining of extracts. Thus, under the conditions used for infection, MnSOD does not appear to play a crucial role for *S. typhimurium* virulence in mice.

Discussion

In this report we demonstrated that high levels of MnSOD can protect *S. typhimurium* against early killing by J774 cells. These expression levels can be achieved by growth of bacteria under iron-limiting conditions, expression of *sodA* from a strong promoter, or by inactivation of the Fur repressor. Growth under iron deficiency, which induces expression of *sodA*, is likely to more closely resemble the in vivo situation since *Salmonella* faces a low iron (transferrin-containing) environment (body fluids) prior to phagocytosis. Genes repressed by Fur have also been shown to be induced during growth in epithelial cells, which precedes contact with the lymphatic fluid during the course of a *Salmonella* infection [12].

Although elevated MnSOD levels were protective in the intracellular survival assay using J774 cells, the *sodA* mutant was only weakly attenuated in the mouse model of infection. One possible explanation for this apparent discrepancy is that bacteria are equipped with multiple enzymes to protect them against oxidative damage. A defect in only one enzyme may thus be insufficient to render the bacterium incompetent to withstand oxidative stress in vivo. In *E. coli*, single mutations in *sodA* or *sodB* resulted in a slightly increased (up to 10-fold in rich medium) sensitivity to paraquat in vitro, whereas the double mutant *sodA sodB* was approximately 1000-fold more sensitive to paraquat than the wild type [7]. The *sodA sodB* mutant also exhibits an aerobic growth defect on minimal medium, which has been attributed to the sensitivity of enzymes necessary for synthesis of branched chain amino acids to oxygen radicals [7]. This growth defect of the double mutant, which we presume would also occur in a *sodA sodB* mutant of *S. typhimurium*, would render it difficult to assess the role of SOD in virulence by determining the LD_{50} of the double mutant.

A second possible reason for the only moderate attenuation of the *S. typhimurium sodA* mutant in mice is that the defense against oxidative killing mechanisms of phagocytes is not crucial for a successful infection. This latter hypothesis is supported by the finding that *Salmonella typhi* and *S. typhimurium* have been reported to elicit little or no oxidative burst upon entry into phagocytic cells [16, 22, 33]. Other macrophage pathogens, such as *Legionella* and *Mycobacterium* have been shown to enter the macrophage via complement receptors [27, 29]. Uptake by this route has been shown not to trigger the release of oxygen intermediates by macrophages, and would thus allow these pathogens to avoid the toxic consequences of the oxidative burst. Similarly, complement receptors have been implicated in the uptake of *S. typhimurium* by macrophages [14]. Complement receptors are expressed at elevated levels in resident macrophages, as compared to activated macrophages [18]. *S. typhimurium* may persist in vivo preferentially in resident macrophages of the liver and spleen which are in a lower state of activation for oxidative killing [19, 24]. Thus, like other intracellular pathogens, *S. typhimurium* may evade the oxidative burst of phagocytes, perhaps by selecting the appropriate set of phagocytic receptors for entry into its intracellular niche. *S. typhimurium* may therefore elicit only a weak oxidative burst upon contact with its target phagocytes, making MnSOD function dispensable. The J774 cells used for the in vitro assays are different from these resident macrophages, which might explain the contribution of oxygen-dependent killing mechanisms in this in vitro model. The contribution of oxygen-dependent microbicidal activity against *S. typhimurium* in macrophages studied outside the native context of host organs may also differ from that occurring in vivo, which might explain the differences between results achieved in our in vivo and in vitro models.

Acknowledgments:

The authors would like to thank D. Touati, K. Hantke, J. Pepe and I. Stojiljkovic for providing bacterial strains, I. Stojiljkovic for suggestions on the manuscript, and S. Anic and J. Lipps for technical assistance.

AJB was supported by a stipend from the Deutsche Forschungsgemeinschaft Ba 1337/1-2.

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Manuscript #2

Fur regulon of *Salmonella typhimurium*: identification of new iron regulated genes

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Published in Journal of Bacteriology, 177:4628-4637 (1995).

Summary

In order to identify genes belonging to the Fur regulon of *Salmonella typhimurium*, a bank of 10,000 independent *S. typhimurium* Mud*J* insertion mutants was screened for *lacZ* fusions regulated by the iron response regulator Fur. In parallel, a plasmid gene bank of *S. typhimurium* consisting of 10,000 independent clones was screened for Fur regulated promoters or iron binding proteins using the Fur titration assay (FURTA). Fur regulated Mud*J* insertions and Fur regulated promoters were mapped. In addition, iron regulated promoter activity of transcriptional fusions from Mud*J* insertions and FURTA positive clones was quantified. The nucleotide sequences of 11 FURTA positive plasmids and of short fragments of DNA flanking three Mud*J* insertions were determined. Using these methods we identified 14 Fur regulated genes of *S. typhimurium*. For 11 of these genes Fur regulated homologues have been described in *Escherichia coli* or *Yersinia enterocolitica*, including *fhuA*, *fhuB*, *fepA*, *fes*, *fepD*, *p43*, *entB*, *fur*, *foxA*, *hemP*, and *fhuE*. In addition, we identified three genes which have not previously been shown to be Fur regulated in other bacteria.

Introduction

Iron availability is an environmental stimulus to which bacteria respond by regulating expression of genes. The set of genes which is regulated in response to changes in iron concentration forms the iron stimulon. Included in this group are genes which may be under the control of several different regulators; of these, the best characterized is Fur. Using ferrous ions as a corepressor, Fur has been shown to sense the intracellular iron concentration[9]. The Fe(II)-Fur complex binds a regulatory DNA sequence, designated Fur-box, which is located in the promoter region of Fur regulated genes [11, 16]. Fur regulation seems to be a general feature of iron metabolism in gram-negative bacteria, as numerous homologues of Fur have been described to date. Genes whose expression are under the control of this regulatory protein form the Fur-regulon.

The Fur-regulon contains genes which are involved in iron acquisition, a function which is essential for bacterial multiplication. For animal pathogens, efficient strategies for iron uptake are important adaptations for growth in the host since iron availability is limited. Nonspecific host defense mechanisms, collectively known as the "iron withholding response", further lower iron levels in the body during bacterial infection [54]. Although it is clear that iron uptake systems are a prerequisite for bacterial multiplication, the genes involved in iron uptake during growth of *S. typhimurium* in its host have not been described. A further host adaptation mechanism which requires Fur for regulation is the acid tolerance response [17]. *S. typhimurium*, in order to cause infection, must withstand the acid pH of the stomach and possibly of the intracellular compartment in which it resides. As a first step in identifying factors involved in iron uptake and acid tolerance within the host, we attempted to identify Fur regulated genes in *S. typhimurium*.

For the identification of genes belonging to a regulon, isolation of operon fusions to a reporter gene created by transposon mutagenesis has been used most frequently. An alternative approach has recently been developed which is useful for the identification of Fur regulated genes of a wide variety of gram negative and even some gram positive bacteria [48]. This technique, called FURTA for Fur titration assay, is based on the observation that a Fur-box introduced on a multicopy plasmid is able to compete with chromosomal Fur-boxes for the Fe(II)-Fur repressor. A plasmid encoded Fur-box is introduced into a strain, carrying a chromosomal reporter gene which is expressed from a Fur-regulated promoter. Upon introduction the plasmid encoded Fur-boxes titrate the Fe(II)-Fur complex, allowing expression of the reporter gene.

In this study we use two approaches, isolation of transcriptional fusions created by transposon mutagenesis, and FURTA, for the identification of Fur-regulated genes of *S. typhimurium*. Our results allow a direct comparison of these approaches and their usefulness in identifying genes of the Fur-regulon.

Materials and Methods

Bacterial strains, media and growth conditions. The strains used are listed in table 1. All bacteria were cultured aerobically at 37°C. Antibiotics, when required, were included in the culture medium or plates at the following concentrations: kanamycin 60 mg/l, chloramphenicol 20 mg/l, carbenicillin, 100 mg/l. In order to create iron limiting or iron sufficient growth conditions, 0.2 mM 2,2'dipyridyl or 0.04 mM $FeSO_4$ were added, respectively.

The bacteria were tested for auxotrophy on M9 agar plates [36]. If required, histidine was included at a concentration of 0.04 mg/ml (M9-his-agar plates).

Iron regulated *lacZ* operon fusions of *S. typhimurium* were screened on MacConkey agar plates containing either 0.04mM $FeSO_4$ (M+Fe) or 0.2 mM 2,2' dipyridyl (M-Fe), as described earlier [22]. MacConkey agar plates used for the Fur titration assay contained 0.04mM $FeSO_4$.

Chrome azurol S (CAS) plates used to assay for siderophore production were prepared according to Schwyn and Neilands [44]. Evans Blue-Uranine (EBU) plates were prepared according to Bochner [6].

The medium for Mn^{2+} selection of *fur* mutants reported previously [24] was prepared with one modification: the chelator Desferal was replaced by 2,2'-dipyridyl since the former can be utilized as a siderophore by *Salmonella* [25].

Utilization of ferrioxamine was detected by an agar diffusion assay. The strain to be tested was poured in 3 ml of 2% noble agar onto a Nutrient Broth-Dipyridyl (NBD) agar plate. Filter paper disks impregnated with ferrioxamine (10 µl of a 1mg/ml solution of Desferal in 0.1 M $FeCl_3$) were laid onto the top agar and, after incubation overnight at 37°C, the zone of growth around the filter disk was measured.

Genetic techniques. P22 was used for generalized transduction of transposon insertions into different genetic backgrounds. Transductants were routinely streaked on EBU plates to detect phage contamination before use in further experiments.

Mud*J* insertion mutants of *S. typhimurium* were isolated using the method of Hughes and Roth [27]. In brief, the strain 14028 was transduced with a P22 lysate of TT10288 and Mud*J* insertions were selected on LB plates containing kanamycin. The donor (TT10288) carries Mud*J* inserted in *hisD*. The transductants were therefore screened for homologous recombination of Mud*J* into the *hisD* gene by comparison of the growth on M9- and M9-histidine agar plates. Only histidine prototrophs were further investigated. To detect differences in β-galactosidase expression under different iron concentrations, mutants were streaked in parallel on M+Fe or M-Fe agar plates. Mutants with differences in color on these two media were characterized further by cross-streaking on M+Fe and overlaying with a filter strip soaked in a solution of 0.02 mM dipyridyl, as described previously [22]. The quantitative determination of β-galactosidase units has been published elsewhere [36].

The selection for *fur* mutants of *S. typhimurium* was carried out according to a protocol of Hantke [24]. Dependence of β-galactosidase expression on Fur was determined by transforming mutants with plasmid pMH152 [9]. Promoter activity of DNA fragments identified by Fur titration was investigated by cloning fragments into the promoter probe vector pUJ10 [14]. The quantitative determination of alkaline phosphatase activity has been described elsewhere [10].

Fur titration Assay (FURTA) Chromosomal DNA of *S. typhimurium* 14028 was digested partially with the restriction enzyme *Sau*3A, or to completion using *Eco*RV and *Hin*dII. Fragments between 0.5 and 3 kb were cloned into the vector pSUKS1 [40] digested with *Bam*HI or *Sma*I, respectively, and transformed into *E. coli* DH5α. A total of ca. 10,000 independent colonies were pooled into four groups and the plasmid DNA

isolated from these pools. Fur titration was assayed as previously described [48]. In brief, plasmid DNA from each pool was used to transform *E. coli* H1717 and transformants that were red rather than white on M+Fe were selected, plasmid DNA isolated and retransformed into H1717 to confirm the FURTA-positive phenotype.

Construction of strain derivatives for mapping with pulsed field gel electrophoresis. For physical mapping of Mud*J* insertion sites and FURTA clones, a set of suicide vectors was used to introduce *Xba*I and *Bln*I restriction sites at the map location of the cloned DNA. A *Bln*I site was introduced into the polylinker of pGP704[37] by creating a linker using the oligonucleotides 5'-TCGATCCTAGG-3' and 5'-TCGACCTAGGA-3'. The oligonucleotides were denatured and annealed and the resulting linker cloned into the *Sal*I site of pGP704 to create pMAP (Fig. 1). For mapping of Mud*J* mutants, a 1.8 kb *Eco*RI *Eco*RV fragment of the *E. coli* *lacZ* gene was isolated from pHSS6 and cloned into *Eco*RI *Eco*RV cut pMAP to yield pMAP1(Fig. 1). *E. coli* S17-1λpir was used for propagation of all suicide vector constructs and as a donor for introduction of these constructs into *S. typhimurium* IR715 [49] by conjugation.

Pulsed field gel electrophoresis. Preparation of agarose embedded chromosomal DNA for pulsed field gel electrophoresis was based on a protocol from Liu and Sanderson [32]. In brief, cells were harvested from 5 ml of an early log phase bacterial culture, resuspended in 0.5 ml prewarmed (37°C) cell suspension buffer (10 mM Tris/HCl pH 7.2, 20 mM NaCl, 100 mM EDTA), and mixed with 0.5 ml 2 % InCert agarose (InCert) which was dissolved in PBS (phosphate buffered saline) and precooled to 37°C. The mixture was poured into molds (Bio-Rad) and allowed to solidify. The molds were digested with lysozyme (1 mg/ml, NEB) in a volume of 7.5 ml for 1h at room temperature. The lysozyme solution was replaced by 7.5 ml proteinase K (1 mg/ml, NEB) and incubated at 55°C for 48h with shaking. Agarose embedded DNA prepared in this manner can be stored

in 7.5 ml TE buffer (10 mM Tris/HCl pH 7.2, 5 mM EDTA) at 4°C for several months without degradation.

For restriction digests, an agarose block equivalent of about 0.05 to 0.1 ml was incubated for 15 minutes at room temperature in 0.2 ml 1xKGB buffer [35] in which sodium acetate was replaced by potassium acetate. The buffer was replaced with 1xKGB buffer containing 20 U of a restriction endonuclease (*Xba*I or *Bln*I), and the samples were incubated for 4 h at 37°C. The agarose blocks were then loaded on an agarose gel for pulsed field gel electrophoresis.

Pulsed field gel electrophoresis of a 1 % agarose gel was performed in 0.5% TBE at 200 V with pulse length of 6 s-150 s for 6 h, 6 s-12s for 6 h, 26 s-36 s for 6 h, and 120 s-180s for 6 h using a CHEF-DRII apparatus from Bio-Rad.

Recombinant DNA techniques. Plasmid DNA was isolated using ion exchange columns from Qiagen. Standard methods were used for restriction endonuclease analyses, ligation and transformation of plasmid DNA [36]. Sequencing was performed by the dideoxy chain termination method according to a protocol of Kraft [30] using α-[^{35}S]-dATP (Amersham) for labeling or using the ALF automated sequencer (Pharmacia).

Inverse PCR was performed using agarose embedded chromosomal DNA from Mud*J* mutants, as described previously [5]. In brief, an agarose block equivalent to approx. 0.05 to 0.1 ml was incubated for 15 minutes at room temperature in 0.2 ml 1xKGB buffer [35] in which sodium acetate was replaced by potassium acetate. The buffer was replaced with 1xKGB buffer containing 50 U of *Alu*I, and the samples were incubated for 4 h at 37°C. The samples were then incubated for 20 minutes at 72°C (to inactivate the restriction

enzyme and to melt the agarose block). ATP (10 mM) and T4-DNA ligase (2 U) were added at 37°C and ligation performed over night at 15°C. Inverse PCR was performed under standard conditions [29] in a total volume of 0.1 ml using 0.005 ml of the above ligation as a template and 0.1 ng of each of the following primers: 5' CCAATTCTGCCCCGAATTAC 3', and 5' GTCGTGTAAAATATCGAGTTCG 3' . These primers are complementary to the left end of Mud*J* [52]. PCR products were cloned into the vector pCRII and transformed into the *E. coli* strain TA One Shot™ (TA-cloning kit, Invitrogen).

Southern hybridization. Southern transfer of DNA onto a nylon membrane was performed as previously described [2]. Labeling of DNA probes, hybridization, and immunological detection were performed using the DNA labeling and detection kit (non-radioactive) from Boehringer-Mannheim. The DNA was labeled by random primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate. Hybridization to the blot was performed at 68°C in solutions without formamide. Hybrids were detected by an enzyme linked immunoassay using an antibody conjugate (anti-digoxigenin alkaline phosphatase conjugate) and the substrate AMPPD (3-(2'-spiroademantane)-4-methoxy-4-(3''-phosphoryloxy)phenyl-1,2-dioxethane, Boehringer-Mannheim). The light emitted by the dephosphorylated AMPPD was detected by X-ray film.

Computer analysis. The nucleotide sequences were compared to SWISS-PROT, PIR(R), and GenPept at the National Center for Biotechnology Information (NCBI) using the program blastX, and to GenBank and EMBL using the program blastN [1, 20].

