Fur Is the Master Regulator of the Extraintestinal Pathogenic Escherichia coli Response to Serum

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ABSTRACT Drug-resistant extraintestinal pathogenic Escherichia coli (ExPEC) strains are the major cause of colisepticemia (coli-bacillosis), a condition that has become an increasing public health problem in recent years. ExPEC strains are characterized by high resistance to serum, which is otherwise highly toxic to most bacteria. To understand how these bacteria survive and grow in serum, we performed system-wide analyses of their response to serum, making a clear distinction between the responses to nutritional immunity and innate immunity. Thus, mild heat inactivation of serum destroys the immune complement and abolishes the bactericidal effect of serum (inactive serum), making it possible to examine nutritional immunity. We used a combination of deep RNA sequencing and proteomics in order to characterize ExPEC genes whose expression is affected by the nutritional stress of serum and by the immune complement. The major change in gene expression induced by serum—active and inactive—involves metabolic genes. In particular, the serum metabolic response is coordinated by three transcriptional regulators, Fur, BasR, and CysB. Fur alone was responsible for more than 80% of the serum-induced transcriptional response. Consistent with its role as a major serum response regulator, deletion of Fur renders the bacteria completely serum sensitive. These results highlight the role of metabolic adaptation in colisepticemia and virulence.

IMPORTANCE Drug-resistant extraintestinal pathogenic Escherichia coli (ExPEC) strains have emerged as major pathogens, especially in community- and hospital-acquired infections. These bacteria cause a large spectrum of syndromes, the most serious of which is septicemia, a condition with a high mortality rate. These bacterial strains are characterized by high resistance to serum, otherwise highly toxic to most bacteria. To understand the basis of this resistance, we carried out system-wide analyses of the response of ExPEC strains to serum by using proteomics and deep RNA sequencing. The major changes in gene expression induced by exposure to serum involved metabolic genes, not necessarily implicated in relation to virulence. One metabolic regulator—Fur—involved in iron metabolism was responsible for more than 80% of the serum-induced response, and its deletion renders the bacteria completely serum sensitive. These results highlight the role of metabolic adaptation in virulence.
sustained bacteremia is the one drawn by the immune system of the host. The complement system serves as the first line of defense against invading bacteria and acts on the outer membrane. In Gram-negative bacteria, the complement complex mediates direct killing by the formation of pores in the cell membrane. To avoid the highly bactericidal effect of serum, pathogens evolved structural features that inhibit complement-dependent killing. Many of these adaptations are in surface-exposed components, such as the outer membrane lipopolysaccharide (LPS) and the bacterial capsule (10). It was previously shown that variation in such as the outer membrane lipopolysaccharide (LPS) and the bacterial capsule (10). It was previously shown that variation in the length of the O antigen, as well as its type (Escherichia coli for instance, has more than 190 types of O antigens), influences the level of serum resistance (11). Even a single nucleotide change that consequently results in a truncated gene coding for O antigen, is sufficient to turn a serum-resistant strain to a serum-sensitive one (12). However, not all of the adaptations are structural; some bacteria can secrete complement-binding proteins and specific complement inhibitors. *Staphylococcus aureus*, for instance, employs an arsenal of proteins to interfere with and block the activation of the innate immune response (13). Successful evasion of the innate immune system, coupled with proper metabolic adaptations, enables pathogenic bacteria to persist and multiply in the bloodstream, a process that often leads to sepsis and, if not treated successfully, death.

Among the leading causative agents of sepsis are extraintestinal pathogenic *E. coli* (ExPEC) strains, which bring about high morbidity and mortality rates. *E. coli* infections also result in a heavy economic burden. ExPEC strains are characterized by the ability to resist the bactericidal effect of human serum, and this study was aimed at understanding the functional genomic basis of this resistance. To this end, we studied *E. coli* O78:H19 sequence type 88 (ST88) isolate 789 (O78-9), an ExPEC isolate able to grow, and even multiply, in serum. We performed comprehensive system-wide analyses of both its transcriptome and its proteome in response to serum. To distinguish between genes that respond to the metabolic challenge presented by serum and those that respond to the bactericidal effect of the complement system, we compared the effect of serum depleted of immune complement by heat treatment (inactive serum) with that of active serum. Here we show that the bacterial response to heat-inactivated serum included a change in the expression of a vast spectrum of known virulence determinants and iron acquisition genes, as well as transcriptional regulators. In contrast, the distinctive response to active serum (complement system) involved a much smaller group of genes, some of which are involved in the response to pH changes and others of which have unknown functions. One clear conclusion that emerged from these studies is that the key regulator of the response to serum—active and inactive—is the iron regulator Fur, which controls the expression of more than 80% of the serum-upregulated genes.

