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The Role of Fur in the Acid Tolerance Response of *Salmonella typhimurium* Is Physiologically and Genetically Separable from Its Role in Iron Acquisition

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The response of *Salmonella typhimurium* to low pH includes a low-pH protection system called the acid tolerance response (ATR). The iron-regulatory protein Fur has been implicated in the ATR since *fur* mutants are acid sensitive and cause altered expression of several acid shock proteins (J. W. Foster, J. Bacteriol. 173:6896–6902, 1991). We have determined that the acid-sensitive phenotype of *fur* mutations is indeed due to a defect in Fur that can be complemented by a *fur*$^+$-containing plasmid. However, changes in cellular iron status alone did not trigger the ATR. Cells clearly required exposure to low pH in order to induce acid tolerance. The role of Fur in acid tolerance was found to extend beyond regulating iron acquisition. A mutation in *fur* converting histidine 90 to an arginine (H90R) eliminated Fur-mediated iron regulation of enterochelin production and deregulated an *iroA-lacZ* fusion but had no effect on acid tolerance. The H90R iron-blind Fur protein also mediated acid shock induction of several Fur-dependent acid shock proteins and acid control of the *hyd* locus. In addition, a Fur superrepressor that constitutively repressed iron-regulated genes mediated normal Fur-dependent acid tolerance and pH-controlled gene expression. The results indicate the acid-sensing and iron-sensing mechanisms of Fur are separable by mutation and reinforce the concept of Fur as a major global regulator in the cell.

Acidic pH is one of many environmental challenges that confront bacteria. Neutralophilic microorganisms, such as *Salmonella typhimurium*, can grow in conditions ranging from pH 5 to 9. However, these organisms periodically encounter more severe acid and alkaline conditions outside these limits. *S. typhimurium*, as a facultative intracellular parasite transmitted via oral-fecal routes, will experience acid in a variety of host and nonhost situations. During pathogenesis, *S. typhimurium* experiences severe acidity in the stomach, an alkaline environment in the small intestine, and fermentative reacidification in the bowel. Upon invading the intestinal epithelia or macrophages, mild acidification occurs in the endocytic vacuole (10, 11). Subsequent phagolysosomal fusion within macrophages may further acidify the vacuole to a potentially deadly pH (34, 35). Outside the host, acid rain, acid mine drainage, and industrial slurries can produce pH conditions marginal for survival of *S. typhimurium* in lakes and rivers (2). Thus, how *S. typhimurium* responds to acid stress has important implications for the host-parasite relationship and the organism's ability to persist in various nonhost environments.

S. typhimurium can withstand exposures to severe acid challenge by inducing the acid tolerance response (ATR) (16; reviewed in reference 15). Acid tolerance is induced when logarithmically growing cells (pH 7.7) are exposed to a mild acidification at pH 5.8 for one generation or to a moderate acid shock of pH 4.4 for 20 min or more. These adapted cells will survive a subsequent acid challenge at normally lethal pH values (3.3 to 3.0) 100- to 1,000-fold better than will unadapted cells. The ATR also provides cross-protection to heat, osmotic, and oxidative challenges (28, 29). Consequently, unraveling the molecular mechanisms involved with inducible acid tolerance should provide insight into how microorganisms endure a variety of stress situations. Stress cross-protection provided by the ATR should also prove important during pathogenesis. For example, cells undergoing acid shock in the stomach will be better prepared to endure environmental stresses subsequently confronted in the intestine.

The process of acid shock induces the synthesis of 51 acid shock proteins (ASPs) thought to contribute to survival at low pH (13, 24, 28). Two global regulators, the alternative sigma factor σ^s, encoded by *rpoS* (33), and the iron-regulatory protein Fur (ferric uptake regulation) (21, 22), control the expression of nonoverlapping ASP subsets and significantly contribute to the development of acid tolerance (13, 28). RpoS, itself an ASP, was shown to direct the expression of eight ASPs. Mutations in *rpoS* dramatically compromise inducible acid tolerance, although a significant, albeit transient, response remains (28). This RpoS-independent transient ATR was shown to be dependent on the Fur regulator, which acts, either directly or indirectly, in the positive control of several RpoS-independent ASPs (17, 18).