Table I: Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source
S. typhimurium		
ATCC 14028	wild type strain	ATCC
IR 715	nalidixic acid resistant derivative of ATCC 14028	I. Stojiljkovic (47)
AR396	14028 Mud*J* mutant	this study
AR895	14028 *sidK::MudJ*	this study
AR1258	14028 *entB::MudJ*	this study
AR3675	14028 *fhuB::MudJ*	this study
AR6687	14028 Mud*J* mutant	this study
AR8412	14028 Mud*J* mutant	this study
AR8439	14028 *ent::MudJ*	this study
SF895	AR895 *fur*	this study
SF1	14028 *fur*	this study
SL1346	*aroA554* (Tc^R)	B. Stocker (SGSC)
CL 1509	14028 *aroA554*	this study
E. coli		
S17-1 *lpir*	*Prp thi recA hsdR*: chromosomal RP4-2 (Tn*1*::ISR*1* *tet*::Mu Km::Tn*7*); *lpir*	lab collection
DH5a	*endA1hsdR17(rk-mk-) supE44t hi-1 recA1 gyrA relA1 D(lacZYA-argF) U169 deoR (f80 dlac D (lacZ)M15)*	lab collection
H1717	*araD139 DlacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR aroB fhuF::l*placMu	K. Hantke (23)
CC118	*araD139 D(ara, leu) DlacY74 phoA20 galE galK thi rpsE rpoB* $argE_{am}$ *recA1*	R. Taylor (32)
Plasmids		
pGP704	R6K ori, *bla*	J. Mekalanos (35)
pMAP	pGP704, *Bln*I site in polylinker	this study
pMAP1	pMAP, *'lacZ'*	this study
pBluescript KS	colE1, *bla*	Stratagene
pMH152	pACYC184, fur-	K. Hantke (9)
pMH15	pACYC184, *E. coli fur*	K. Hantke (22)
pUJ10	*'lacZ 'phoA, bla*	V. deLorenzo (14)
pSUKS1	pSU19 with *Hae* II fragment of pBCSK+	R. Schönherr (38)

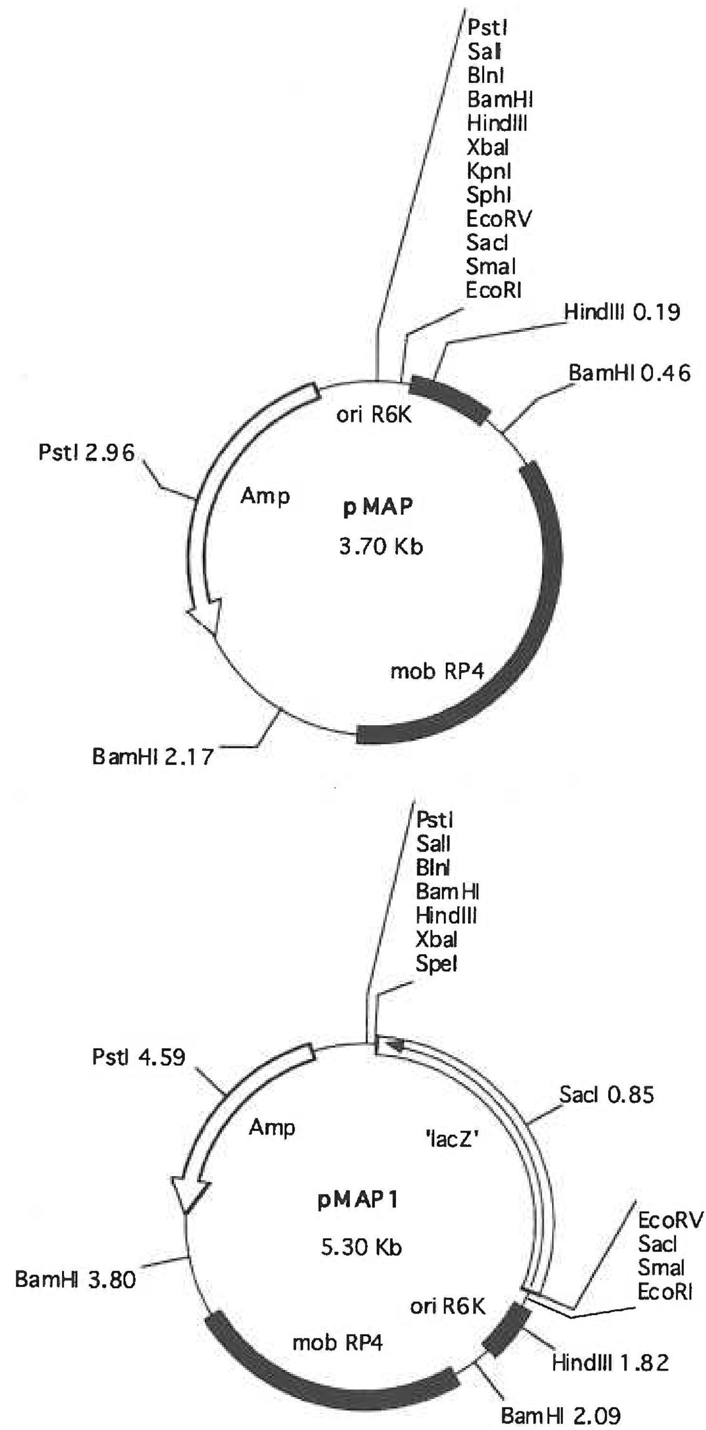

Figure 1: Restriction maps of suicide vectors pMAP and pMAP1.

Results

Screening for Mud*J* insertions in Fur-regulated genes

A bank of 10,000 independent Mud*J* insertion mutants of *S. typhimurium* 14028 was screened for differences in *lacZ* expression in response to variation of the available iron concentration on MacConkey agar plates. Of these mutants, 75 showed elevated levels of β-galactosidase expression in response to iron restriction. These fusions define genes belonging to the iron stimulon.

In order to identify fusions regulated by Fur, we compared expression of β-galactosidase in Fur positive and Fur negative strains. Negative complementation, which has been described by Hantke and Braun [9], was used to create a Fur negative phenotype in the Mud*J* mutants analyzed. By introducing a mutated *fur* gene carrying a point mutation in the DNA binding domain on a multicopy plasmid (pMH152), inactive heterodimers are formed which are unable to bind DNA [9]. As a result, the merodiploid strain behaves as a *fur* mutant with respect to expression of *fur*-regulated genes. Plasmid pMH152 was introduced by electroporation into each of the iron responsive Mud*J* mutants. Both the Fur negative transformant and the original mutant were streaked across dipyridyl on M+Fe plates in order to compare β-galactosidase expression, as described previously [48]. We identified seven mutants in which the expression of β-galactosidase appeared to be Fur-dependent. To confirm these data, expression of β-galactosidase was quantified according to Miller (Table 2). Comparison of β-galactosidase activity in the presence and absence of pMH152 showed that these seven transcriptional fusions were indeed regulated by Fur. Regulation of these seven translational fusions was affected to varying degrees by iron availability or the introduction of pMH152 (Table 2). Expression of fusions in two strains,

AR1258 and AR8439 were regulated about 30-fold in response to iron availability. Experiments described below show that these mutations are in genes involved in siderophore biosynthesis. In contrast, fusions in the mutants AR895 and AR3675, later shown to be in the ferrichrome uptake locus (*fhu*), were found to be regulated only three- to fourfold in response to iron concentration. Experiments described below show that in these two mutants, Mud*J* is inserted in homologues of the first and last gene of the *E. coli fhuACDB* operon, respectively.

Since pMH152 contains the *E. coli fur* gene, the effect of the negative complementation method on gene expression was compared with the effect of inactivation of the chromosomal copy of *fur* in *S. typhimurium*. For this purpose, a *S. typhimurium fur* mutant was selected by growth of strain 14028 on media containing high concentrations of Mn^{2+} and low concentrations of iron [24]. Under these conditions, a Fur/Mn^{2+} repressor is formed so that *fur*$^{+}$ cells cannot grow because they are starved for iron. In contrast, *fur* mutants can express their iron uptake systems and can therefore form colonies. One such mutant was designated SF1. The Mud*J* insertion in strain AR895 was introduced into SF1 by P22 transduction, yielding strain SF895. The *fur* mutation in SF895 was complemented with the cloned *fur* gene of *E. coli* (pMH15) [24]. The expression of β-galactosidase in strain SF895, and SF895(pMH15) was quantified and compared with β-galactosidase expression in strains AR895, and AR895 (pMH152) (Table 2). Creation of a Fur^{-} phenotype by negative complementation with pMH152 and by mutational inactivation of *fur* both abolished regulation of the β-galactosidase fusion in AR895 and SF895, respectively. In the case of SF895, regulation could be restored by introduction of the cloned *E. coli fur* gene on plasmid pMH15, confirming that SF895 carries a mutation in *fur*.

Table 2: Iron-dependent expression of *lacZ* in Mud*J* mutants in fur- positive and fur-negative backgrounds

Strain	Relevant genotype	β-galactosidase activity (Miller Units)[a]			
		-pMH152		+pMH152	
		+Fe[b]	-Fe[c]	+Fe	-Fe
S. typhimurium					
AR396	nd[d]	16 ± 6.2	182 ± 77	93 ± 44	248 ± 107
AR895	*sidK*:Mud*J*	81 ± 18	329 ± 22	316 ± 79	379 ± 53
AR1258	*entB*::Mud*J*	13 ± 2	375 ± 64	94 ± 37	352 ± 71
AR3675	*fhuB*::Mud*J*	33 ± 13	138 ± 23	83 ± 21	91 ± 30
AR6687	nd[d]	38 ± 0.7	163 ± 24	104 ± 8.5	118 ± 3.5
AR8412	nd[d]	4.8 ± 0.8	134 ± 22	38 ± 19	140 ± 39
AR8439	*ent*::Mud*J*	9.8 ± 3.2	319 ± 79	152 ± 76	541 ± 363
SF895	*fur sidK*::Mud*J* [e]	212 ± 59	227 ± 65	nd	nd
SF895 (pMH15)	*fur sidK*::Mud*J* [e] *E. coli fur* gene on plasmid	99 ± 26	158 ± 48	nd	nd
E. coli					
H1717	*fhuF*::Mud*J*	60±44	699±539	668±49	630±23

[a] calculated according to Miller, 1972. Values given are averages of at least 3 independent experiments ± standard error
[b] grown in LB + 40μM $FeSO_4$
[c] grown in NB + 0.2mM 2,2'-dipyridyl
[d] uncharacterized Mud*J* insertion
[e] chromosomal *fur* mutant
nd=not determined

Cloning and sequence analysis of DNA flanking Fur-regulated Mud*J* insertions

Inverse PCR was used to clone short fragments of DNA flanking the left end of Mud*J* of three mutants. The sequence of DNA immediately flanking the transposon insertion site was determined and compared with sequence entries in the databases at NCBI. The nucleotide sequence of 247 bp of tranposon flanking DNA of AR895 was determined and the deduced amino acid sequence was found to have homology to amino acids 459-528 of *E. coli* FhuA, the outer membrane receptor for ferrichrome (Fig. 2A). The deduced amino acid sequence of 77 bp flanking Mud*J* in AR3675 was found to share homology with amino acids 278-302 of a second component of the ferrichrome uptake system in *E. coli*, FhuB, which forms the cytoplasmic membrane permease (Fig. 2B). For mutant AR1258, 189 bp of sequence flanking Mud*J* was determined. The deduced amino acid sequence was found to share homology with the enterobactin synthesis enzyme EntB of *E. coli* (Fig. 2C). This comparison revealed that all three Mud*J* insertions were in genes with known homologues in *E. coli*: *fhuA*, *fhuB*, and *entB*. A homologue of *fhuA* is known to exist in *Salmonella*, where it was designated *sidK* [8, 42]. For four of the mutants we were unable to clone DNA flanking the insertion site.

Figure 2A-N: Alignments of deduced amino acid sequences of *S. typhimurium* genes found in this study with the closest homologue from the data bases at NCBI. Alignments were performed using the ClustalV program.

Fig. 2A:

```
S.t. SidK        MNESKQTGLYVQDQAEWNKWVFTLGGRYDWSKQATTVRQNSTTP
E.c. FhuA   459  LNKQKQTGVYVQDQAQWDKVLVTLGGRYDWADQESLNRVAGTTD
                 .*  ****.******.*.* . ********. * .  *  .**

S.t. SidK        TEGYIERNDHQFTWRGGVNYVFDNGISPYFS
E.c. FhuA        -----KRDDKQFTWRGGVNYLFDNGVTPYFS   528
                       *.* **********.****..****

Identity:    60%
Similarity: 16%
```

Fig.2B:

```
S.t. FhuB        MEVSTGSVTALIGAALLLWLLPRLK
E.c. FhuB   278  MEVSTGSVIALIGAPLLLWLLPRLR   302
                 ******** *****.*********.

Identity:   88%
Similarity:8%
```

Fig.2C:

```
S.t. EntB        CKEHHIPVYYAAQPKEQSDEDRALLNDMWGPGLTRSPERQKVVEAL
E.c. EntB    67  CKQHNIPVYYTAQPKEQSDEDRALLNDMWGPGLTRSPEQQKVVDRL
                 **.*.*****.***************************.****. *

S.t. EntB        TPDEADTVLVKWRYS
E.c. EntB        TPDADDTVLVKWRYS   127
                 ***  **********

Identity:   86%
Similarity:8%
```

Fid. 2D

```
S.t. FoxA        MFAT---------TRMALLIG-GAIGGATFPLFAQETTKND--TVIVTSP
Y.e. FoxA    1   MFSAFIIKRSAILCSLAMFIPLASIADDTIEVTAKAGHEADLPTSGYTAT
                 **..           .*..*   ..*.. *. . *.      *  *   *.

S.t. FoxA        VQSGATKLATPDIETPQSVSIITRQQFEEQGATSVRQAVSYTPGVYSNQI
Y.e. FoxA        TTKGATKTDQPLILTAQSVSVVTRQQMDDQNVATVNQALNYTPGVFTGFS
                    ****    * * *.****..****  ..*   ..* **..*****..

S.t. FoxA        GASNRFDYIVLRGFSDGSLDNVYLDGLKMMGDTNSHSSLVVDPWFLEDIE
Y.e. FoxA        GGATRYDTVALRGFHGGDVNNTFLDGLRLLSDGGSYNVLQVDPWFLERID
                 *.. *.* . **** .*  ..* .****....*   *  . * ******* *.

S.t. FoxA        VVRGPASVLYGRSSPGGIVALTSRKPAFDAGGEVKLFAGNNNQRGAAFDV
Y.e. FoxA        VIKGPSSALYGQSIPGGVVMMTSKRPQFTSEGHFRLTAGNNNTQVAAFDY
                 *..**.* ***.* ***.*  .**..* *  . *. .* *****  . ****

S.t. FoxA        TGPLDDNERVAARLSGMTRYADSQFTPLKEERYALMPSLTWRITDRTRLD
Y.e. FoxA        TDAI--SEHWAFRLTGITRNSDTMYDHQREERYAIAPSLLWQPDENTSLL
                 *...   .*.  * **.*.** .*. .     .*****. *** *.    . * *
identity     45%
similarity   27%
```

Fig. 2E:

```
S.t. HemP        M--------------SRMDNTELPHPKEIDNETLLPAAERR---VNSQA
Y.e. HemP        MIDNAYHIDSGYHYLVCNMDKQLNKAPTMNDEPAAKPPAGNKPLSVSSEQ
                 *                **.      *    *. .   *.*    .    *.*.

S.t. HemP        LLGPDGKVIIDHNGQEYLLRKTQAGKLLLTK
Y.e. HemP        LLGEHGVAFIIHQGECYQLRQTKAGKLILTK
                 ***  .*   .* *.*.  * **.*.****.***
```

```
identity     42%
similarity   22%
```

Fig.2F:

```
S.t. FepA        MNKKIHSLTLLVNLGIYGAALPVMAEDKTDSAALTNEDTIVVTAAQQNLQ
E.c. FepA    1   MNKKIHSLALLVNLGIYGVA---QAQEPTDT-PVSHDDTIVVTAAEQNLQ
                 ********.********* *      *  . **.   .....********.****

S.t. FepA        APGVSTITADEI
E.c. FepA        APGVSTITADEI
                 ************
identity     73%
similarity   15%
```

Fig. 2G:

```
S.t. Fes         M-RVTFWWRDPQGNETHSPIRRVWVYITAVTD
E.c. Fes         MFEVTFWWRDPQGSEEYSTIKRVWVYITGVTD
                 *   **********.*   * *.*******.***
identity     77%
similarity    4%
```

Fig.2H:

```
S.t. P43     1    MNRQSWLLNLSLLKRHPAFRAVFLARFISIVSLGLLGVAVPVQI
E.c. P43          MNKQSWLLNLSLLKTHPAFRAVFLARFISIVSLGLLGVAVPVQI
                  **.*********** *****************************

identity     95%
similarity    2%
```

Fig. 2I:

```
S.t. FepD         MSCSFSVTRAFAVPGLLLLLSLAAVLSLVIGAKPLPAAVVLEAFTGVCQS
E.c. FepD    1    MSGSVAVTRAIAVPGLLLLLIIATALSLLIGAKSLPASVVLEAFSGTCQS
                  ** * .****.********* .*. ***.****.***.******.* ***

S.t. FepD         ADCTIVLDARLPR
E.c. FepD         ADCTIVLDARLPR
                  *************

Identity:    78%
Similarity:  13%
```