**RESULTS AND DISCUSSION**

**ExPEC growth in serum.** Septicemic bacteria are relatively resistant to the bactericidal effect of serum. We studied a septicemic strain, *E. coli* serotype O78:H19 ST88 isolate 789 (O78-9), that is capable of growing in serum. The results presented in Fig. 1A show the growth of *E. coli* O78-9 in 40% serum in comparison with that of a nonpathogenic *E. coli* strain (K-12) that undergoes lysis under the same conditions (Fig. 1B). The serum component significant in growth inhibition is the immune complement, as its inactivation by heat (inactive serum, 40 min at 56°C) enables the growth of *E. coli* K-12 (Fig. 1B, inset).

**Transcriptome analyses of ExPEC genes upon exposure to serum.** As pointed out, the response to serum is driven by two major stress conditions—nutritional stress and the bactericidal effect of complement. To study the nutritional stress, we investigated the response of ExPEC O78-9 to inactive serum. Because serum inactivation is achieved by mild heat conditions that destroy the complement system without affecting other properties such as pH and nutrient availability, the response to inactive serum represents the response to nutritional immunity. The experiments were carried out with cultures grown in minimal, salt-glucose, media. In these media, all of the biosynthetic genes are expressed, and this enables us to assess the nutritional stress. We compared the response of ExPEC O78-9 to active serum with that of inactive serum. The results indicated that the nutritional stress is significantly greater in active serum than in inactive serum. This finding suggests that the bactericidal effect of serum is not the only factor that contributes to the response of ExPEC to serum. Rather, the nutritional stress induced by serum depletion is also a critical factor in the response of ExPEC to serum. This study provides insight into the complex interplay between the bactericidal effect of serum and the nutritional stress, which are two important factors that contribute to the response of ExPEC to serum.
To obtain transcriptional expression data for the complete genome, we used the Illumina transcriptome sequencing (RNA-seq) platform. RNA was obtained from biological replicates of bacteria grown in minimal morpholinepropanesulfonic acid (MOPS) medium supplemented with glucose and with or without exposure to 40% inactive serum. Total RNA was extracted and rRNA depleted (as described in Materials and Methods). Two independent biological replicates and deep sequencing analyses were performed, and the data were analyzed by using strict criteria, as described in Materials and Methods. We examined only genes that were expressed the same way in the two independent biological experiments by using a 2-fold cutoff. The expression data of selected genes were confirmed by quantitative reverse transcription-PCR.

To obtain a comprehensive overview of the transcriptional response to serum, we compiled Voronoi tree maps (14, 15) from the quantitative transcriptomic data. In these tree maps, the transcripts are clustered according to their functions as obtained from the TIGR classification. Thus, functionally related elements are localized in close proximity to each other. The categories can then be subdivided sequentially down to the level of a single gene. A general representation of all functional pathways is shown in Fig. 2, upper panel. The log ratios (i.e., treated/control) of expression data were color coded by using a divergent color gradient (from blue for downregulated to red for upregulated). The Voronoi tree maps of the transcriptome induced by inactive serum and the pathways derived from it are presented in Fig. 2, lower panel, left side, which shows that serum affects the expression of a broad spectrum of genes with a variety of functions. Most of the serum-induced changes are metabolic; the upregulated pathways were mainly those of cations, iron-carrying compounds, amino acids, and amine biosynthesis, especially in the aspartate family. In contrast, pyruvate, glutamate, and aromatic amino acid biosynthesis pathways were downregulated. Other significantly downregulated genes included those for sulfur metabolism, the trichloroacetic acid cycle, and metabolism of polysaccharides. Most striking is the downregulation of biosynthetic genes involving the synthesis of several amino acids, mainly cysteine. The downregulated genes (Table 1) appeared to be affected directly by

![FIG 2](image_url)
two major transcription factors, CysB and BasR. The cysB gene product downregulates 11 genes involved in cysteine metabolism in addition to that for the transcription factor Cbl, which is responsible for sulfite starvation. The downregulated genes also include the six members of the sugar nucleoside metabolism ann operon (16–18) (annB, annC, annA, annD, annT, annE), which are under the control of the BasRS two-component system. The latter consists of two proteins that are involved in iron metabolism and protect against high concentrations of external iron (19, 20). Generally, it appears that the downregulated genes encode mainly metabolic enzymes (aminotransferases, glycosyltransferases, and formyltransferases).

The metabolic changes in response to serum are characterized by a massive upregulation of operons attributed to various iron receptors, binding proteins, transporters, and siderophore genes (Table 2). We show that the vast majority of these genes are involved in the maintenance of iron equilibrium between the bacterium and its surroundings. Interestingly, many of the upregulated genes have basic metabolic functions. For instance, the entire operon encoding iron hydroxamate (fhuACDB), iron dicarboxylate (fecABCDE), FeS cluster assembly protein (sufABCDSE), and electron transport system (nrdHIEF), and enterobactin (fepA-entD) formed the scaffold of the ExPEC O78-9 iron response to serum. Notably, these operons are also found in commensal E. coli strains such as K-12.