The 17-kDa Fur protein exerts control over a series of genes in *S. typhimurium* involved with the synthesis, excretion, and recovery of the iron-chelating siderophore enterochelin (54). Because excess intracellular iron can be detrimental to the cell (12), tight regulation of iron uptake is required for protection. When intracellular Fe(II) concentrations are high, iron-metallated Fur complex binds to a 19-bp DNA consensus sequence, the Fur box, in the promoter region of the iron acquisition genes to repress their transcription (1, 5, 9, 21, 43). However, reports from several laboratories, including our own, suggest that the role of Fur in cellular physiology extends beyond that of regulating iron utilization (18, 19, 23, 32, 37, 47, 49, 51, 53). The focus of this study was to further define the roles of Fur and iron in regulating the ATR. We have cloned and sequenced *fur* from *S. typhimurium* and have, through random mutagenesis, separated acid tolerance from iron-regulatory

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
Plasmids		
pMH15	*E. coli fur*$^+$ in pACYC184, Cm^r	K. Hantke
pMON2064	*E. coli fur*$^+$ in pBR327, Ap^r	S. Wee
S. typhimurium strains		
SF1	LT2 *rpoS*	K. Sanderson (28)
SF381	*ent-1*	B. A. D. Stocker
SF530 (UK1)	Virulent wild type	R. Curtiss III (7)
SF588 (χ4971)	UK1 *fur-1 zbf-5123*::Tn*10*	R. Curtiss III (7)
JF1534	SF1 *hyd*::MudJ	19
JF1992	SF1 *iroA1*::MudJ	Laboratory stock
JF2021	SF1 *iroA1*::MudJ *fur-1*	18
JF2023	SF1 *fur-1 zfi-5121*::Tn*10* (50% to *iroA*$^+$)	19
JF2032	SF1 *iroA1*::MudJ *zbf-5123*::Tn*10* (33% to *fur*$^+$)	This study
JF2036	SF1 *iroA1*::MudJ *zbf-5127*::Tn*10* (60% to *fur*$^+$)	This study
JF2037	SF1 *iroA1*::MudJ *zbf-5128*::Tn*10* (90% to *fur*$^+$)	This study
JF2056	SF1 *iroA1*::MudJ *fur-5*	Spontaneous
JF2058	SF1 *iroA1*::MudJ *fur-7*	Spontaneous
JF2059	SF1 *iroA1*::MudJ *fur-8*	Spontaneous
JF2060	SF1 *iroA1*::MudJ *fur-9*	Spontaneous
JF2061	SF1 *iroA1*::MudJ *fur-10*	Spontaneous
JF2062	SF1 *iroA1*::MudJ	18
JF2070	SF1 *iroA1*::MudJ *fur-11 zbf-5127*::Tn*10*	Hydroxylamine
JF2110	SF1 *iroA1*::MudJ *fur-14*	Hydroxylamine
JF2209	SF1 *iroA1*::MudJ *fur-1*/pTZ 19R Ap^r	This study
JF2391	SF1 *fur-14*	This study
JF2392	SF1 *hyd-1088*::MudJ *fur-2 zfi-5121*::Tn*10*	This study
JF2420	SF1 *fur-5 zfi-5121*::Tn*10*	This study
JF2421	SF1 *fur-7 zfi-5121*::Tn*10*	This study
JF2422	SF1 *fur-8 zfi-5121*::Tn*10*	This study
JF2423	SF1 *fur-9 zfi-5121*::Tn*10*	This study
JF2484	SF1/pMON2064 (*fur*$^+$ Ap^r)	This study
JF2485	SF1 *iroA1*::MudJ *fur-1*/pMON2064 (*fur*$^+$ Ap^r)	This study
JF2486	SF1 *fur-14*/pMON2064 (*fur*$^+$ Ap^r)	This study
JF2703	SF1 *fur-1*/pMH15 (*fur*$^+$ Cm^r)	This study
JF2709	SF1 *ent-1 fur-1 zbf-5123*::Tn*10*	This study
JF2851	SF1 *iroA1*::MudJ *fur*^{S}*-15 zbf-5123*::Tn*10*	Hydroxylamine
JF2856	SF1 *hydA1088*::MudJ *fur*^{S}*-15 zbf-5123*::Tn*10*	Hydroxylamine
JF2861	SF1 *iroA1*::MudJ *fur-16 zbf-5128*::Tn*10*	Hydroxylamine
JF2862	SF1 *iroA1*::MudJ *fur-17 zbf-5128*::Tn*10*	Hydroxylamine
JF2863	SF1 *iroA1*::MudJ *fur*^{S}*-18 zbf-5128*::Tn*10*	Hydroxylamine
JF2864	SF1 *iroA1*::MudJ *fur*^{S}*-19 zbf-5128*::Tn*10*	Hydroxylamine
JF3158	SF1 *hyd-1088*::MudJ *fur-9 zfi-5121*::Tn*10*	This study