Fig.2J:

```
S.t. FhuE      1  MSFIQYRRDKHLPSTAAPSLLAMGMAMAFMP-AAFAA---EDTV
E.c. FhuE         MLSTQFNRDNQYQAITKPSLLAGCIALALLPSAAFAAPATEETV
                  *   *. **..  . . *****  .*.*..* *****   *.**

S.t. FhuE         IVEGETTADAVNREEQDYSMKTTAAGTKMPMTQRDI
E.c. FhuE         IVEGSATAP--DDGENDYSVTSTSAGTKMQMTQRDI
                  **** .**   .  *.***. .*.***** ******

identity     44%
similarity   17%
```

Fig. 2K:

```
S.t. Pmg          MAVTKLVLVRHGESQWNKENRFTGWYDVDLSEKGVSEAKAAGK
E.c. PMG1         MAVTKLVLVRHGESQWNKENRFTGWYDVDLSEKGVSEAKAAGK
                  *******************************************

S.t. Pmg          LLKEEGFSFDFAYTSVLKAAIHTLWNVLDELD
E.c. PMG1         LLKEEGYSFDFAYTSVLKRAIHTLWNVLDELD
                  ******.*********** *************

Identity:    97%
Similarity:   1%
```

Fig. 2L:

```
S.t. Fur        IDMGEEIGLATVYRVLNQFDDAGIVTRHNFEGGKSVFELTQQHHHDHLIC
E.c. Fur    44  IDMGEEIGLATVYRVLNQFDDAGIVTRHNFEGGKSVFELTQQHHHDHLIC
                **************************************************

S.t. Fur        LDCGKVIEFSDDSIEARQREIAAKHGIRLTNHSLYLYGHCAEGDCREDEH
E.c. Fur        LDCGKVIEFSDDSIEARQREIAAKHGIRLTNHSLYLYGHCAEGDCREDEH
                **************************************************

S.t. Fur        AHDDATK
E.c. Fur        AHEG--K
                **..  *
identity     96%
similarity    2%
```

Fig.2M:

```
S.t. ORF o162    MSEIVIRHAEPKDYDAIRQI
E.c. ORF o162    MSEIVIRHAETRDYEAIRQI
                 ********** .**.*****
Identity:    85%
Similarity:  10%
```

Fig. 2N:

```
S.t. CybB       MQFKNTPQRYGVVSAALHWLTPWWSMACLRWAYGWSTLSYYDGWYHQAPE
E.c. CybB   1   MLWKINIQGYKSAFTG--WSFYWLSQRIAQWRFR----GFFPRSDRPLIN
                *  *   * *    ..  *   * *    .* .     ...    .   .

S.t. CybB       -IHKSIGILLMMALIVRIIWRL-YSPPPVALTSYSRLTRIGAAAGHLLLY
E.c. CybB       MIHVSCGISILVLMVVRLLLRLKYPTPPIIPKPKPMMTGL-AHLGHLVIY
                 ** * ** ... ..**.. ** *. **.   . . .* . *  ***..*

S.t. CybB       LLLFAIIISGYLISTADGKPISVFGWFEIPATLT-DAGAQADIAGTLHLW
E.c. CybB       LLFIALPVIGLVMMYNRGNP-----WFAFGLTMPYASEANFERVDSLKSW
                **..*. . * ..    *.*     ** .  *.   . *. .  ..*  *

S.t. CybB       FA----WSLVIISLSHGVMALKHHFIDKDDNSETYDRNVSHLTMELKNEK
E.c. CybB       HETLANLGYFVIGL-HAAAALAHHYFWKDNHTSTHD--------------
                       .  .*.* *.  ** **.. **... * *

S.t. CybB       KPAGIHPRILVIHDRFRRGGGV
E.c. CybB       --AA
                  *.
identity     23%
similarity   22%
```

Mapping and phenotypic characterization of Mud*J* insertions

Pulsed Field Gel Electrophoresis was used to determine the locations of the transposon insertions on the *Salmonella* chromosome. Macrorestriction maps of the *S. typhimurium* genome with the enzymes *Bln*I and *Xba*I have been published recently [32, 55]. These restriction endonucleases cut the *S. typhimurium* chromosome 11 and 23 times, respectively. In order to map Mud*J* insertions on the *Salmonella* chromosome we constructed a suicide vector derivative of pGP704, termed pMAP, which contains *Xba*I and *Bln*I restriction sites for physical mapping and can be propagated in the *E. coli* host S17-1λpir (Fig. 1)[46]. We then cloned a fragment of the *lacZ* gene into pMAP, giving rise to pMAP1 (Fig. 1). The suicide vector pMAP1 was conjugated into *S. typhimurium* Mud*J* mutants. This vector will integrate into the chromosome by homologous recombination between its internal *lacZ* fragment and the *lacZ* gene of Mud*J*, thereby introducing new *Xba*I and *Bln*I restriction sites into the genome. After a restriction digest of chromosomal DNA from a Mud*J*::pMAP1 mutant, the fragment in which the transposon is inserted will therefore disappear while two new, smaller fragments can be detected after separation with pulsed field gel electrophoresis. The map positions calculated for AR895, AR3675, and AR1258 corresponded to the map positions for *fhuA*, *fhuB*, and *entB*, on the *E. coli* chromosome, respectively[3]. Although these genes have not been sequenced in *Salmonella*, homologues are known to be located at 4-5 minutes (*sidK*) and at approximately 14 minutes (*ent*-locus), which is confirmed with the Mud*J* insertions at these loci [42](Table 3).

All mutants were tested for siderophore production by growth on CAS-agar. Colonies which produce the siderophore enterobactin produce a yellow halo on CAS agar plates [44]. Only AR1258 and AR8439 failed to produce a halo around colonies on CAS agar plates. These two mutants mapped at 14 minutes, the location of the enterobactin synthesis

genes [42]. Phenotype and map location were further supported by sequence analysis from transposon flanking DNA from the Mud*J* insertion in AR1258, which shared homology with *entB* from *E. coli* [31, 38]. AR6687 formed colonies with halos strongly reduced in size on CAS agar plates and the corresponding insertion was located at 15 minutes.

Table 3: Mapping data of *S. typhimurium* Mud*J* insertions yielding *fur*-regulated *lacZ* fusions

Mud*J*- mutant	*Xba*I-restriction map[a]		*Bln*I-restriction map[b]		Sequence homology[c]	Phenotype	Map position (min)
	Fragment missing	Size (kb) of new fragments	Fragment missing	Size (kb) of new fragments			
AR396	-	24	C	400	nd[d]	nd	30
AR895	B	400+310	A	700	*fhuA*	FhuA missing	5
AR1258	A	120	-	ca.1200	*entB*	sid-	14
AR3675	B	400+310	A	700	*fhuB*	fox utilization	5
AR6687	A	150+650	A	-	nd	nd	15
AR8412	J	130	B	400	nd	nd	46
AR8439	A	120	A	ca.1200	nd	sid-	14

[a] based in Liu and Sanderson, 1992
[b] based on Wong and McClelland, 1992
[c] homology to *E. coli* genes
[d] nd=not determined

Table 4: Mapping data of *S. typhimurium* FURTA-positive clones

FURTA clone	XbaI-restriction map[a]		BlnI-restriction map[b]		Sequence homology[c]	Map position (min)
	Fragment missing	Size (kb) of new fragments	Fragment missing	Size (kb) of new fragments		
pFT1E	A	185	C	650	*fhuE*	25
pFT1H	-	210	-	-	*foxA*	nd
pFT1S	nd	nd	nd	nd	*foxA*	nd
pFT5	A	510	C	760	-	23
pFT13	nd	nd	nd	nd	*fur*	15
pFT16	A	-	A	-	PMG1	12-22
pFT17	A	120	A	ca. 1200	*fes-fepA*	14
pFT30	nd	nd	nd	nd	ORF o162	74[d]
pFT32	A	120	A	ca. 1200	p43-*fepC*	14
pFT35	nd	nd	nd	nd	nh	nd
pFT36	nd	nd	nd	nd	*hemP*	36[d]

[a] based on Liu and Sanderson, 1992
[b] based on Wong and McClelland, 1992
[c] see text for explanation
[d] map position was calculated fron map position in *E. coli* based on Riley and Krawiec, 1987
nd=not determined

In order to identify the FhuA (SidK) receptor, outer membranes of *S. typhimurium* 14028 and AR895, were purified and the protein profile compared by SDS-PAGE. A protein of 78 kDa present in the parent strain was missing from the outer membrane of AR895 (data not shown). This result indicated that in AR895, the Mud*J* insertion had inactivated the *Salmonella fhuA* homologue.

Sequence data indicated that mutant AR3675 carries Mud*J* inserted in another homologue of the *E. coli* ferrichrome uptake operon, *fhuB*. FhuCDB also mediates the transport of ferrioxamine through the cytoplasmic membrane in *E. coli* [7]. Since *S. typhimurium* can utilize this siderophore efficiently [33] , we examined the effect of a *fhuB* mutation on utilization of ferrioxamine as a sole iron source. In order to study ferrioxamine uptake in the absence of enterobactin production, an *aroA::*Tn*10* insertion from SL1346

was transduced into strain 14028 and AR3675 giving rise to strains CL1509 and AJB29, respectively. Strain AJB29 and its isogenic parent CL1509 were tested for ferrioxamine utilization using an agar diffusion assay. While strain CL1509 formed halos of 25 mm in diameter around ferrioxamine-soaked filter discs on Nutrient Broth (NB) + Dipyridyl agar, no growth stimulation was observed for strain AJB29. These data confirm the involvement of FhuB in ferrioxamine transport in *S. typhimurium* .

In conclusion, using Mud*J* mutagenesis we identified seven distinct Fur regulated *lacZ* fusions, four of which resulted either in a known phenotype (AR8439), or were located in known Fur regulated genes (AR 895, AR1258, and AR3675). The three other Mud*J* insertion mutants showed no known phenotype. AR6687 mapped to 15 minutes, the location of *fur* in *Salmonella*, but the observed reduction in siderophore production does not agree with an insertion in *fur*. AR8412 mapped to 46 minutes, the location of *cir*, an outer membrane protein of *E. coli* which has been shown to mediate uptake of siderophore breakdown products [26]. AR396 was located at 30' on the chromosome where no Fur regulated loci have been described so far in *E. coli* or *S. typhimurium*.

Screen for Fur regulated promoters by FURTA

Fur regulated promoters or iron binding proteins carried on a plasmid can be identified by transformation of this plasmid into the *E. coli* strain H1717, which carries *fhuF::lacZ*, a Fur regulated gene fusion sensitive to changes in repressor concentration [48]. Transformants carrying plasmids that contain a Fur binding site or encode an iron binding protein form red colonies on M+Fe, while other colonies will be white. A *Salmonella* gene bank consisting of 10,000 independent plasmid clones was screened by this FURTA. Initially, a total of 40 colonies were FURTA positive (red colonies on M+Fe) and were further analyzed. After eliminating duplicate clones based on restriction patterns (data not

shown), 11 clones were chosen for further characterization. In five clones, pFT5, pFT30, pFT32, pFT35, and pFT36, the insert size was larger than 1 kb and they were subcloned into pBluescriptSK using the enzymes *Rsa*I, *Hin*dII, *Sal*I or *Eco*RV. The FURTA positive subclones, pFT501, pFT302, pFT324, pFT3522, and pFT361, respectively, were identified by transformation into H1717 and plating on M+Fe plates before further characterization.

Sequence analysis and mapping of DNA regions identified by FURTA

The DNA sequence of 11 FURTA positive clones was determined. The insert of two FURTA positive clones, pFT1S (not shown) and pFT1H, contained the promoter region and the 5'-end of an ORF with homology to *foxA* , a gene encoding the outer membrane ferrioxamine receptor of *Yersinia enterocolitica* (Fig. 2D; Fig. 3)[4]. A potential Fur binding site matching the consensus sequence in 12 of 19 bases was located upstream of this ORF (Fig. 4). In contrast to *E. coli, Salmonella* can utilize ferrioxamine very efficiently [33]. However, the ferrioxamine receptor of *S. typhimurium* has not yet been characterized at the nucleotide sequence level. One FURTA positive clone, pFT36, contained a small open reading frame (189 bp), which was preceded by a sequence matching the consensus site for Fur binding in 16 of 19 bases. This ORF shared homology with *hemP* of *Y. enterocolitica* (Fig. 2E; Fig. 3). This gene is the first open reading frame in the Fur regulated heme uptake operon of *Y. enterocolitica* [50]. Clone pFT17 contained the 5' ends of two divergently oriented ORFs with homology to *fes* and *fepA*, encoding enterochelin esterase and the ferrienterochelin receptor, respectively, of *E. coli*. The bidirectional promoter region between these two genes has recently been characterized in detail [28]. and a sequence identical to the Fur-box determined in this study was also present in the putative promoter region on pFT17 (Fig. 2F,G; Fig. 3, Fig. 4). The sequence of clone pFT324 exhibited homology to a second bidirectional promoter

region in the *ent-fep* cluster of *E. coli*, the p43-*fepD* region (Fig. 2H,I). The function of P43 is unknown, although it has been suggested to be a cytoplasmic membrane protein [12, 45]. FepD forms part of the cytoplasmic membrane permease for ferrienterobactin. Truncated ORFs encoding homologues to these two proteins as well as a putative Fur binding site with two mismatches to the one suggested by Shea and McIntosh[45] and Chenault and Earhart [12] were identified on pFT324. Plasmid pFTE1 contained an ORF with homology to *E. coli fhuE*, the outer membrane receptor for coprogen [23, 43]. A putative Fur-box identical to the one identified in the *E. coli fhuE* promoter was present upstream of the *fhuE* homologue on pFTE1(Fig. 2J, Fig. 3; Fig. 4)[43]. Upstream of the *fhuE* homologue, and oriented in the same direction, is the 3' end of a second ORF (designated `orf1) which had no homology to any entries in the data base.

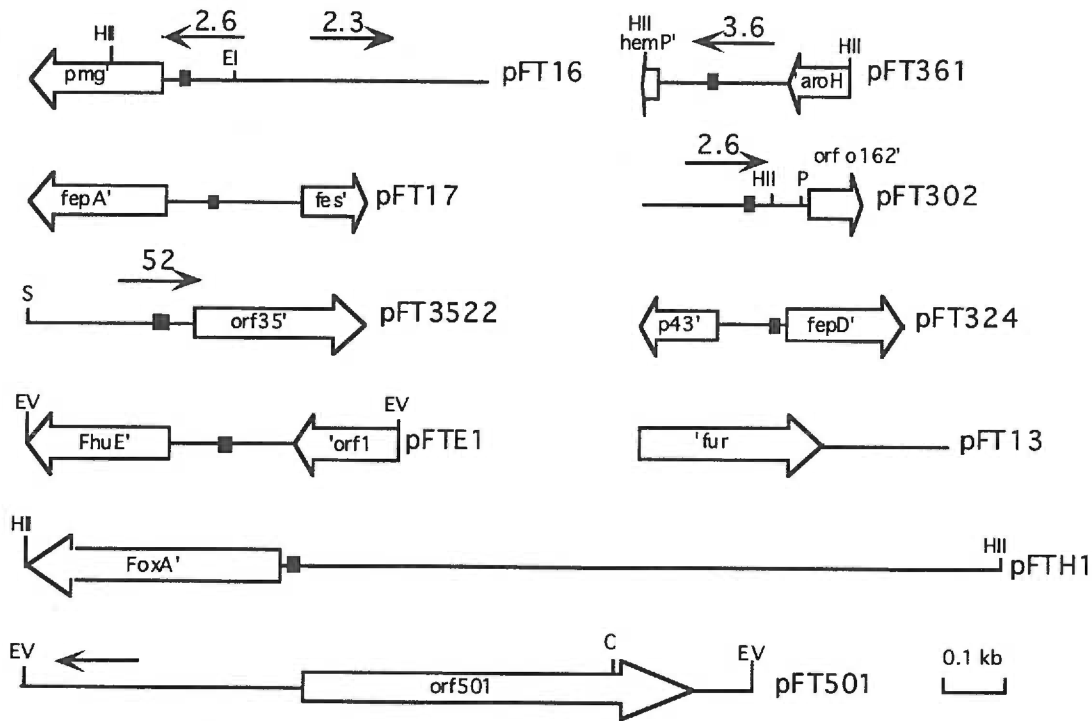

Figure 3: Inserts of FURTA-positive clones from *S. typhimurium*. Open arrows indicate open reading frames. Thin arrows denote the direction of promoter activity as determined in Table 5. If promoter activity is affected by iron, the fold induction is shown above the arrow. Black boxes indicate the presence of a consensus Fur binding site.

```
Consensus:          GATAATGAT A ATCATTATC

pFT361:     124     GATAATAAT A ATCATTGAG   106
pFT324:     229     GATAATGAT A GTAATTATC   211
pFT16:              273   TATAATGAG A ATTATTATC   255
pFT3522:            210   GATAATCAT T ATCACTAAC   228
pFT3522:            204   TGAAGTGAT A ATCATTATC   222
pFT302:             165   AATAATCAT T CTCATTCGC   183
pFT17:              294   GCAAATGCA A ATAGTTATC   312
pFTE1:              334   GCGTATATT T CTCATTTGC   316
pFTH1:              434   GGTAATAAT T CTTATTTAC   416
```

Figure 4: Comparison of potential Fur binding sites found in FURTA-positive clones with the consensus sequence found by deLorenzo, et al. (16).