Most interesting is the upshift in the expression of bgfl, a component of the yjjQ-bgfl transcription unit. These genes detoxify the α-oxoaldehyde methyglyoxal (MG), an intracellular metabolism product; overproduction of MG results in cell death. The gene products contain the helix-turn-helix motif typical of the LuxR transcription regulator family (21) and are therefore suspected of being transcription regulators. It was previously suggested that the yjjQ gene of E. coli serotype O2:H5 (22) and S. enterica serovar Typhimurium (23) may be important for virulence in long-term systemic infections of mice.

Another upregulated gene is hokE, a homologue of hok (host killing) that is part of the hok-sok toxin-antitoxin system, an associated gene member of the SOS regulon (24). Five additional genes that are upregulated in the presence of serum have unknown functions (yrbL, pqql, 78901042, 78903310, and 78904620).

### Complement-dependent transcriptomic response

The genes responding to the bactericidal effect of serum could be identified by exposure to active serum and comparison of the transcriptome data to those obtained in the presence of inactive serum. This comparison revealed a small set of genes that were induced only in the presence of active serum—probably by the presence of complement. In contrast to the metabolic stress (Fig. 2, lower panel, left side), the complement-induced gene fraction is characterized by upregulation of the serine family, sulfur metabolism, and pyruvate and glutamate biosynthesis (Fig. 2, lower panel, right side). The complement-induced response consists of genes involved in iron metabolism (Table 3)—the ferric enterobactin transport system gene fepE and the iron storage bacterioferritin gene bfr. The presence of complement also induces galP, a gene that codes for a transporter, a member of the major facilitator superfamily of transporters. The rest of the upregulated genes have unknown functions and code for the putative proteins YcfJ (25, 26), YpfG (27), and YgaC (28, 29). Exposure to complement repressed the transcription of the enzyme-encoding genes yfaO, sodB, sorC, and fimA in addition to one conserved hypothetical protein with high homology to iraM. The observation that FimA, the major E. coli fimbrial subunit protein, is downregulated in response to serum is in line with previous findings suggesting that under low-iron conditions, E. coli decreases the expression of type I fimbriae (30, 31). The iraM gene is induced under magnesium starvation and serves as a stabilizer of RpoS, the sigma factor of the general stress response (32, 33). The sodB gene, which encodes the Fe-dependent superoxide dismutase FeSOD, is also downregulated in the presence of serum and is connected with iron metabolism (34).

It should be noted that in addition to the genes whose expression changes in response to complement, there are genes for other systems essential for complement survival, such as LPSs or capsule. These genes were studied in recent papers (10, 35) that looked at the effect of serum on an E. coli strain involved in urinary tract infections. Clearly, these surface components are essential for pathogenesis and survival in the bloodstream. Some of these genes are constitutive, and therefore, their expression is not affected by the presence of serum. However, the genes involved in the biosynthesis of the exopolysaccharide colanic acid are induced by serum (35) but were not detected in our system. These genes are
under the control of the Rcs regulator, whose level is greatly affected by the growth medium (36). Thus, the experiments of Mijajlovic et al. were conducted with cultures grown in Luria-Bertani (LB) medium, where the expression levels of the Rcs-regulated genes are low. Our experiments were carried out with cultures grown in minimal medium, where the Rcs-regulated genes are already induced and the addition of serum probably does not result in significant further induction.

Proteome analyses of the serum response. The effect of serum on gene expression was determined at the proteome level by using mass spectrometry (MS)-based proteomics as described in Materials and Methods. As in the transcriptome experiments, the proteins were extracted from cultures exposed to active or inactive serum and an untreated reference culture. A total of 1,037 proteins were identified (see Appendix SA in the supplemental material), and many of them could be detected only under one condi-

### TABLE 2 Genes upregulated (at least 2-fold) in response to inactive serum

| Operon | Gene(s) differentially expressed | Function(s) | Regulator(s) |
|--------|---------------------------------|-------------|--------------|
| fhuACDB | fhuA, fhuC, fhuD, fhuB | Iron-hydroxamate transporter | Fur |
| fepA-entD | fepA, entD | Enterobactin | Fur, CRP |
| fes-ybdZ-entF-fepE | fes, ybdZ, entF | Enterobactin | Fur, CRP |
| fepDC | fepC, fepC | Enterobactin transporter | Fur |
| entCERAH | entC, entE, entB, entA, entH | Enterobactin | Fur, CRP |
| sufABCDE | sufA, sufB, sufC, sufD, sufS | FeS cluster assembly protein | Fur, IIF, IscR, NsrR, OxyR |
| ndrHIEF | ndrH, ndrI, ndrE, ndrF | Electron transport system | Fur, IscR, NdrR |
| fesABCD | fesA, fesB, fesC, fesD | Iron-dictator protein | Fur, CRP, PdhR, Yjie |
| fepB | fepB | Enterobactin transporter | Fur, RutR |
| ybiX | ybiX | Fe(II)-dependent oxygenase | Fur |
| Fiu | Fiu | Catecholate siderophore receptor | Fur |
| bfy-bfr | bfy | Bacterioferritin, iron storage, detoxification | Fur* |
| yncE | yncE | Unknown | Fur, MarA |
| ybcA | ybcA | Yersiniaibactin transcriptional regulator | Fur* |
| iron | iron | Outer membrane siderophore receptor | Fur* |
| yddAB | yddA, yddB | Multidrug transporter | Fur* (yddA) |
| efeU | efeU | Ferrous ion transporter | Fur, CpxR |
| efeOB | efeO, efeB | Ferrous ion transporter | Fur |
| yviQ-bglJ | bglJ | DNA-binding transcriptional activator | H-NS, LeuO |
| hokE | hokE | Toxic polypeptide | LexA |
| ybiL | ybiL | Unknown | BasR, PhoP |
| pqL | pqL | Predicted peptidase | |
| 78900613 | 78900613 | Enhancement of lycopene biosynthesis protein 1 | Down ArcA, FNR, PhoP |
| 78901042 | 78901042 | Ferric enterochelin esterase | Fur* |
| 78903310 | 78903310 | Unknown | |
| 78904620 | 78904620 | Unknown | |

* Fur binding box computationally detected in promoter region.

### TABLE 3 Complement-dependent genes

| Operon | Genes differentially expressed | Function(s) | Response to serum | Regulation |
|--------|--------------------------------|-------------|-------------------|------------|
| fes-ybdZ-entF-fepE | fepE | Enterobactin | Up | Fur, CRP |
| ygaC | ygaC | Response to cytoplasmic pH stress | Up | Fur |
| yppG | yppG | Unknown | Up | Fur |
| yvfJ | yvfJ | Biofilm formation | Up | |
| bfr-bfr | bfr | Bacterioferritin, iron storage, detoxification | Up | |
| galP | galP | Galactose-proton symport of transport system | Up | CRP, GalR, GalS, NagC |
| 78900128 | 78900128 | Enhancement of lycopene biosynthesis protein 1 | Down | ArcA, FNR, PhoP |
| fimABCDFGH | fimA | Major type 1 fimbrial subunit | Down | H-NS, IIF, Lrp |
| sodB | sodB | Superoxide dismutase | Down | NsrR, CRP, IIF |
| yfaO | yfaO | Putative nuxid hydradase YfaO | Down | |
| sorC | sorC | Transcriptional regulator of sorbitol uptake and utilization | Down | |

* Fur binding box computationally detected in promoter region.

* Up, upregulation. Down, downregulation.
tion—in the presence of serum or in the untreated culture. Two hundred proteins were significantly differentially expressed, at least 2-fold, in the presence of serum (113 were upregulated, and 87 were downregulated). Of these proteins, 155 were also differentially expressed in response to inactive serum (70 were upregulated, and 85 were downregulated). The proteins that are present at very low levels in the presence of serum are probably encoded by genes that were significantly repressed in the presence of serum. However, it is possible that the level of some of them goes down in the presence of serum because of degradation. In an effort to achieve a global view of the functions altered in response to innate or nutritional immunity, we mapped all of the proteins to their corresponding pathways on the basis of the Voronoi tree map representation (Fig. 3, top panel).

The group of proteins that were induced by serum included a number of conserved hypothetical proteins. However, many of the proteins that were present in very low concentrations without serum and induced by serum (inactive and active) were involved in iron acquisition. This finding is not surprising, as serum is devoid of free iron because of the presence of human iron-binding proteins. There were also proteins that could not be detected once serum (active or inactive) was added. This group includes proteins that participate in various biosynthesis pathways, the 

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\text{arn} \quad (\text{arnC}, \text{arnA}, \text{and} \text{arnD} \ [16–18]), \text{transcription regulatory factor} \text{BasR} \ [19, 20], \text{and conserved hypothetical proteins.}
\]

A comparison of the genes differentially expressed in the presence of active serum versus inactive serum is shown in the bottom part of Fig. 3, right side. This group contained only a few proteins, as most of the responding proteins behave the same in active and inactive serum. The unique group of complement-induced proteins contained proteins involved in energy metabolism, amino acid synthesis, and the biosynthesis of cofactors, purines pyrimidine, and nucleosides. This group also contained proteins involved in cell envelope maintenance (MurD, MurE, MreB, GlmU, Alr, GalU). The latter observation is in line with the knowledge that the innate immune system causes membrane stress and that the integrity of the bacterial cell envelope is crucial for survival in serum and circumvention of the host’s membrane attack complex (37–39).