Plasmid pFT16 contained the 5' end of an open reading frame with homology to various phosphoglycerate mutases. The predicted protein encoded by this ORF shared homology with PGM1 of *E. coli*. Upstream of this ORF, a potential Fur-box was located which matched the consensus sequence in 16 of 19 bases (Fig. 2K, Fig. 3, Fig. 4).

Interestingly, one of the FURTA-positive clones contains a gene fusion between the α-fragment of the *lacZ* gene (contained on the cloning vector) and the *S. typhimurium fur* gene lacking the first 129 bp (Fig. 2L). The resulting LacZ-Fur fusion protein could have caused the FURTA-positive phenotype of this clone. Since the C-terminal part of Fur contains the domain necessary for repressor dimerization, but not for DNA binding [13] [51], the LacZ-Fur protein may form defective dimers with wild-type *E. coli* Fur in H1717, thereby causing a Fur^- phenotype. This negative complementation would explain the derepression of the *fhuF::lacZ* fusion leading the FURTA-positive result seen in our screen.

The remaining three FURTA positive plasmids showed no homology to known genes. Plasmid pFT3522 contained 282 bp of the 5' end of an ORF, designated orf35. Approximately 50 bp upstream of this ORF was a potential Fur binding site matching the consensus sequence in 15 of 19 bases. Plasmid pFT302 contained 78 bp of the 5' end of an ORF, designated orf30, with no homology to entries in the data base. A primer walk was performed on the parent plasmid pFT30. With this additional sequence data, 342 bp of this ORF were found to be homologous to ORF o162 (NCBI seq ID 606376) found in the *E. coli* chromosomal region from 67.4 to 76 minutes (Fig. 2M). A FURTA-positive clone was also found in a previous study at the corresponding region of the *E. coli* chromosome, at 71 minutes [48]. The potential Fur-box upstream of this ORF on pFT302 matched the consensus sequence in 13 of 19 bases, and an identical sequence was found in *E. coli* (Fig. 4). Thus, the truncated ORF contained on pFT302 may define a Fur-

regulated gene product of yet unknown function which is common to *E. coli* and *Salmonella*. Plasmid pFT501 encoded an ORF (orf501) of 645 bp, whose translated product exhibited homology to the *E. coli* cytochrome b561. No consensus binding sites for Fur were found upstream of this ORF (Fig. 3; Fig. 2N). Together, these data suggest that pFT501 may encode an iron-binding protein.

In summary, the nucleotide sequence of five FURTA positive clones was homologous to Fur regulated promoters described previously in *Salmonella* or other Enterobacteria (Fig. 4). The nucleotide sequence of three FURTA positive clones contained Fur binding motifs but showed no homology to previously described Fur regulated promoters. One FURTA positive clone may encode an iron binding protein.

Physical mapping of FURTA-positive clones on the *S. typhimurium* chromosome

In order to find the location of these clones on the *Salmonella* chromosome, *Eco*RV fragments of the inserts of pFT1H, pFT5, and pFT16 were cloned into the suicide vector pMAP (Fig. 1), giving rise to plasmids pMAP1H, pMAP5 and pMAP16, respectively. In order to confirm the reliability of the mapping technique we also constructed mapping derivatives for *Salmonella* genes which have been mapped previously. For this purpose *Eco*RV fragments of the inserts of pFT1E, pFT17, pFT13 and pFT32 were cloned into the suicide vector pMAP, giving rise to plasmids pMAP1E, pMAP17, pMAP13 and pMAP32, respectively. All pMAP derivatives were introduced into the *S. typhimurium* strain 14028 by conjugation and exconjugants carrying the suicide vector inserted in the chromosome were selected by plating on carbenicillin. The insertion site of the plasmid was then determined by PFGE analysis of chromosomal DNA of these derivatives digested with *Xba*I and *Bln*I (Table 4). The map locations calculated for *fhuE, fes-fepA, fur* and *p43-*

fepD agreed with data published for *Salmonella* or were at positions which corresponded to known loci in *E. coli* [3, 40, 41], thus confirming the reliability of the mapping technique. Using these mapping data or the sequence information we were able to determine the map locations of a total of 8 FURTA positive clones (Table 4).

Iron-responsive promoter activity of FURTA-positive clones

In order to distinguish between plasmids containing Fur-regulated promoters or encoding iron binding proteins the inserts of FURTA positive plasmids were cloned, using the restriction enzymes *Xba*I and *Sal*I, into the promoter probe vector pUJ10 [14]. This vector contains a multiple cloning site flanked by promoterless β-galactosidase (*lacZ*) and alkaline phosphatase (*phoA*) genes oriented in opposite directions. Since the 5'-end of each of these genes flanks the multiple cloning site, introduction of a promoter at this site creates a transcriptional fusion to either *lacZ* or *phoA*. Inserts from FURTA-positive clones were cloned into pUJ10 and the resulting strains assayed for iron-responsive promoter activity in the *lac*$^-$ *phoA*$^-$ *E. coli* host CC118 [34]. By measuring the activities of β-galactosidase and alkaline phosphatase under both iron-replete and iron-limited conditions, the degree and direction of iron-responsive promoter activity were determined (Table 5).

Although pUJ501 had promoter activity driving *phoA* expression, the level of induction under iron limitation was not higher than in the vector control. This promoter is therefore not regulated by iron. Of the five constructs examined, four could be shown to exhibit iron-responsive promoter activity (Table 5). An iron-regulated promoter was found to drive expression of *phoA* in pUJ361 and pUJ161. Plasmids pUJ161, pUJ3522, and pUJ302 showed iron-responsive promoter activity driving expression of the *lacZ* gene. The direction of promoter activity of these clones is shown in Fig. 3. Together with the sequence data showing the presence of a Fur box (Fig. 4), these data indicate that clones

pUJ161, pUJ3522, pUJ302 and pUJ361 each carry a Fur-regulated promoter. The absence of a iron regulated promoter in pUJ501 agrees with sequence analysis data, which indicated a lack of a consensus Fur binding site. The presence of an open reading frame with a low degree of homology to cytochrome B561, an iron-binding protein of *E. coli,* indicated that this clone may encode an iron binding protein which leads to a FURTA positive phenotype [39]. In conclusion, using FURTA we identified 11 distinct clones, nine of which contained iron regulated promoters. In addition one potential iron binding protein and the *Salmonella fur* gene itself were isolated (Fig. 3).

Table 5: Promoter activity of FURTA positive fragments[a]

	β-galactosidase activity (Miller Units)[b]		Alkaline phosphatase activity (Units)[c]	
Clone	+Fe[d]	-Fe[e]	+Fe[d]	-Fe[e]
pUJ501	1.59 ± 1.29	3.23 ± 2.22	160 ± 30	228 ± 45
pUJ161	35.5 ± 11	94.8 ± 35	58 ± 54	189 ± 37
pUJ302	5.3 ± 7.5	14 ± 0.7	7.6 ± 2.9	41 ± 24
pUJ352	1.35 ± 0.83	73 ± 41	42 ± 14	43 ± 29
pUJ361	0.36 ± 0.41	2.1 ± 0.67	547 ± 34	1975 ± 326
pUJ10	2.7 ± 2.53	2.3 ± 0.99	17 ± 1.7	39 ± 11

[a]averages of at least 3 independent experiments ± standard error
[b] calculated according to Miller, 1972.
[c]calculated based on Brickman and Beckwith, 1975.
[d] grown in LB + 40μM $FeSO_4$
[e] grown in NB + 0.2mM 2,2'-dipyridyl

Discussion

In this report we describe the analysis of the *S. typhimurium* Fur regulon using two different techniques, Mud*J* mutagenesis and FURTA [27, 48]. With these two methods we screened 10,000 *Salmonella* transposon insertion mutants and 10,000 clones of a *Salmonella* plasmid bank for Fur regulated genes or promoters, respectively. This approach allowed for a direct comparison of both techniques in identifying members of the *Salmonella* Fur regulon. By screening Mud*J* mutants for Fur regulated genes we were able to isolate seven Fur regulated insertions while eight different Fur regulated promoters were isolated by FURTA. In addition, a truncated *fur* gene and a putative iron binding protein were identified using FURTA. Although both approaches were therefore equally efficient in identifying Fur regulated genes, we found each to have different advantages. Mud*J* mutagenesis had the advantage of immediately providing mutants for phenotypic studies, however, the mutant screen was more labor-intensive than the positive selection for clones used in the FURTA. In contrast, screening of plasmids by FURTA was rapid and provided clones for immediate sequence analysis. However, this technique would not be suitable for mutational analysis of a larger number of loci.

The Fur regulon has been best studied in *E. coli* K12, where more than 36 Fur regulated genes have been described [7]. A thorough analysis by two dimensional gel electrophoresis identified 19 proteins of *S. typhimurium* whose expression is negatively regulated by Fur[17] Using FURTA and Mud*J* mutagenesis we identified 14 *S. typhimurium* genes which are repressed by Fur. Six of these genes, *fhuA*, *fhuB*, *fes*, *fepA*, *fepD*, and *entB*, are located in operons consisting of several genes in *E. coli*. Assuming the same situation in *Salmonella*, the Fur regulated loci identified in this study contain approximately 24 genes. However, since we were unable to find *tonB* and *sodA*, two known Fur regulated genes of *S. typhimurium*, the number of Fur repressed genes is

likely to be larger [21] [53]. Our results show that *S. typhimurium* possesses homologues for many Fur repressed *E. coli* genes located at similar positions on the genetic map (Fig. 5). However, some differences between *E. coli* and *S. typhimurium* iron uptake systems exist. For example, *E. coli* has been shown to possess an iron-dicitrate uptake system, encoded by *fecIR fecABCDE*, whereas *Salmonella* cannot utilize this compound as an iron source [19, 33, 47]. Furthermore, *S. typhimurium* is able to utilize the siderophore ferrioxamine very efficiently while in *E. coli* only a low level of ferrioxamine uptake is mediated by the outer membrane receptor FhuE [33, 43]. A *S. typhimurium* gene identified in this study was found to be homologous to *foxA*, the gene encoding the outer membrane ferrioxamine receptor of *Y. enterocolitica* [4]. Although it is tempting to speculate that this gene might encode the *Salmonella* ferrioxamine receptor, the possibility that this function is mediated by the *Salmonella fhuE* homologue isolated during this study (Fig. 3), or a third unidentified gene cannot be excluded. However, our data show that, as in *E. coli*, FhuB is necessary for transport of ferrioxamine across the cytoplasmic membrane of *S. typhimurium* [7]. In addition to the *foxA* homologue, several other Fur regulated loci which have previously not been described in *E. coli* or *S. typhimurium* were identified during this study. One such gene showed homology to *hemP*, the first gene in the *Y. enterocolitica* operon encoding a heme uptake system [50] However, *S. typhimurium* is unable to utilize heme as an iron source (our unpublished results). Therefore, the *hemP* homologue could either be part of a defective heme uptake system, or it may be the first gene in a related but different operon. Interestingly, this open reading frame was also found in *E. coli* K12, in the 3' region of *aroH* and maps close to the *btuCED* locus, encoding a cytoplasmic membrane permease for vitamin B12[18].

By comparison of the proteins encoded by the fragments of these *Salmonella* genes with their homologues in *E. coli* we found that cytoplasmic membrane proteins and cytoplasmic enzymes were highly conserved, with overall similarities (% identity plus % conservative

amino acid changes) between 81% and 98%. In contrast, the outer membrane receptors were less conserved, with overall similarity scores ranging from 61% to 88%. The bacterial surface is exposed to the immune system of the host, which might have been a selective pressure leading to increased variability of surface-exposed proteins among Enterobacteria. Differences in outer membrane receptors of *E. coli* and *Salmonella* have been described previously as differences in susceptibility to phages and colicins [8].

At least one Mud*J* mutation and three FURTA clones appear to define new Fur regulated loci. However, we found no homology to known proteins, thus the sequence data do not suggest a function for the proteins encoded by these loci. Since Fur-regulated genes described so far fall into several groups, including genes involved in iron uptake, in the acid tolerance response [17], in sugar metabolism, defense against oxygen radicals, and genes encoding bacterial toxins, it is premature to speculate about the function of these genes.

Of particular interest was the finding that *pmg*, encoding the phosphoglycerate mutase, was regulated by Fur. First evidence for the involvement of Fur in regulation of general metabolic pathways was provided by the finding that *fur* mutants of *E. coli* are unable to utilize succinate as a C-source [25]. Furthermore, the gene encoding aconitase of *E. coli* , an enzyme of the Krebs cycle, contains a Fur binding site in its promoter region [41]. Fur may thus participate in the control of major catabolic pathways thereby linking bacterial growth to the supply of limiting nutrients like iron. In return, the CRP binding site found in the *fur* promoter region may link expression of this regulator to the availability of C-sources [15]. That this complex regulatory network has yet other facets is indicated by the observation that *fur* mutants of *Salmonella* are defective in the acid tolerance response [17].

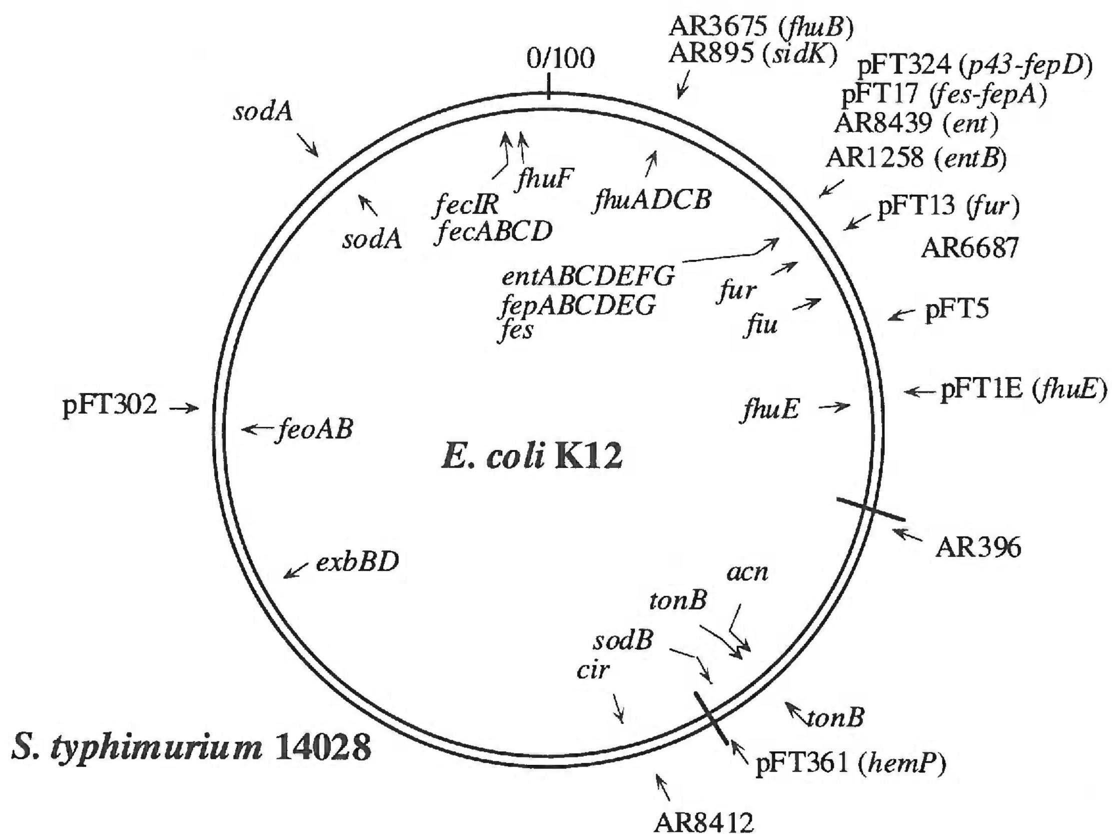

Figure 5: Comparison of the locations of Fur-regulated genes on the genetic maps of *E. coli* (inner circle)and *S. typhimurium*(outer circle). Lines intersecting the map indicate the endpoints of a region (26'-36' in *S. typhimurium*) which is inverted between the two species. Fur regulated genes of *S. typhimurium* found in this study are indicated .

Acknowledgments:

We thank S. Anic for expert sequencing with the ALF automated sequencer and J. Lipps for technical assistance. We also thank K. Hantke, R. Schönherr, V. deLorenzo, J. Mekalanos and R. Taylor for strains and B. Ahmer, P. Valentine and the reviewers for helpful comments on the manuscript. This work was supported by US Public Health Service grant AI 22933 to F.H. from the National Institutes of Health. A.J.B. was supported by stipend Ba 1337/1-2 from the Deutsche Forschungsgemeinschaft.

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Manuscript #3

Contribution of TonB- and Feo-mediated iron uptake to growth of *Salmonella typhimurium* in the mouse

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Abstract:

We examined the role of iron(II) and iron(III) uptake, mediated by FeoB and TonB, respectively, in infection of the mouse by *S. typhimurium*. The *S. typhimurium feoB* homologue, encoding a cytoplasmic membrane iron(II) permease, was cloned and a mutant generated by allelic exchange. In addition, a *S. typhimurium tonB* mutant was constructed. These mutants were characterized for their ability to grow in vitro and in different compartments of the host. Mutants in *feoB* were out competed by the wild type during mixed colonization of the mouse intestine but did not attenuate *S. typhimurium* for oral or intraperitoneal infection of mice. The *tonB* mutation attenuated *S. typhimurium* for infection of mice by the intragastric route, but not the intraperitoneal route, and was recovered in reduced numbers from the Peyer's patches and mesenteric lymph nodes than the wild type. These results indicate that TonB-mediated iron uptake is required for growth within Peyer's patches and mesenteric lymph nodes but not the liver and spleen of the mouse. Feo-mediated iron uptake contributes to growth in the intestine of the mouse, but is not required for virulence in this model of infection.

Introduction:

In order to cause infection in mammalian hosts, bacterial pathogens must overcome the iron limitation imposed by the host's iron withholding defense (46). The first line of this defense includes the iron binding proteins transferrin and lactoferrin, present in body fluids. The second line of the iron withholding defense is induced in response to infection. Upon contact with a pathogen, the iron saturation of serum transferrin is reduced, raising its iron binding capacity. The reduction of the serum iron level is, in part, due to increased storage of iron in the macrophages of the liver and the spleen (46). Pathogens growing in the host are able to circumvent these iron withholding mechanisms by scavenging of iron from host iron binding proteins during extracellular growth. Some bacteria achieve this by use of siderophores, high affinity iron binding compounds, which can out compete transferrin and lactoferrin for binding of iron (for reviews see (10, 14, 47)). Others produce outer membrane receptors for transferrin, lactoferrin and heme compounds, which are able to bind these proteins directly and extract iron from them (reviewed in(35)). These mechanisms all have two features in common: first, they supply bacteria with iron(III) and second, energetization of this iron uptake is dependent on the TonB protein(9). TonB transmits energy from the cytoplasmic membrane by direct contact with outer membrane receptors(5, 18, 40) . For some pathogens which grow in an extracellular location, iron (III) uptake systems have been shown to contribute to virulence (15, 27, 32).

During growth of *E. coli* in the intestine, an iron(II) transport system has been shown to supply the bacteria with iron. This system, designated Feo, functions as a ferrous iron pump in the cytoplasmic membrane of *E. coli* (24). Mutations eliminating the function of this system reduced the ability of *E. coli* to colonize the mouse intestine (43).

In contrast to extracellular pathogens, it is not known how pathogens which grow intracellularly, such as *Legionella pneumophila* and *Salmonella typhimurium*, obtain iron within the host (7, 38).

S. typhimurium causes a disease similar to typhoid when introduced into mice. After infection by the oral route, *S. typhimurium* invades the epithelium of the ileum, preferentially at the Peyer's patches. After 2-3 days, bacteria can be found in the lymph nodes draining the Peyer's patches, from where they disseminate via the lymphatics into the blood(13) . In later stages of infection, bacteria are present in the liver and spleen, the filtering organs of the blood. In these organs, *S. typhimurium* has been shown to replicate intracellularly (16, 33). The cell type in which this intracellular replication is thought to occur is the resident macrophage(17). When *S. typhimurium* is administered intravenously or intraperitoneally, bacteria reach the liver and spleen more rapidly and bypass the initial intestinal phase of infection. A thorough examination of effects of mutations in siderophore synthesis on parenteral routes of infection has shown that for the final, intracellular phase of *S. typhimurium* infection, synthesis and uptake of the siderophore enterobactin are dispensable (7). In order to determine whether other iron uptake systems might contribute to the supply of this vital trace element during growth in the host, we examined the effects of mutations in two types of iron assimilation systems. We tested a mutant in *tonB*, which in gram-negative bacteria has been shown to be required for receptor-mediated iron(III) uptake, including assimilation of iron from siderophores and from host iron compounds (9, 21, 44) In addition, a mutation in *feoB* was constructed to determine whether this iron(II) uptake system plays a role in *S. typhimurium* infection of the mouse.

Materials and Methods

Bacterial strains, media and growth conditions. The strains used are listed in Table 1. Bacteria were cultured aerobically at 37°C. Media used were Luria-Bertani Broth (30) and Nutrient broth (8 g/L Nutrient Broth, 5 g/L NaCl),. Solid media contained 15 g/L agar. Antibiotics, when required, were included in the culture medium or plates at the following concentrations: tetracycline 10 mg/L, kanamycin 100 mg/L, chloramphenicol 20 mg/L, carbenicillin, 100 mg/L. In order to create iron limiting growth conditions, 0.2 mM 2,2'dipyridyl was added to solid or liquid media. Growth in liquid media was quantified by measuring optical density of cultures at 578 nm. Growth on agar plates was determined by measurement of colony diameter under a dissecting microscope using an objective micrometer.

Utilization of ferrioxamine was detected by an agar diffusion assay (3). The strain to be tested was poured in 3 ml of 2% noble agar onto a Nutrient Broth-Dipyridyl (NBD) agar plate. Filter paper disks impregnated with ferrioxamine (3 µl of a 1 mg/ml solution of Desferal in 0.1 M $FeCl_3$) were laid onto the top agar and, after incubation overnight at 37°C, the zone of growth around the filter disk was measured.

Genetic techniques. P22 HT*int* was used for generalized transduction of transposon insertions into a different genetic background. Transductants were routinely streaked on Evans Blue-Uranine (EBU) plates to detect phage contamination before use in further experiments (8). Quantification of β-galactosidase activity was performed according to (30).

Recombinant DNA techniques. Plasmid DNA was isolated using ion exchange columns from Qiagen. Standard methods were used for restriction endonuclease analyses, ligation and transformation of plasmid DNA (30). Sequencing was performed using the

ALF automated sequencer (Pharmacia). A fragment of the *S. typhi feoB* gene was amplified using the primers 5'-TCCGAATTCTGGCAAGACAACG-3' and 5'-CCGAATTCAGCGCCTGCATCAGACG-3'. Primers used for amplification of *feoB* from *S. typhimurium* were 5'-TCACCGTCGAGCGTAAAGAGG-3' and 5'-GCCAATCCATTGAATGCCATG-3'. Primers used to amplify fragments of *tonB* were 5'-GGAATTCGGTGAGCGTGCCCACTG-3', 5'-GGTCGACCTAAACCTCGCCCGCTCCC-3', 5'-CGGGATCCCGACGATTATGACTTCAATGACCCTTG-3', and 5'-GGAATTCCATGCGGTGCTGCTTGTTGG-3'. PCR was performed under standard conditions using *Pfu* polymerase (Stratagene).

Southern hybridization Southern transfer of DNA onto a nylon membrane was performed as previously described (1). Labeling of DNA probes, hybridization, and immunological detection were performed using the Renaissance DNA labeling and detection kit (non-radioactive) from New England Nuclear.

Tissue Culture HEp-2 cells were obtained from American Type Culture Collection (ATCC) and maintained in Eagle's Minimal Essential Medium with Earle's balanced Salt solution (Bio Whitaker), 1 mM L-Glutamine and 10% fetal calf serum. Intracellular growth was assayed essentially as described by Leung and Finlay (28) . Briefly, 10µl of a standing overnight culture was added to each well of a 24-well microtiter plate containing a monolayer of HEp-2 cells. Bacteria were allowed to invade for 1 hr, then the wells were rinsed 5 times with 1 ml of phosphate-buffered saline. Cells were overlaid with fresh medium containing 12 µg/ml gentamicin to kill extracellular bacteria. At this point (t=0h) and after 18 hours, cells were lysed in 0.5% sodium deoxycholate and plated to determine the number of intracellular bacteria. The fold growth was determined as the ratio of bacterial numbers per well at 18 hours to the number present at 0 hours.

Mouse experiments

LD_{50} and in vivo stability of mutations Female BALB/c ByJ mice (6 to 8 weeks old) obtained from Jackson Laboratories (Bar Harbor, ME) and housed under specific pathogen-free conditions were used for all experiments. Virulence of mutants was tested by administering 0.2 ml of serial tenfold dilutions in LB (ranging from 10^9 to 10^4 CFU/ml) intragastrically (i.g.) to groups of four mice. For intraperitoneal infection, dilutions ranging from 10 to 1000 CFU/ml were made in phosphate-buffered saline (PBS) and 0.1 ml injected. Mortality was recorded at 28 days post infection and the 50% lethal dose (LD_{50}) values for each route of infection calculated by the method of Reed and Muench (37).

Stability of mutations or plasmids in vivo was assessed by determining colony counts in Peyer's patches, mesenteric lymph nodes, livers and spleens of moribund infected mice. Liver and spleen were each homogenized in 1 ml of PBS using a Stomacher (Tekmar, Cincinnati, OH) and 0.1 ml of diluted homogenate plated on agar containing appropriate antibiotics as well as plates without antibiotics. Viable counts were compared on each plate to determine stability of the mutations tested. Plasmid stability was tested by plating in duplicate on agar with and without the antibiotic used to maintain the plasmid.

Determination of bacterial counts in organs Groups of ten mice were infected i.g. with a 1:1 mixture of wild type and mutant *S. typhimurium* containing approx. 1 x 10^7 bacteria. At five days post infection, mice were sacrificed, internal organs (the three Peyer's patches proximal to the ileum, mesenteric lymph node, spleen and liver) collected and homogenized in 5 ml PBS using a Stomacher (Tekmar, Cincinnati, OH). Dilutions were plated on LB plates containing the appropriate antibiotics and significance of differences observed was determined with the Wilcoxon signed rank test for a paired experiment. For colonization studies, groups of 10 mice were infected i.g. with a 1:1

mixture of wild type and mutant *S. typhimurium* containing approx. 1 x 10^7 bacteria and two fecal pellets were collected daily for enumeration of bacterial counts. Fecal pellets were weighed, homogenized in 1 ml PBS and plated on appropriate antibiotic plates to determine CFU/mg feces for each strain.

Growth in serum Balb/c mice (retired breeders) were anesthetized and blood collected from the axillary vessels. Blood from 15 mice was pooled and allowed to coagulate 1 hr at room temperature, then kept over night on ice. Serum was filtered and used immediately for bacterial growth experiments. To assess bacterial growth, overnight cultures of strains to be tested were washed in PBS, then diluted to 10^6 CFU/ml in PBS. 10μl was added to duplicate wells containing 0.5 ml of serum in a 24-well microtiter plate. To assess growth, 20 μl was removed at appropriate time points, diluted and plated on antibiotic plates for enumeration of bacteria.

Table 1: Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source
S. typhimurium		
ATCC 14028	wild type strain	ATCC
IR 715	nalidixic acid resistant derivative of ATCC 14028	(42)
AR1258	14028 *entB::MudJ*	(45)
AIR15	IR715, *feoB*::*tet*	this study
AIR17	AR1258, *feoB*::*tet*	this study
AIR36	IR715, *tonB*::*km*	this study
AIR62	AIR15, *tonB*::*km*	this study
CL1509	14028 *aroA*::Tn*10*	(12)
AIR51	AIR36, *aroA*::Tn*10*	this study
SA3675	*fhuB*::Mud*J*, *aroA*::Tn*10*	(45)
E. coli		
S17-1 λ*pir*	*Prp thi recA hsdR*: chromosomal RP4-2 (Tn*1*::ISR*1 tet*::Mu Km::Tn*7*); λ*pir*	lab collection
DH5α	*endA1hsdR17(rk-mk-) supE44t hi-1 recA1 gyrA relA1 Δ(lacZYA-argF) U169 deoR (φ80 dlac Δ (lacZ)M15)*	lab collection
CC118	*araD139 Δ(ara, leu) ΔlacY74 phoA20 galE galK thi rpsE rpoB* $argE_{am}$ *recA1*	R. Taylor
H5128 (24)	*aroB tsx malT tonB feoB*:Tn*5*	K. Hantke
Plasmids		
pEP185.2	pGP704, pBluescript MCS, *cat*	J. Pepe (25)
pBluescript KS	colE1, *bla*	Stratagene
pUH18 (24)	pT7-6, *E. coli feoB*	K. Hantke
pIRS618	pSUKS1, *S. typhimurium tonB*	this study

Results

Partial cloning of the *S. typhimurium feoB* homologue and construction of a *feoB* mutant

In *E. coli, feoAB* has been shown to encode a transport system which mediates uptake of ferrous ions (24). In order to examine the role of iron(II) uptake during *S. typhimurium* infection of mice, we cloned a fragment of the *S. typhimurium feoB* homologue and used this fragment to construct a defined mutant in the *S. typhimurium feoB* gene. To this end, we first attempted to amplify a fragment of *feoB* from *S. typhimurium* IR715 (42)using primers designed from the *E. coli feoB* sequence (24). Using these primers we amplified an 1.0 kb product from *S. typhi*, but we were unable to amplify a fragment of *feoB* from *S. typhimurium*. The *S. typhi* PCR product was cloned in the vector pCRII (Invitrogen) and was confirmed by sequencing to contain a *S. typhi feoB* homologue (data not shown). Using this sequence, new primers were designed and an 895 bp DNA fragment was amplified from *S. typhimurium*. This product was cloned into pCRII. Sequence analysis of this construct indicated that it contained a fragment of the *S. typhimurium feoB* homologue corresponding to bp 79 to 952 of the *E. coli feoB* gene (24). The deduced amino acid sequence of the *S. typhimurium* FeoB protein possessed 87% identity and 96% similarity at the amino acid level to the *E. coli* protein (24) (Fig. 1). In order to create a construct for allelic exchange, the pAK1900 tetracycline resistance cassette (K. Poole, unpublished results) was introduced into an internal *Hind*II restriction site in the cloned fragment of the *S. typhimurium feoB* gene. The fragment of the *S. typhimurium feoB* gene containing the tetracycline resistance gene was excised from pBluescript using the enzymes *Xba*I and *Kpn*I and inserted into the suicide vector pEP185.2 (25). The resulting construct was propagated in the host strain S17-1λ*pir* and introduced into *S. typhimurium* IR715 (Nal^r) by conjugation. Exconjugants were plated on agar containing nalidixic acid and tetracycline to select for recipients of the suicide vector.

These were then restreaked on plates containing chloramphenicol to test for loss of the suicide vector and stable integration of the tet cassette into the *feoB* gene. Recombinants resistant to tetracycline but sensitive to chloramphenicol originate from allelic exchange between the chromosomal *feoB* and the mutated copy carried on pEP185.2. One recombinant was selected for further study and designated AIR15. DNA from this strain was isolated and the insertion of the tetracycline resistance cassette in *feoB* was confirmed by Southern Blot (Fig. 2A).

Confirmation of the *feoB* phenotype of AIR15

In order to confirm the inactivation of the *S. typhimurium feoB* gene, AIR15 (*feoB*) was tested for a phenotype described for *E. coli feo* mutants. Mutations in the *feo* locus of *E. coli* have been shown to result in internal iron starvation, even in the presence of exogenously supplied iron (22). This iron starvation can be seen as constitutive expression of an Fur regulated transcriptional fusion to *lacZ*. In order to confirm this phenotype for the *S. typhimurium feoB* mutant (AIR15), the *feoB* mutation was introduced into a *S. typhimurium* reporter strain carrying an iron responsive *lacZ* fusion. This reporter strain, AR1258 carries Mud*J* inserted in the *entB* enterobactin synthesis gene (45). The resulting *entB*::*lacZ* fusion is regulated by the Fur repressor and is therefore expressed only under iron deficient conditions. Expression of the *entB*::*lacZ* fusion was compared in both AR1258 (*entB*::*lacZ*) and AIR17(*feoB entB*::*lacZ*) by measuring β-galactosidase levels after growth under both iron-rich and iron-deficient conditions (Table 2). While expression of the *entB*::*lacZ* fusion was derepressed only under iron deficient conditions in AR1258, similar levels of β-galactosidase were found under both growth conditions in AIR17. This result indicated that the *feoB* mutation causes the same phenotype in *S. typhimurium* as in *E. coli*. Iron regulation of the *entB*::*lacZ* fusion in *S. typhimurium* AIR17 could be restored by introduction of the *E. coli feoB* gene on plasmid pUH18 (Table 2)

```
                                               32
E.c. FeoB     1 MKKLTIGLIGNPNSGKTTLFNQLTGSRQRVGNWAGVTVERKEGQFSTTDH
S.t. FeoB                                      NSA-FTVERKEGQFATTDH
                                               * *  *********.****

E.c. FeoB       QVTLVDLPGTYSLTTISSQTSLDEQIACHYILSGDADLLINVVDASNLER
S.t. FeoB       QVTLVDLPGTYSLTTISSQTSLDEQIACHYILSGDADLLINVVDASYLER
                ********************************************** ***

                                                +Tet^r
E.c. FeoB       NLYLTLQLLELGIPCIVALNMLDIAEKQNIRIEIDALSARLGCPVIPLVS
S.t. FeoB       NLYLTLQLLELGIPCIVALNMLDIAEKQQVRIDVDALSTRLGCPVVPLVS
                ****************************..**..****.******.****

E.c. FeoB       TRGRGIEALKLAIDRYKANENVELVHYAQPLLNEADSLAKVMPSDIPLKQ
S.t. FeoB       TRGRGIEALKLAIDRHNANDNVELVHYAQPLLREADFLADAMAQEMPLQQ
                *************** .**.************ *** **  *. ..**.*

E.c. FeoB       RRWLGLQMLEGDIYSRAYAGEASQHLDAALARLRNEMDDPALHIADARYQ
S.t. FeoB       RRWLGLQMLEGDIYSRXYAGEAAQNLDTSLARLKDEMDDPALHIADARYQ
                **************** *****.*.**..****..***************

E.c. FeoB       CIAAICDVVSNTLTAEPSRFTTAVDKIVLNRFLGLPIFLFVMYLMFLLAI
S.t. FeoB       CIAAICDVVSNTLTAEPSRFTRAVDKIILNRFLGLPIFLFVMYLMFLLAI
                ********************* *****.**********************

E.c. FeoB       NIGGALQPLFDVGSVALFVHGIQWIG  326
S.t. FeoB       NVGGALQPLFNAGSVAIFIHGIQWIG
                *.********. ****.*.*******
```

Fig. 1: Alignment of the predicted proteins of the *S. typhimurium* and *E. coli feoB* genes. The alignment spans from amino acids 1 to 326 of the *E. coli* protein. Identical amino acids are indicated by asterisks, and points denote conservative amino acid changes. The insertion site of the tetracycline resistance cassette is indicated above the sequence (+Tet^r). The alignment was performed using the ClustalV program.