For the complete list of proteins whose expression depends on the presence of serum, see Appendix SA in the supplemental material.

Comparison of mRNA levels and protein abundance. An analysis of the mRNA levels and protein concentrations in response to exposure to inactive and activated serum (Fig. 4A and B, respectively) shows that under both conditions, there is a linear
relationship between RNA levels and protein abundance (active serum, $R^2 = 0.3$ and Spearman’s rank correlation coefficient $= 0.45$; inactivated serum, $R^2 = 0.25$ and Spearman’s rank correlation coefficient $= 0.47$). These results are consistent with previous observations that mRNA levels explain one- to two-thirds of the variance in protein levels (40).

Fur is the major regulator of the serum response. In an attempt to discover the regulatory network underlying serum adaptation, we systematically analyzed which transcription factor controls each of the differentially expressed genes involved in ExPEC’s serum response. To this end, we used a literature survey and RegulonDB, a database of the regulatory interactions of E. coli K-12 (41). As shown in Table 2, in accordance with the proteome results, Fur appears to be the key regulator of serum adaptation, controlling more than 70% of all upregulated genes. Fur, the ferric uptake regulator, serves as the main iron regulator in a wide range of bacterial species (29, 42–51). Fur was found to play a role in the virulence of many bacteria, including Staphylococcus aureus, Vibrio cholerae, Neisseria species, Yersinia species, and E. coli (52, 53). Fur repression occurs under iron-depleted conditions and is achieved by binding of Fur dimers to Fur boxes of target promoters. In the absence of free iron, the repression is removed and the genes are transcribed, as occurs in serum, an iron-limiting environment.

While RegulonDB provides comprehensive data on transcription factor-gene interactions in E. coli K-12, it cannot detect new regulatory interactions that appear in other strains. To overcome this obstacle, we scanned the region upstream of each of the serum-responsive genes to identify the presence of the 19-bp Fur binding consensus box (Fig. 5, upper part) by using weight matrices taken from PRODORIC (54). The Fur binding box is composed of three adjacent hexamers that bind dimeric Fur (55). Fur binding boxes were detected in seven additional genes that were upregulated in response to inactive serum, efuU, iroN, ymcE, yddA, ybtA, ypfG, and bfd, and the ferric enterochelin esterase-encoding gene 78900613 (Fig. 5, lower part), and in one gene, ypfG, that was induced only in the presence of complement. As expected, most of these genes are attributed to iron transporters, acquisition systems, and storage. It may be significant that two out of the eight genes found to have a Fur box, iroN and ybtA, were acquired by lateral gene transfer from other pathogens (S. enterica and Yersinia pestis, respectively) (56, 57). Thus, all in all, Fur is predicted to control more than 80% of the genes involved in the adaptation of E. coli to serum (Fig. 6 and Table 2).

Fur is essential for survival in serum. As Fur is clearly a major regulator of growth in serum, we constructed a deletion of the fur gene to study its effect on survival in serum. The results presented in Fig. 7 indicate that the removal of Fur had no effect on growth.

**FIG 4** Comparative analysis of RNA and protein profiles of E. coli O78-9 in response to human serum. The results show the correlation between changes in protein (log$_2$ fmol/ng ratio) and transcript (log$_2$ RPKM ratio) levels in bacteria grown in 40% serum over bacteria grown in MOPS minimal medium. A 2-fold change between the conditions was used as the cutoff for up- and downregulation of RNA and protein levels. (A) After exposure to inactive serum. (B) After exposure to active serum.

**FIG 5** The Fur binding box motif and predicted E. coli O78-9 genes with Fur binding boxes in their promoter regions. The Fur binding box 19-bp consensus sequence was created in WebLogo (upper part). Eight O78-9 gene sequences containing a Fur binding box (highlighted) that were computationally derived from the PRODORIC database are shown at the bottom.
in minimal medium. In contrast, the deletion-containing bacteria grew very poorly in the presence of serum. Growth in serum was restored by complementation with the fur gene on a plasmid. Although there was an induction of many proteins involved in iron metabolism, the concentration of Fur was unchanged upon exposure to serum. These results further support the notion that the effects of Fur are not solely concentration dependent and there must be additional factors involved, such as iron-dependent changes in its activity (58, 59).