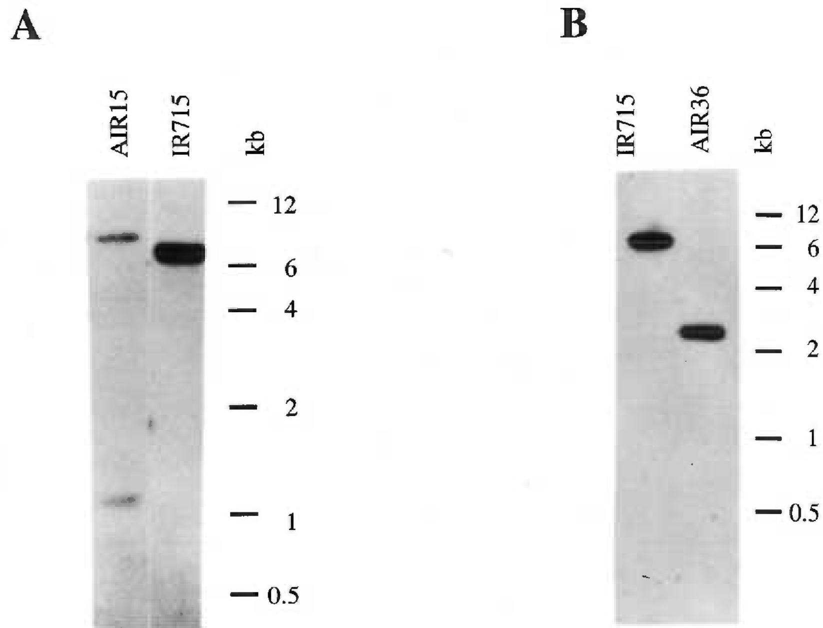

Fig. 2: Southern Blots confirming insertions in *S. typhimurium tonB* and *feoB* genes. **A:** Chromosomal DNA of AIR15 (*feoB*::tet) and IR715 (parent strain) digested with *Eco*RI/*Hind*III and probed with an 895 bp fragment of *S. typhimurium feoB*. **B:**Chromosomal DNA of AIR36 (tonB::Km) and IR715 digested with *Eco*RI and probed with a fragment containing bp 106-547 of *S. typhimurium tonB*.

Table 2: Effect of a mutation in *feoB* on the expression of an iron regulated gene

Strain	Relevant genotype	β-galactosidase activity (Miller Units)[a]	
		+Fe[b]	-Fe[c]
AR1258	*entB::lacZ*	12.4 ± 1.4[d]	260 ± 77
AIR17	*entB::lacZ feoB*	348 ± 76	334 ± 29
AIR17 (pUH18)	*entB::lacZ feoB* (*E. coli feoB* gene on plasmid)	17.3 ± 2.7	226 ± 55

[a] calculated according to Miller, 1972.
[b]grown in LB + 40mM $FeSO_4$
[c] grown in NB + 0.2mM 2,2'-dipyridyl
[d]averages of at least 4 independent experiments ± standard error

Construction of a *tonB* mutant

TonB has been shown to be required for assimilation of iron (III) from a variety of compounds, including siderophores and host iron proteins (10, 44). Therefore, mutation of *S. typhimurium tonB* should lead to inactivation of a wide range of potential iron(III) uptake mechanisms. In order to examine the role of iron (III) uptake on growth of *S. typhimurium* in its host we therefore constructed a defined mutation in *tonB* by allelic exchange. In order to amplify fragments of the *S. typhimurium tonB* gene, primers were designed using the sequence of *S. typhimurium tonB* which has been published previously (21). Restriction sites for *Eco*RI, *Sal*I and *Bam*HI were incorporated into the primers for subsequent cloning steps. Two fragments of the *tonB* gene were amplified by PCR. The 5'-fragment, containing bp 106-547 of *S. typhimurium tonB* was flanked by sites for *Bam*HI and *Eco*RI, and the 3' fragment, containing bp 566-939 by *Eco*RI and *Sal*I sites. These two fragments were digested with the enzymes *Eco*RI, *Sal*I and *Bam*HI and cloned together into *Bam*HI/*Sal*I digested pBluescript KS, to yield pTB1. Thus, pTB1 contains a fragment of *tonB* with an *Eco*RI site introduced into an internal deletion from bp 547-566.

The correct orientation of these fragments was confirmed by sequence analysis (data not shown). The kanamycin resistance cassette KSAC (Pharmacia) was introduced into this internal *Eco*RI site. The entire insert containing KSAC cassette flanked by *tonB* sequence was excised using the enzymes *Xba*I and *Kpn*I and inserted into the suicide vector pEP185.2 (25) to yield pTB3. This construct was propagated in the host strain S17-1λ*pir* and introduced into *S. typhimurium* IR715 by conjugation. Exconjugants were selected on plates containing nalidixic acid and kanamycin. These were then restreaked on plates containing chloramphenicol to test for loss of the suicide vector. Recombinants resistant to kanamycin but sensitive to chloramphenicol originate from allelic exchange between the chromosomal *feoB* and the mutated copy on pTB3. One such recombinant was designated AIR36 and studied further. The insertion in AIR36 was confirmed by Southern Blot of chromosomal DNA isolated from AIR36 and its parent (Fig. 2B).

Confirmation of the *tonB* phenotype of AIR36

In order to confirm the *tonB* mutation in AIR36 phenotypically, we tested the effect of the mutation on the ability of *S. typhimurium* to utilize ferrioxamine B as an iron source. In other enteric bacteria, uptake of ferrioxamines has been shown to be *tonB* dependent (3). Since *S. typhimurium* is able to utilize ferrioxamines as an iron source (29), a *S. typhimurium tonB* mutant should be unable to take up and utilize iron from ferrioxamine B. In order to prevent scavenging and uptake of ferrioxamine-iron by the siderophore enterobactin, ferrioxamine-iron utilization was assayed in an *aroA* strain background using *S. typhimurium* CL1509 (12). Because of its inability to produce a precursor common to both enterobactin and aromatic amino acids, the *aroA* mutant is unable to produce enterobactin. As a negative control, a *fhuB aroA* mutant (SA3675) was assayed for ferrioxamineB utilization. This mutant is defective in a cytoplasmic permease necessary for internalization of ferrioxamineB and ferrichrome, and is therefore unable to grow on ferrioxamineB as a sole iron source (3, 26, 45). Strains were tested in an agar

diffusion assay for growth on ferrioxamine B (Table 3). In contrast to its parent CL1509 (*aroA*), AIR51 (*aroA tonB*) did not grow around a ferrioxamineB-soaked disk (Table 3). Growth could be restored by introduction of plasmid pIRS618, carrying an intact copy of *S. typhimurium tonB* (Table 3). Plasmid pIRS618 contains a 3.5 kb *SacI/EcoRI* fragment of *S. typhimurium* ATCC14028 in the vector pSUKS1(34). It was obtained during an attempt to clone *S. typhimurium feoB* by complementation of *E. coli* H5128 (*aroB tonB feoB*) for growth on NB agar (24). This plasmid was confirmed by sequencing to contain the *S. typhimurium tonB* gene (data not shown).

These results show that the *tonB* mutation in AIR36 and AIR51 exhibits a phenotype shown for *tonB* mutants in other species.

Table 3: Utilization of ferrioxamine B on NBD agar

Strain	Genotype	Growth zone (mm)[a]
CL1509	*aroA*	24
SA3367	*fhuB aroA*	0
AIR51	*tonB aroA*	0
AIR51 (pIRS618)	*tonB aroA* (*tonB* on plasmid)	26

[a] growth zone around filter disk containing 3 μg Ferrioxamine B (Fe-Desferal).

Growth of *feoB* and *tonB* mutants in vitro

To further characterize our mutants, we examined the effects of *feoB* and *tonB* mutations singly and in combination with other mutations affecting iron uptake on growth in laboratory media. Under iron-rich conditions, no effect of these mutations on growth in either liquid or solid media was observed (Table 4, Table 5). Under iron-limiting conditions, mutations in both *tonB* and in *entB* , which encodes an enzyme necessary for enterobactin biosynthesis, led to a growth defect on solid and liquid media. Since *tonB* and

entB mutations both affect the same pathway of siderophore-mediated iron uptake, it was surprising that the *tonB feoB* mutant grew better than the *entB feoB* mutant on LB plates. One possible explanation for this finding is that the *tonB feoB* mutant is still able to produce and secrete the enterobactin precursor 2,3-dihydroxybenzoic acid (DHB) into the medium (36). In contrast, the *entB* mutant is blocked at a step before DHB in the enterobactin biosynthesis pathway (31). *E. coli* is able to utilize DHB as an iron source in a TonB-independent manner (20). Thus, DHB may mediate the better growth of the *tonB feoB* mutant as compared to the *entB feoB* mutant. A similar finding was reported when the growth phenotype of an *E. coli tonB feoB* mutant, which is able to synthesize enterobactin, was compared with that of an *E. coli tonB feoB aroB* mutant, which is defective for enterobactin synthesis(24). The growth defect of the *entB feoB* mutant on LB could be reversed by supplementation with the siderophore ferrioxamineB (Table 4).

Comparable results were seen when strains were grown aerobically in liquid media. NBD contains dipyridyl, a strong iron (II) chelator. The *feoB* mutant grew to nearly wild type levels, whereas strains carrying mutations in *tonB* and *entB* showed only limited growth (Table 5).

Intracellular growth

The ability to grow within epithelial cells has been shown to be required for virulence of *S. typhimurium* (28). Bacterial growth in the liver and spleen of the mouse has also been shown to be intracellular (16, 33). We therefore assessed the ability of the *tonB* and *feoB* mutants to grow intracellularly. Interestingly, neither the single mutants nor the double mutant differed from the wild type in their ability to grow within HEp-2 cells (Table 6). Similar results were obtained with HeLa cells and J774 cells (data not shown). Thus, the TonB and Feo uptake systems appear not to be necessary for intracellular growth of *S. typhimurium*.

Table 4: Growth on Solid Media

Strain	Mutations	Colony diameter (mm) on[a] LB+ Ferrioxamine	LB	NBD
IR715	wild type	+++[b]	+++	+++
AR1258	*entB*	+++	+++	+
AIR15	*feoB*	+++	+++	++
AIR36	*tonB*	+++	+++	+
AIR17	*entB feoB*	+++	+ +	
AIR62	*tonB feoB*	+++	+++	+
AIR62 (pIRS618)	*tonB feoB* (*tonB* on plasmid)	+++	+++	+++

[a]average diameter of five colonies. Error of the measurement was ≤ 10%.
[b] +++=1.4 - 2.0 mm; ++=0.7 - 1.3 mm; += 0.2 - 0.6 mm.

Table 5: Growth in iron-limited liquid media:

Strain	Genotype	OD_{578} after 24h in[a] Nutrient Broth / Dipyridyl[b]
IR715	wild type	0.748 ± 0.024
AR1258	*entB*	0.220 ± 0.032
AIR15	*feoB*	0.656 ± 0.044
AIR17	*feoB entB*	0.270 ± 0.042
AIR36	*tonB*	0.270 ± 0.006
AIR62	*feoB tonB*	0.266 ± 0.022
AIR62 (pIRS618)	*feoB tonB* *tonB* on plasmid	0.477 ± 0.021
AIR62 (pUH18)	*feoB tonB* *feoB* on plasmid[d]	0.206 ± 0.011

[a] mean of three experiments ± standard error
[b] Nutrient Broth containing 0.2 mM Dipyridyl
[d] *E. coli feoB*

Table 6: Intracellular growth in HEp-2 cells

Strain	Genotype	Fold growth after 18 hours[a]
IR715	wild type	10.8 ± 1.5
AIR15	*feoB*	8.9 ± 1.6
AIR36	*tonB*	10.9 ± 1.9
AIR62	*tonB feoB*	11.7 ± 2.2

[a] mean of three experiments ± standard error

Virulence studies

In order to determine whether mutations in *tonB* and *feoB* affect the ability of *S. typhimurium* to cause disease in the host, we examined the ability of these mutants to cause lethal infection in the mouse. Previous studies on the role of iron uptake in *S. typhimurium* infection of the mouse have been performed using the i.v. or i.p. routes of infection (7, 48). These routes of infection bypass the intestine, where the initial phase of infection takes place. In order to include this stage of infection in our study, we determined the LD_{50} of mutants administered i.g. as well as i.p. (Table 7). Strains carrying single as well as double mutations were tested. We confirmed the previous result from Benjamin et al, that a mutation in the enterobactin biosynthesis pathway had no effect on virulence(7). Similarly, a mutation in *feo* did not reduce virulence. No additive effects of mutations in *entB*, *feoB*, or *tonB* on mouse virulence were observed by either route of infection. Interestingly, only strains AIR36 and AIR62, carrying *tonB* mutations, exhibited an increased LD_{50} when given i.g. In addition, the average time to death of mice infected i.g. at doses close to the LD_{50} was examined. Both AIR36(*tonB*) and AIR62(*tonB feoB*) required, on the average, two to three days longer to kill mice than the parent strain (Table 7). The LD_{50} of AIR62 could be lowered to wild type level by introducing an intact copy of *tonB* on pIRS618 (Table 7). All strains tested were as virulent as the wild type when

administered i.p. These results indicate that the *tonB* mutant is affected during growth in a compartment encountered only during i.g. infection but not during i.p. infection.

In order to determine at what point in infection the *tonB* mutants are affected, bacteria were enumerated in Peyer's patches, mesenteric lymph nodes, livers and spleens of mice 5 days after intragastric infection (Table 8). In order to compensate for large differences between individual animals, each mouse was infected with a 1:1 mixture of *tonB* mutant and wild type bacteria. Since the two strains carry different antibiotic resistance genes, it is possible to determine the numbers of each strain in the organs by plating on the appropriate antibiotics. Significant differences between the numbers of *tonB* and wild type bacteria were found in the Peyer's patches ($p<0.01$) and the mesenteric lymph nodes ($p<0.025$; Table 8). Numbers of the two strains recovered from the liver and spleen showed no significant differences. These results show that the *tonB* mutant has a decreased ability to reach or to grow in the Peyer's patches and the mesenteric lymph nodes, but is able to grow in the liver and spleen like the wild type. A further indication that TonB contributes to growth in these organs was obtained during in vivo complementation of the *tonB feoB* mutant AIR62 with a plasmid pIRS618 carrying *tonB*. At 4 days post-infection, the spleen of a mouse infected with AIR62(pIRS618) was examined to determine the stability of pIRS618 in vivo. We found that although pIRS618 could complement the defect in virulence of AIR62 to the wild type level, less that 1% of bacteria isolated from the spleen still carried the plasmid. In contrast, 50% of the bacteria in the Peyer's patches and 10% of the bacteria in the mesenteric lymph nodes still carried the plasmid pIRS618 (data not shown). This result suggests that, in the spleen, there is no selective pressure to maintain the *tonB* plasmid. These data are in agreement with the LD_{50} values showing that the *tonB* mutants are not attenuated during i.p. infection (Table 7) and with the work of Benjamin and Stocker showing that *tonB* deletion mutants are not attenuated by parenteral routes of infection (6, 41).

Table 7: LD_{50} of iron uptake mutants

Strain	Relevant intraperitoneal genotype	intragastric infection		
		LD_{50}	mean time to death (days)[a]	LD_{50}
IR715	wild type	1.3×10^5	7.2 ± 0.98	<10
AR1258	*entB*	1.4×10^5	10 ± 4.4	<10
AIR15	*feoB*	1.5×10^5	8.3 ± 1.1	<10
AIR36	*tonB*	7.8×10^5	13.3 ± 5.9	<10
AIR17	*entB feoB*	2×10^5	8.0 ± 1.4	<10
AIR62	*tonB feoB*	1.1×10^6	11.8 ± 3.5	<10
AIR62 (pIRS618)	*tonB feoB* (*tonB* on plasmid)	1.9×10^5	7.0 ± 0.0	nd[b]

[a] mean $\pm$ standard error for mice infected at doses above and below the LD_{50}.
[b] nd=not determined

Table 8: Bacterial load in organs of mice five days after mixed infection[a]

Organ	mean of CFU / organ ± standard error[b]		Significance of difference between IR715 and AIR36[c] (probability)
	IR715 (wild type)	AIR36 (*tonB* mutant)	
Peyer's patches[d]	$6.1 \times 10^2 \pm 3.0 \times 10^2$	$1.2 \times 10^2 \pm 67$	significantly different ($p<0.01$)
Mesenteric lymph node	$3.4 \times 10^4 \pm 2.7 \times 10^4$	$8.5 \times 10^3 \pm 2.4 \times 10^3$	significantly different ($p<0.025$)
Liver	$1.2 \times 10^5 \pm 1.0 \times 10^5$	$8.8 \times 10^4 \pm 4.7 \times 10^4$	not significantly different ($p>0.05$)
Spleen	$7.5 \times 10^5 \pm 7.5 \times 10^5$	$3.2 \times 10^5 \pm 1.8 \times 10^5$	not significantly different ($p>0.05$)

[a] Mice were infected with a 1:1 mixture of IR715 and AIR36 containing 1×10^7 bacteria and organs were collected after 5 days.

[b] mean of between 9 and 16 mice

[c] Significance of differences observed were determined using the Wilcoxon signed rank test.

[d] Three Peyer's patches proximal to the cecum were collected and pooled.

Colonization of the mouse intestine

In order to assess the contribution of TonB and Feo-mediated iron uptake in colonization of the mouse intestine, competitive infections of mice were performed. Mixtures (at a 1:1 ratio) of AIR36(*tonB*)/IR715 and AIR15(*feoB*)/IR715 were administered i.g. to groups of 10 mice. Between 1 and 4 days post-infection, bacterial counts in the feces of infected mice were determined. Although the numbers of *tonB* mutant and wild type bacteria recovered between 1 and 4 days post-infection differed only on day 2 post infection, the *feoB* mutant was recovered in lower numbers at days 3 and 4 (Figure 3). Numbers of the *tonB feoB* double mutant in the feces were consistently lower than the wild type during all four days of infection assessed (Figure 3). These data show that *feoB* mutants are defective in growth in the intestine and that this growth defect can be potentiated by a second mutation in *tonB*.

Growth in normal mouse serum

We next examined the role of TonB and Feo during growth in normal mouse serum. Since *ent* mutants have been shown to be deficient for growth in serum, growth of the *entB* mutant was quantified as a negative control (7). The kinetics of growth of the *tonB* mutant reveals two growth phases. From 0 to 8 hours, *tonB* fails to grow, however by 12 hours, bacteria grow to almost wild type levels. Reversion of the *tonB* mutation was ruled out by plating on antibiotic plates. One possibility for the observed growth kinetics is the accumulation of DHB as a siderophore precursor. During the first 8 hours, the *tonB* mutant is iron starved and produces large amounts of enterobactin. By 8 hours, however, there may be a sufficient level of DHB to permit growth of the *tonB* mutant (19, 24). As in all other media tested, the *feoB* mutant grew like the wild type. Since *entB* mutants were unable to grow in serum but were fully virulent in mice, growth of *S. typhimurium* in the mouse does not appear to occur in the blood.

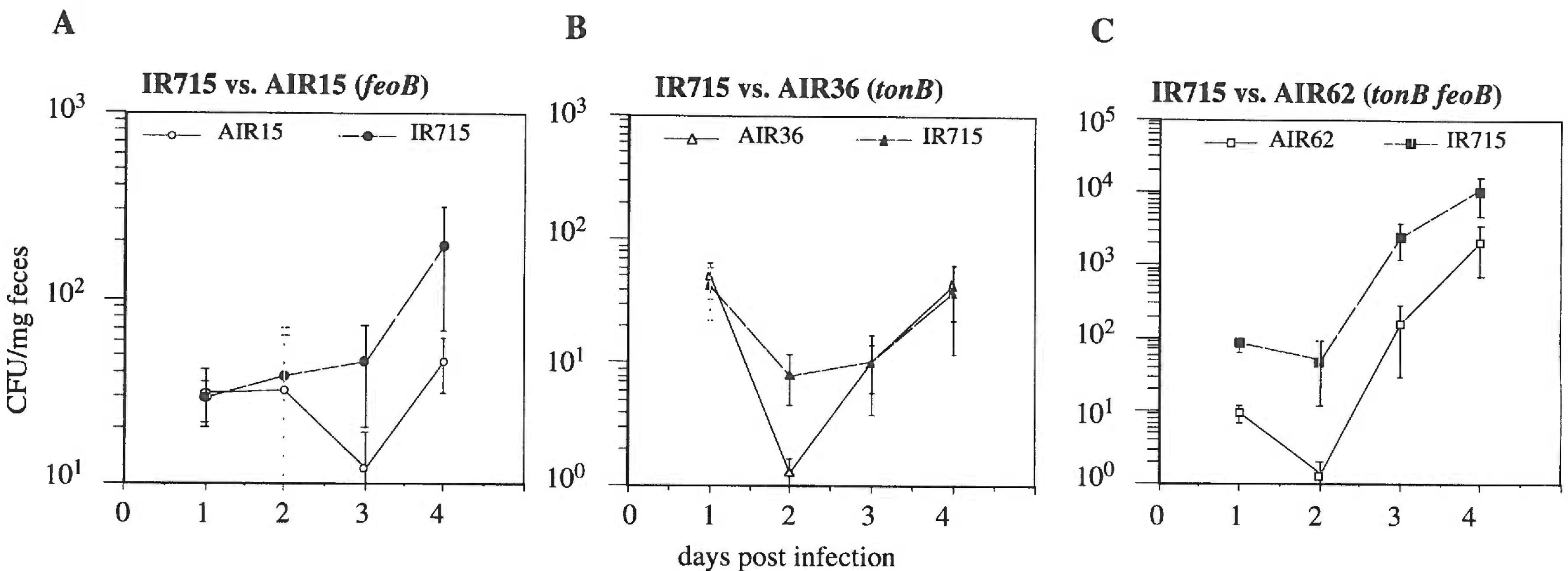

Fig. 3: Comparison of the ability of S. typhimurium mutants (A) AIR15 (*feoB*), (B) AIR36(*tonB*), and (C) AIR62(*tonB feoB*) to colonize the mouse intestine when administered in a 1:1 mixture with the parent strain (IR715). Data points represent the average of 10 mice ± standard error.

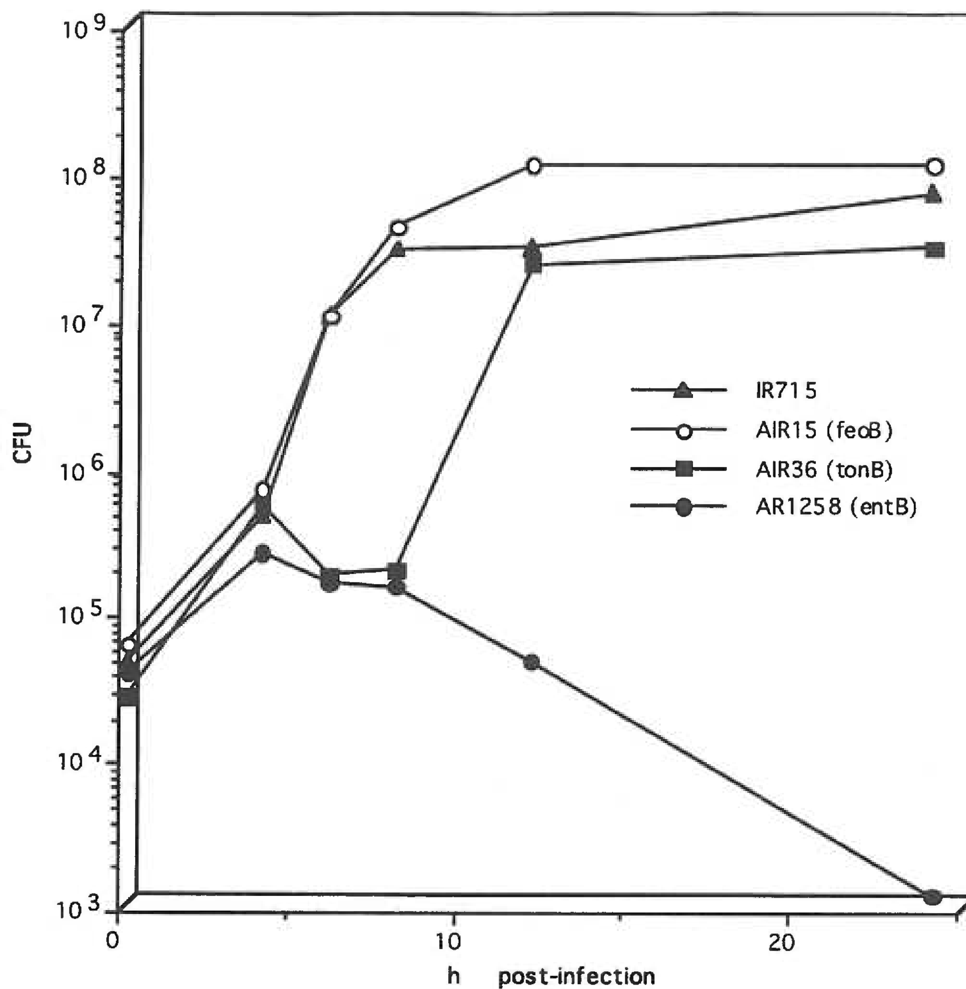

Fig. 4: Comparison of the ability of *S. typhimurium* mutants to grow in pooled normal mouse serum. Serum from 15 mice was pooled for this experiment. Data point represent the average of duplicate wells.

Discussion

This study examined the role of iron (II) uptake, mediated by the Feo uptake system, and the role of iron(III) uptake, mediated by TonB, on the ability of *S. typhimurium* to grow in its murine host. Using allelic exchange we constructed defined *S. typhimurium* mutants in *feoB* and *tonB*. As has been shown for *E. coli*, the mutation in *feoB* resulted in derepression of Fur-regulated genes in *S. typhimurium*. Similarly, we found that, as in other enteric bacteria, TonB is necessary for uptake of ferrioxamine B. These results confirmed that *feoB* and *tonB* have similar roles in iron uptake in *S. typhimurium* as in other bacteria. We further characterized the effect of these mutations and mutations in *entB*, a gene involved in enterobactin biosynthesis, on growth under different in vitro conditions. Under iron-rich conditions, these mutations had no effect on growth in liquid or solid media. Under iron-limiting conditions, mutations in both *tonB* and *entB* led to a growth defect on both solid and liquid media. This was expected, since enterobactin is the only siderophore produced by this strain and TonB is necessary for enterobactin uptake. However, a precursor of enterobactin, 2,3-dihydroxybenzoic acid (DHB), can be used as an iron source in *E. coli* and is internalized independently of TonB (20). The *entB* mutant is blocked before DHB in the enterobactin synthesis pathway (31), but the *tonB* mutant is still able to produce DHB. This defect in production of DHB might be the reason that a *S. typhimurium entB feoB* mutant has a growth defect on LB, while the *tonB feoB* mutant grows like the wild type.

The *feoB* mutant and *tonB* mutant differed in their ability to grow in vivo, in different compartments of the mouse. Whereas the *feoB* mutant was capable of growing like the wild type in vitro, it was at a disadvantage when compared to its parent strain during colonization of the mouse intestine. These results indicate that the iron(II) uptake mediated by *feo* contributes to growth of *S. typhimurium* in the mouse intestine. The data are

therefore consistent with earlier studies which show that *E. coli feo* mutants are impaired in their ability to colonize the mouse intestine (43). This decreased colonization of the *S. typhimurium feoB* mutant, however, had no effect on its ability to cause lethal infection in the mouse. Although the *ton B* mutation alone had no significant effect on colonization of the mouse intestine, it further attenuated the growth of the *feoB* mutant in this environment.

The *S. typhimurium tonB* mutant was, however, attenuated for intragastric infection of mice. The *tonB* strain displayed not only an fivefold increased LD_{50}, but infected mice also required longer to die from infection with this mutant. Since the *tonB* mutant was fully virulent by the i.p. route of infection, these findings suggested that TonB is necessary for growth in a compartment of the mouse other than the liver and the spleen, since these are the sites of multiplication common to both routes of infection. Rather, this compartment appears to be in either the Peyer's patches or the mesenteric lymph nodes, as significantly ($p<0.01$) lower numbers of the *tonB* mutant than the wild type were recovered from these organs during infection. The *S. typhimurium tonB feoB* mutant exhibited the same degree of attenuation as the *tonB* mutant, and could be complemented to the i.g. LD_{50} of the wild type by introduction of a plasmid-encoded copy of *tonB*. These results show that even in the absence of TonB-mediated ferric iron uptake, ferrous iron uptake via Feo does not contribute to growth in mouse tissues. Interestingly, during in vivo complementation, the plasmid carrying the *tonB* gene was maintained better at sites at which TonB was necessary for growth. For example, in Peyer's patches and in the mesenteric lymph node, where mutations in *tonB* caused a growth defect, the plasmid was maintained in 10-50% of the bacteria recovered. In contrast, only about 1% of the bacteria recovered from the liver and spleen had retained the plasmid carrying *tonB*. Together these data indicate that the attenuation of the *S. typhimurium tonB* mutant is caused by a growth defect in the Peyer's patches and mesenteric lymph nodes. This growth defect reduces the growth rate in the mouse and causes a delayed time to death of infected mice. The growth

defect of the *S. typhimurium tonB* mutant is most likely not caused by a defect in multiplication within epithelial cells or in the blood, as the *tonB* mutant was able to grow like the wild type within cultured epithelial cells and in mouse serum.

In addition to mediating iron(III) uptake, TonB is also necessary for transport of vitamin B_{12} into the cell (2). *S. typhimurium* is also able to synthesize vitamin B_{12} under anaerobic conditions (23). However, neither mutations affecting synthesis nor those affecting uptake of vitamin B_{12} attenuated *S. typhimurium* for virulence in the mouse model of infection (39). Thus the virulence defect of the *tonB* mutant is not caused by a defect in vitamin B_{12} uptake.

TonB is necessary for uptake of enterobactin but a *tonB* mutant reached the same numbers in mouse serum as the wild type (Fig. 4). Since we and others (Fig. 4) (7) have shown that *ent* mutants are unable to grow in normal mouse serum, this result was at first confusing. However, *tonB* mutants are still able to produce 2,3-dihydroxybenzoic acid (DHB), which is able to chelate iron and can be taken up in a TonB-independent manner (20, 24). It is therefore likely that, in mouse serum DHB can mediate uptake of iron in the *tonB* mutant. However, since the *entB* mutant was defective for growth in serum but not in the mouse, it appears that growth in the blood or in a serum-like compartment does not contribute to virulence.

Taken together, these data suggest that during the course of a *S. typhimurium* infection, bacteria pass through a series of compartments, each of which contains different iron sources for bacterial growth. Consequently, in order to grow in a particular compartment *S. typhimurium* must possess different uptake systems to exploit these iron sources. In the anaerobic environment of the gut, ferrous iron appears to be available and contributes to growth. In the Peyer's patches and the mesenteric lymph nodes, a form of iron (III) appears to be the available source, as *tonB* mutants had difficulty in multiplying at these sites. Once the bacteria pass through the lymph nodes into the lymph vessels, they enter the bloodstream. Growth in the bloodstream does not appear to contribute to

virulence, since mutants in enterobactin biosynthesis, which are unable to grow in serum, are fully virulent (7). In the liver and spleen bacteria are filtered out of the blood by resident macrophages. These are the cells in which mouse pathogenic *Salmonella* species are thought to grow (11). The resident macrophages of the liver and spleen are also rich in iron, as they have the function of removing effete red blood cells from the circulation. Thus, one possible strategy for *S. typhimurium* to obtain iron in the host is to multiply within these sites of iron storage (4). *S. typhimurium* apparently is able to obtain iron in this intracellular niche independently of Feo and TonB-dependent iron uptake systems. These findings suggest that a third type of iron uptake system may be involved in growth of *S. typhimurium* in the liver and spleen.

Acknowledgments:

The authors thank S. Anic for sequencing with the ALF automatic sequencer, K. Hantke for providing us with advice as well as plasmid pUH18 and strain H5128, K. Poole for providing plasmid pAK1900, and S. Hoffmann for assistance with the mouse experiments. This work was supported by US Public Health Service grant AI 22933 to F.H. from the National Institutes of Health.

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Discussion and Conclusions:

The pathogenesis of *Salmonella typhimurium* infection requires bacterial growth in the host. Although the bacteria can synthesize most compounds required for their growth, iron is a nutrient which must be acquired from the external environment. Mammalian hosts exploit this weakness by withholding iron to inhibit the growth of microorganisms (23). Bacteria, in turn, use this low iron concentration as a signal for the induction of virulence genes (6). In fact iron concentration has been suggested to be the second most frequent signal (after temperature) used to regulate expression of virulence genes. These virulence genes may encode toxins, which release cellular iron compounds by lysis of host cells, or specialized uptake systems for host iron proteins (6). In some cases, however, they appear to be only indirectly related to iron acquisition, such as the acid tolerance genes of *Salmonella* (11).