Our results reflect the complex role of Fur in bacterial metabolism. As Fur is the regulator of iron metabolism, its removal would disrupt the delicate balance of the function involved in this important system. Actually, one would expect that a fur deletion would have a significant effect under all growth conditions. Yet,
the bacteria appear to be able to control growth in the absence of Fur but are not capable of doing so under extreme iron concentration changes, such as in the presence of serum. The effect of fur deletion is not restricted to E. coli, as it was also previously shown in Neisseria meningitidis, deletion mutants of which acquired serum sensitivity (60).

Conclusions. Here we present data, obtained from proteomic and transcriptomic experiments, on the global effect of exposure to serum on septicemic E. coli. We focused on genes required to overcome the nutritional immunity of serum and therefore conducted our studies with minimal media, in which the metabolic genes are active. Under such conditions, it is possible to see the serum-induced metabolic changes and the regulatory networks that control them. The most striking finding is the identification of Fur as the major regulator during serum exposure. Thus, >80% of the upregulated genes are under the control of Fur, the major regulator of iron metabolism (61, 62).

Genes that encode virulence factors are essential for pathogenesis. However, recent studies indicate that bacterial virulence is highly dependent on the regulation of metabolic genes (63–66), which are no less important than genes that encode virulence factors. The results presented here strongly support this notion and indicate that the major changes in bacterial gene expression upon exposure to serum involve metabolic genes, and these changes are essential for survival in serum.

MATERIALS AND METHODS

Bacterial strains and growth media. All of the E. coli strain and plasmids used in this study are listed in Table 4. Unless stated otherwise, all E. coli strains were grown while shaking at 30°C in minimal defined MOPS medium (with 0.2% glucose added). When required, antibiotics were added to the medium (ampicillin at 300 μg/ml, kanamycin at 50 μg/ml, chloramphenicol at 20 μg/ml). To determine growth curves, log-phase cultures were diluted to an optical density at 600 nm (OD₆₀₀) of 0.04 as determined with a BioTek Eon plate reader, and turbidity at 600 nm was measured every 20 min. Viable counts were determined after 1 h of incubation with serum or inactive serum by plating 10-μl drops of increasing dilutions on LB medium plates and incubating them overnight.

RNA isolation and purification. Overnight cultures were diluted to an OD₆₀₀ of 0.04 and allowed to grow to an OD₆₀₀ of 0.3; cells were then introduced to serum, inactive serum, or saline to a final volume of 40%. Cells were pelleted by 10 min of centrifugation at 3,000 rpm at 4°C. Pellets were resuspended in 6 μl of freshly prepared lysozyme solution (1 mg/ml) in 10 mM Tris-HCl, pH 8.0, and incubated at 37°C for 7 min with occasional mixing. One milliliter of TRIReagent was added to each sample, and the homogenate was stored at room temperature for 5 min. A 200-μl volume of chloroform was added for 15 s of incubation, and the resulting mixture was stored at room temperature for 2 to 15 min and centrifuged at 12,000 rpm for 15 min at 4°C. RNA was precipitated from the aqueous phase by mixing with isopropanol (0.5 ml/ml of the TRIReagent used for the initial homogenization), stored at room temperature for 5 to 10 min, and centrifuged at 12,000 rpm for 8 min at 4 to 25°C. The RNA pellet was washed with 75% ethanol, centrifuged at 7,500 rpm for 5 min, and air dried. The pellet was dissolved in 30 to 50 μl of ultrapure water (or treated with diethyl pyrocarbonate). RNA was depleted by using the MICROBExpress kit (Ambion) according to the manufacturer’s protocol. DNase treatment was done according to the manufacturer protocol with the Ambion TURBO DNA-free kit.

RNA-seq. Removal of 16S and 23S rRNAs from total RNA was performed with the MICROBExpress bacterial mRNA purification kit (Ambion) according to manufacturer’s protocol (67). RNA concentrations and quality were determined by using the Bioanalyzer 2100 (Agilent). For whole-transcriptome sequencing (RNA-seq), cDNA libraries were prepared by the Illumina mRNA-seq TruSeq protocol according to the manufacturer’s protocol without the poly(A) isolation stage. Sequencing was performed with an Illumina HiSeq 2500 sequencing machine. Sequencing generated 20 to 28 million raw reads per sample, of which 2 to 6 million were mapped to protein-encoding genes. The RNA-seq data were analyzed by a standard protocol based on the number of reads per kilobase of coding sequence length per million reads analyzed (RPKM) (68).