The result that led us to examine the role of the iron-responsive Fur regulon of *S. typhimurium* in pathogenesis was obtained while examining the behavior of a *fur* mutant in *Salmonella*-infected macrophages. It was found that this *fur* mutant was recovered in higher numbers from murine macrophages than the parent *Salmonella* strain. Since survival in macrophages is essential to the pathogenesis of mouse typhoid, this was a very interesting finding, and it led us to ask whether Fur might regulate genes required for macrophage survival and possibly other virulence genes of *S. typhimurium.* In order to address this question, we took two different approaches to identify Fur regulated genes involved in virulence: (i) cloning and inactivation of Fur regulated genes known to exist in other enteric bacteria, and (ii) screening the *S. typhimurium* genome for new genes regulated by Fur.

The increased survival of the *fur* mutant was evident as early as 1 h after infection of macrophages. Killing of bacteria by macrophages early in infection is dependent on the oxidative burst. Therefore, the improved survival of the *fur* mutant may be the result of a

preadaptation to oxidative stress caused by the *fur* mutation. One gene involved in detoxification of oxygen radicals, *sodA* , is repressed by Fur (10). Thus, we reasoned that derepression of *sodA* might explain the elevated macrophage survival of the *fur* mutant. To test this hypothesis, we inactivated the *sodA* gene encoding MnSOD and characterized the *S. typhimurium sodA* mutant. Whereas the *fur* mutation increased resistance of *S. typhimurium* to killing early during infection of J774 macrophages, introduction of a *sodA* mutation into the *fur* background abrogated this effect. These results showed that, in the *fur* mutant, derepression of the *sodA* gene led to increased expression of MnSOD and protection against macrophage killing. Interestingly, the *sodA* mutant was only two-to threefold attenuated in the mouse model of infection. Since *S. typhimurium* is able to survive in both PMNs and macrophages which generate a burst of reactive oxygen species upon phagocytosis, this finding was surprising. These data therefore suggested that MnSOD does not play a crucial role in infection. One possible explanation for the lack of attenuation of the *sodA* mutant is that *S. typhimurium* possesses at least one other SOD, FeSOD, which may be sufficient to protect against oxidative stress encountered by *S. typhimurium* in vivo. This possibility could be tested by measuring the in vitro sensitivity of the *sodA* mutant to superoxide. An alternative explanation is that *S. typhimurium* may be able to enter PMN and macrophages without triggering the oxidative burst, or it may elicit only a weak oxidative burst. This hypothesis is supported by the finding that the closely related serotype *S. typhi* fails to trigger the oxidative burst in macrophages (16, 22). Furthermore, mutants of *S. typhimurium* lacking catalase activity are also fully virulent (7). Another pathogen which grows within alveolar macrophages, *Legionella pneumophila*, enters these cells without triggering the oxidative burst. In fact, *L. pneumophila* is exquisitely sensitive to reactive oxygen species in vitro (12). *Legionella* is able to avoid damage by toxic oxygen species by entering the macrophage via complement receptors which mediate phagocytosis but do not trigger the oxidative burst (17). Complement receptors have also been proposed to be involved in uptake of *S. typhimurium*

by macrophages (13). In fact, these receptors are expressed at high levels on the surface of resident macrophages, the cell type preferred as a habitat by *S. typhimurium*. Thus, the apparent lack of dependence on oxygen detoxifying enzymes during infection may result from an ability of *S. typhimurium* to avoid the products of the oxidative burst in vivo. This hypothesis could be addressed experimentally by quantification of the oxidative burst in different populations of murine macrophages upon phagocytosis of *S. typhimurium.*

Concurrent with the studies on *sodA*, we attempted to find additional Fur-regulated genes which might be involved in virulence. Interestingly, although the *S. typhimurium fur* mutant was able to survive better than the wild-type in macrophages, it was attenuated approximately 100-fold in the mouse (A. Bäumler, unpublished results). In light of this result and also of Foster's work indicating that a large number of genes are regulated by Fur in *Salmonella* (11), we decided to look for new Fur-regulated genes. Two different techniques were used to search the *Salmonella* genome for genes regulated by Fur. The first, mutagenesis with the transposon Mud*J*, created transcriptional fusions to *lacZ*. These transcriptional fusions were then screened for Fur regulation. The second approach was to use the Fur titration assay (FURTA) to screen a *Salmonella* plasmid bank for Fur binding sites (20). With these two methods we screened 5,000 *Salmonella* transcriptional fusions to *lacZ* and 10,000 clones of a *Salmonella* plasmid bank for Fur regulated genes or promoters, respectively. As a result, we identified 14 genes which were regulated by Fur. If one considers that homologues of some of the genes found in our study are present in operons in *E. coli*, then the loci identified in this study contain approximately 24 Fur-regulated genes.

The genes found in this study fell into three different groups: genes involved in iron uptake, metabolic genes and unknown genes. Of these, the genes encoding cytosolic or cytoplasmic membrane proteins were the most highly conserved when compared to *E. coli.* (Table 1). The greatest degree of variability was found in the outer membrane siderophore receptors. This finding was not surprising, since surface-exposed proteins contribute to

the antigenicity of bacteria, and the host immune response may thus exert some selection for variation in these surface domains. Siderophore receptors also serve as phage receptors, for example FhuA in *E. coli* is the receptor for the phages T1, T5 and $\phi80$ (4). In *S. typhimurium*, FhuA is the receptor for phage ES18, which does not infect *E. coli* K-12 (5). The ability of phages to bind to one FhuA protein and not another may be a result of this variability in the receptors. These lytic phages may actually, during the evolution of these two species, have exerted a selective pressure on the bacteria to develop variants to which phages can no longer bind.

Table 1: Conservation between *S. typhimurium* and *E. coli* proteins

Protein	% similarity
Outer membrane receptors	
FepA	88
FoxA	78*
FhuE	61
FhuA	76
Cytoplasmic membrane proteins	
P43	97
FhuB	96
FepD	91
Cytoplasmic proteins	
EntB	94
FepC	95
Fur	98
Fes	81
PGAM1	98

*similarity between *S. typhimurium* and *Y. enterocolitica*

Of the unknown genes, we wanted to determine which of these could be virulence factors for *S. typhimurium*. Our reasoning was that *S. typhimurium* may possess specific

adaptations to an intracellular lifestyle that would be absent from the closely related *E. coli*, which is primarily extracellular. Three genes were found which did not have an *E. coli* counterpart in the GenBank database. These were used to probe the *E. coli* genome for similar sequences. Of these, only *foxA*, possessing homology to the *Yersinia enterocolitica* ferrioxamine receptor, was unique to *Salmonella* (1). By inactivation of the *S. typhimurium foxA* gene we found that, like its *Yersinia* homologue, it also mediates uptake of ferrioxamine B (ferrated Desferal) (1). This finding was of clinical relevance because Desferal is the only iron chelator commercially available for use in patients suffering from iron overload diseases. In addition, the *foxA* gene is specific to *Salmonella* serovars and could therefore be used in PCR or hybridization-based approaches to identify *Salmonella* in food or clinical specimens (These two methods are species-specific and require a higher degree of DNA homology than was found between *S. typhimurium* and *Y. enterocolitica foxA* genes). However, the *foxA* gene could not contribute to the pathogenesis of mouse typhoid caused by *S. typhimurium*. FoxA is a TonB-dependent receptor, and the *tonB* gene has previously been shown not to be required for intracellular growth in the liver or spleen of the mouse, the hallmark of mouse typhoid (2, 21).

In retrospect, the use of hybridization to *E. coli* to eliminate candidate virulence genes may have had one drawback. It is possible that a particular gene could be present in both *E. coli* and *S. typhimurium*, but be expressed only in *Salmonella*, where it contributes to pathogenesis. Thus, we could potentially have missed the identification of a virulence gene in this step.

The Fur regulon has been characterized most extensively in *E. coli*, where more than 36 Fur regulated genes have been described (6). Assuming similar sizes of the Fur regulon in *E. coli* and *S. typhimurium*, about two-thirds of the *Salmonella* Fur regulon was identified in this study. Further evidence that the *Salmonella* Fur regulon contains more than the 24 genes found in our study is the fact that we did not find *feo*, *tonB*, or *sodA* genes, which have been shown to exist in *S. typhimurium*. These other genes may not have been found

because we did not screen a sufficient number of mutants. Since we screened 5,000 *lacZ* fusions and 10,000 plasmids (or a total of 15,000 strains) and found two-thirds of the estimated number of Fur-regulated genes, we would have had to screen approximately 22,500 strains (or 50% more) to find the remaining genes. Another explanation for not identifying more Fur-regulated genes is that some of the genes may be under the control of more than one regulator. For example, expression of *feoAB* is controlled by Fnr, a regulator of genes expressed during anaerobiosis, in addition to Fur (14). The *sodA* gene is under the control of six different regulators (10). Thus, other stimuli in addition to iron concentration control the regulation of these genes, and these signals may be required for gene expression. It is therefore possible that we did not detect those genes in our screen because our screen was designed to detect only iron-regulated genes, and lacked these additional signals necessary for gene expression.

Two genes in which we were particularly interested, but did not identify in our search for Fur-regulated genes, were the *tonB* and *feoB* genes. Although at the time the Feo system had only been identified in *E. coli*, we reasoned that since Iron(II) is present within eukaryotic cells, an iron (II) uptake system such as *feo* might provide *Salmonella* with this trace element during its intracellular growth. We therefore made independent mutations in the *tonB* and *feoB* genes of *S. typhimurium.*

The role of the *tonB* gene had previously been examined in an intravenous model of infection (2). This study showed that mutants lacking the *tonB* gene were able to grow as well as the wild-type in the liver and spleen. However, the study did not use a defined mutant, nor did it examine the role of TonB mediated uptake in the initial steps of infection, which take place in the intestinal lumen, Peyer's patches and lymph nodes. For these reasons, we wanted to create a defined *tonB* mutant and to examine its effect on these initial steps of infection. In addition, by combining mutations in *tonB* and *feo*, all iron(II) and iron(III) uptake systems described to date in *Salmonella* would be inactivated. With this

strategy, it was possible to determine whether inactivation of multiple systems had an effect on virulence.

We examined the effects of *tonB* and *feoB* mutations, singly and in combination, on growth of *S. typhimurium* in different compartments of the mouse. Our results indicate that *S. typhimurium* uses different iron uptake systems in these various compartments (Fig. 1). In the intestine, both *tonB* and *feoB* contributed to growth. Since the intestine is anaerobic, and should therefore contain Iron(II), it seems logical that an Iron(II) uptake system should play a role in this environment. Furthermore, since we observed an additive effect of mutations in *tonB* and *feoB* on intestinal growth, we concluded that Iron(III) is also utilized in this niche.

In contrast, the *tonB* mutant was defective for growth in the Peyer's patches and the mesenteric lymph nodes (Fig. 1). The importance of growth in these organs is indicated by the five-fold attenuation of the *tonB* mutant by the oral route of infection. Little is known about the environment to which *Salmonella* is exposed in these lymphatic organs. This result was therefore interesting, because it indicated that *S. typhimurium* utilizes an Iron(III) source for growth in the Peyer's patches and the mesenteric lymph node. Furthermore, this iron source is most likely not enterobactin, as an *entB* mutant defective in enterobactin synthesis was fully virulent by the oral route of infection (3, 21). Mutants in enterobactin synthesis are unable to grow in mouse serum (3). Thus, the ability of *S. typhimurium* to grow in serum does not contribute to mouse typhoid. In bacteremic disease caused by other *Salmonella* serovars, however, enterobactin-mediated iron uptake may play a role in infection.

Growth of *S. typhimurium* in the liver and spleen was found to be independent of TonB-mediated iron uptake. This result indicated that Iron(III) uptake is not required in this stage of infection. One explanation for the dispensability of TonB-mediated iron uptake might be the presence of other "backup" systems for iron acquisition. In order to determine whether *tonB* and *feoB* mutants are able to grow intracellularly because of the

redundancy of iron uptake systems, we combined these mutations with each other and with an *entB* mutation (which eliminated siderophore synthesis) and tested the effect of double mutants on the ability of *Salmonella* to grow in the liver and spleen. Although both *entB feoB* and *tonB feoB* double mutants were defective for growth on laboratory media, these strains were unaffected in their ability to multiply intracellularly. Therefore, these results indicate that *S. typhimurium* possesses yet other systems for acquiring iron in this niche.

How does *Salmonella* obtain iron during intracellular growth? It has been shown that the compartment in which *Salmonella* resides in the cell is acidified, and that acidification is necessary for intracellular growth (18). Thus, this vacuole may have characteristics of an early endosome. If this is the case, free iron would be soluble and more available to bacteria than in the extracellular environment, where its concentration is only 10^{-18}M. In fact, the early endosome is the compartment in which iron is released from transferrin, a process which requires acidification. It has been proposed that *Listeria* and *Legionella* are able to grow intracellularly by utilization of this labile intracellular iron pool, and that this growth requires an acidic endosomal compartment (8, 9). *S. typhimurium* may thus be able to utilize iron released from transferrin by a low-affinity uptake system. Such a system has been described in *E. coli* but not characterized (15). One obvious difficulty in studying low-affinity iron uptake is that all of the high affinity systems (such as the ones investigated here) must first be inactivated, as they would mask the iron uptake via the low affinity system. However, the *entB feoB* double mutant, which has two high affinity systems inactivated, was severely impaired for growth on laboratory media. Mutation of the low-affinity uptake system in this strain background might therefore be lethal to the strain. Thus, it may be impossible to identify a low affinity iron uptake system with this strategy. However, since other macrophage pathogens such as *Listeria* and *Legionella* apparently lack high-affinity uptake systems, a low-affinity iron uptake system might represent a general mechanism for acquisition of iron by intracellular pathogens that grow within host cells.

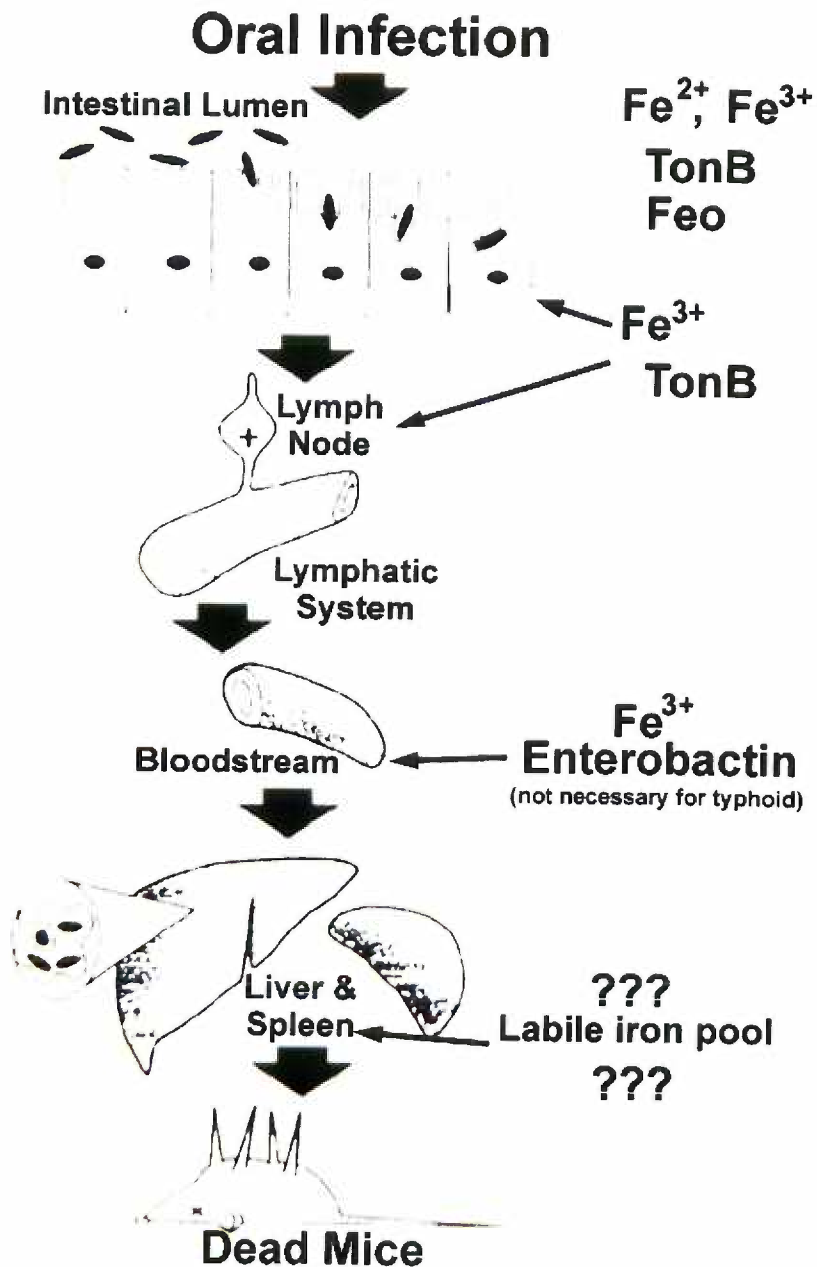

Figure 1: Iron uptake systems of *S. typhimurium* used during different stages of oral infection in the murine typhoid model

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