Construction of deletions and recombinant plasmids. All site-specific gene knockouts were obtained as described by Datsenko and Wanner (69). Briefly, competent wild-type O78-9 bacteria were transformed with plasmid pKD46. The transformants were grown in ampicillin-containing LB medium, induced with arabinose, made competent for electroporation, and stored at −70°C until used. A linear PCR product was made on the template of a kanamycin resistance cassette flanked by FLP recognition target (FRT) sequences from the pKD4 plasmid according to the region to be deleted. The primers were designed to contain 36 nucleotides from the flanking region of the sequence to be deleted from strain O78-9. Kanamycin-resistant recombinants were screened by means of colony PCR with primers k2 and k3. Unless stated otherwise, the pKD46 plasmid was cured by growth on LB medium at 42°C. The resulting bacteria were transformed with pCP20 and grown on LB medium at 42°C to promote both FRT-specific recombination and curing of the plasmid. The final deletion was verified by sequencing. Recombinant plasmids for complementation were constructed by cloning PCR-amplified DNA fragments into a pBAD24 vector with Fast-Link DNA ligase (Epicentre). The ligation products were transformed into competent O78-9 bacteria, and the resulting clones were screened by colony PCR.

Human serum. Sterile and filtered human male AB plasma was acquired from Sigma-Aldrich. Human serum was inactivated by incubation for 40 min at 56°C to eliminate all of the active immune complement system.

Clustering and enrichment analysis. E. coli O78-9 is still available in a roughly annotated version. HMMER (70) was used for the prediction of gene functions according to the TIGRFAM classification. For this purpose, HMMER used the Hidden Markov file containing the definitions derived from sequences of already assigned TIGR protein families from other

| TABLE 4 Plasmids and strains used in this study |
|-----------------------------------------------|
| **Plasmids or strain** | **Description** | **Reference** |
| Plasmids | | |
| pKD4 | Template for kanamycin resistance cassette | 76 |
| pBAD24 | AmpR, arabinose-inducible plasmid | 69 |
| pBADfurfur | AmpR, arabinose-inducible plasmid containing fur ORF | This study |
| E. coli strains | | |
| MG1655 | Wild-type K-12 strain | This study |
| O78-9 | Wild-type ExPEC O78 strain isolated from a turkey with sepsis | This study |
| O78-9 Δfur | | |
| O78-9 Δfur/pBADfurfur | | |

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bacteria, which can be downloaded from the website ftp://ftp.jcvi.org/pub/data/TIGRFAMs. In the resulting list, locus tags (RACXXX) were assigned to one or more TIGRFAMs, which were assigned to (metabolic) subroles, which were then summarized in hierarchicallyparent main roles. TIGRFAMs that were assigned to generic categories such as hypotheticalproteins, general, and so on were, if possible, manually reclassified into more-specific categories. By using Vororoni tree maps (14, 15), allfunctionally assigned genes were shown in a hierarchically organized and space-filling manner, as shown in Fig. 2. The space-filling approach divides the two-dimensional plane into subareas according the main rules, which are then subdivided into subsurfaces of the subroles and so forth. The polygons on the last level represent all of the functionally assigned genes. Transcriptional data were mapped by using a divergent color gradation starting with gray in the middle and ending with blue (for repression) and orange (for induction) on the sides.

Identification of Fur binding sites. Fur binding site position weight matrices (PWMs) were downloaded from PRODORIC version 8.9 (54). The score of each sequence is the log of the ratio of the likelihood of the sequence given the PWM model to the likelihood of the sequence given a background model. The background model assumes that at each position, the probability of each character equals its frequency in a concatenated regulatory sequence of 078-9. A significant hit is defined as a score of ≥4.6. The position-specific scoring matrix for the consensus logo was created with WebLogo.

Gel-free absolute proteome quantification. E. coli 078-9 was grown in 50 ml of MOPS medium in shaking flasks under agitation at 37°C. At an OD_{600} of 0.5, human serum, inactivated human serum, or saline was added to a final concentration of 40% and the bacteria were incubated for 45 min. Cells were harvested by centrifugation (8,000 × g, 15 min, 4°C). The resulting cell pellet was resuspended and washed twice in 50 mM triethylammonium bicarbonate. Cell disruption was carried out in a Ribolyzer (Thermo Fisher Scientific, Waltham, MA) for 2 × 30 s at 6,800 rpm. Two centrifugation steps were used to remove glass beads and cell debris. The supernatant was used for the following analysis.

Protein extracts (250 µg) were digested with trypsin as described by Muntel et al. (71); this was followed by a desalting step via stage tips using a standard protocol described by Rappsilber et al. (72). For absolute quantification, an internal standard protein (tryptic digest of alcohol dehydrogenase; Waters, Milford, MA) was used to spike the samples at a final concentration of 50 fmol/µl.

Data acquisition via LC-IMS® setup. Peptide samples were analyzed with a nanoACQUITY ultrasensitive liquid chromatography (UPLC) system (Waters) coupled to a Synapt G2 mass spectrometer (Waters). For each sample condition, three biological replicates were acquired with a nanoACQUITY ultraperformance liquid chromatography (UPLC) system (Waters) coupled to a Synapt G2 mass spectrometer (Waters). For absolute quantification, an internal standard protein (tryptic digest of alcohol dehydrogenase; Waters, Milford, MA) was used to spike the samples at a final concentration of 50 fmol/µl.

Supplemental Material

Analysis of LC-IMS® data. For identification and quantification of proteins, raw data wereimported into ProteinLynx Global Server (PLGS) 2.5.3 and processed via an Apex3D algorithm. The following processing parameters were used. The chromatographic peak width and MS time-of-flight resolution were set to automatic, the lock mass for charge 2 was 785.8426 Da/e, the lock mass window was 0.25 Da, the low-energy threshold was 250 counts, the elevated-energy threshold was 30 counts, the retention time window was set to automatic, and the precursor/fragment ion cluster intensity threshold was 1,000 counts. A database search was carried out by the ion accounting algorithm implemented in PLGS (67) by using a database that consisted of 4,628 E. coli 078-9 entries in a raw database (73-75; Huja et al., unpublished data) and 51,767 human entries in a human reviewed database uploaded from UniProtKB. This database was complemented with common laboratory contaminants and the yeast ADH1 sequence. The following parameters were used for positive protein identification. The peptide tolerance was set to automatic; the protein tolerance was set to automatic; the minimum number of fragment ion matches per peptide was set to 1; the minimum number of fragment ion matches per protein was set to 5; the minimum number of peptide matches per protein was set to 1; the primary digest reagent was trypsin; the number of missed cleavages was 2; the variable modifications were carbamidomethylation C (+57.0215), deamidation N, Q (+0.9840), and oxidation M (+15.9949); the false-discovery rate (FDR) was 5%; and the calibration protein was yeast ADH1.

All of the proteins identified in only one or two of the nine replicates (three technical replicates of each of the three biological replicates) of a sample were discarded. This filter procedure has revealed protein results with an FDR of <2% on the protein level. Proteins were considered to have significant expression differences if they showed at least a 2-fold change in serum or inactivated serum relative to the MOPS control. Only genes that were detected by both LC-IMS® and RNA-seq were used for the comparison of mRNA levels and protein abundance.

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References

1. Bearman GM, Wenzel RP. 2005. Bacteremia: a leading cause of death. Arch. Med. Res. 36:646–659. http://dx.doi.org/10.1016/j.arcmed.2005.02.005.
2. Dombrovskiy VY, Martin AA, Sunderram J, Paz HL. 2007. Rapid increase in hospitalization and mortality rates for severe sepsis in the United States: a trend analysis from 1993 to 2003. Crit. Care Med. 35:1244–1250. http://dx.doi.org/10.1097/01.CCM.0000218686.1311E9.
3. Weinberg ED. 2009. Iron availability and infection. Biochim. Biophys. Acta 1790:600–605. http://dx.doi.org/10.1016/j.bbaben.2008.07.002.
4. Holden KM, Browning GF, Noormohammadi AH, Markham PF, Mendovska MS. 2012. TonB is essential for virulence in avian pathogenic Escherichia coli. Comp. Immunol. Microbiol. Infect. Dis. 35:129–138. http://dx.doi.org/10.1016/j.cimid.2011.12.004.
5. Keen SA, Torres AG, Pays MJ. 2000. TonB is required for intracellular growth and virulence of Shigella dysenteriae. Infect. Immun. 68:6329–6336. http://dx.doi.org/10.1128/IAI.68.11.6329-6336.2000.
6. Gorbacheva VY, Faundez G, Godfrey HP, Cabell LF. 2001. Restricted growth of Ent(−) and tonB mutants of Salmonella enterica serovar Typhi in human Mono Mac 6 monocytic cells. FEMS Microbiol. Lett. 196:7–11. http://dx.doi.org/10.1016/S0378-1097(01)00026-X.
The ferric uptake regulator (Fur) is an iron-responsive protein that regulates the expression of many bacterial genes involved in iron acquisition and metabolism. Fur has been shown to be a virulence determinant in various bacterial pathogens, including Salmonella enterica and Yersinia pestis. In Salmonella, Fur regulates the expression of genes involved in iron scavenging, such as the serine repeat ATPase gene, sraA, which is essential for iron acquisition in vitro and in vivo.

In Yersinia pestis, Fur regulates the expression of genes involved in iron acquisition, such as the yersiniabactin receptor, YbtS, and the siderophore receptor, SdrG. The expression of these genes is essential for the bacteria to acquire iron from the host and to establish infection.

In addition to its role in iron regulation, Fur has been shown to regulate the expression of genes involved in other biological processes, such as stress response and virulence. For example, in Yersinia pestis, Fur regulates the expression of genes involved in the virulence island Y20, which is required for the bacteria to cause plague.

Overall, Fur is a critical regulator of bacterial metabolism and pathogenesis, and its role in iron regulation is an important consideration in the development of strategies to combat bacterial infection.