functions. A mutation converting His-90 to Arg (H90R mutation) severely diminished iron regulation but did not affect the role of Fur in acid tolerance.

MATERIALS AND METHODS

Strains. Bacterial strains used in this study were derivatives of *S. typhimurium* LT2 (SF1) or the virulent UK1. Characteristics of the strains and plasmids are presented in Table 1. The *Escherichia coli fur*$^+$ clones (pMH15 [43] and pMON2064 [56]) were gifts kindly provided by R. D. Perry (pMON2064 was provided with permission from Monsanto Corporation, St. Louis, Mo.).

Media. The basic media used were Luria-Bertani (LB) (8) and E salts minimal medium (55) with 0.4% glucose (EG). Ampicillin, tetracycline, and chloramphenicol, when used, were added to 60 μg/ml, 20 μg/ml (complex media), and 30 μg/ml, respectively. To assess the effect of iron availability, ferrous sulfate or ferric chloride and the iron chelator diethylaminetriaminepentaacetic acid (DTPA; Sigma Chemical Company) were used to create iron-replete and iron-deficient conditions, respectively. Concentrations used for each are provided in figure legends. Chrome azurol S (Aldrich Chemical) blue agar was used to estimate siderophore production (44).

Measurement of the ATR. Induction of acid tolerance was accomplished in one of two ways, preshock adaptation at pH 5.8 or acid shock adaptation at pH 4.4. The ATR preshock protocol was followed as described earlier (16). Briefly, cultures destined for adaptation were grown to the specified optical density at 600 nm (OD_{600}) in EG (pH 7.7) to 10^8 CFU/ml, shifted to pH 5.8 with HCl for one doubling of cells, and then readjusted to the challenge pH. Acid challenges for UK1- and SF1-derived strains were performed at pH 3.0 and 3.3, respectively (pH adjusted with HCl). Unadapted cultures were grown at pH 7.7 to 2×10^8 CFU/ml and directly acid challenged. $FeCl_3$ or DTPA was added at the times indicated in figure legends.

Acid shock adaptation involved growing cells in EG at pH 7.7 to a cell density of 2×10^8 CFU/ml, adjusting the medium pH with HCl to pH 4.4 for 20 or 60 min (adaptation), and then readjusting the pH for acid challenge as noted above (14). Viable counts were determined by plating dilutions (EG broth) of the acid-stressed cultures on LB agar. Data shown are representative of triplicate experiments in which viable counts were reproducible to within 50% of a stated value. $FeCl_3$ and DTPA were added at least 45 min prior to the shift to pH 4.4.

β-Galactosidase assays. β-Galactosidase assays were performed as described by Miller (36). Cells were grown for β-galactosidase assays in LB buffered with morpholinopropanesulfonic acid (MOPS; pH 8) or morpholinoethanesulfonic acid (MES; pH 5) (45). Supplements included 15 to 60 μM $FeCl_3$ and 200 μM DTPA as indicated. Values given are averages of triplicate experiments.

Isolation of *fur* mutants. Mutations in *fur* that affected iron regulation were screened in two ways involving either the overexpression of an iron-regulated *lacZ* operon fusion (*iroA*::MudJ) in the presence of excess iron or the underexpression of the same fusion under limiting-iron conditions. Hydroxylamine-treated P22 lysates of JF2037 or JF2082, each containing a Tn*10* insertion near *fur*$^+$, were used to transduce JF1992 (*iroA*::MudJ). Tetracycline-resistant colonies were screened on MacConkey medium containing 100 μM $FeSO_4$ (excess iron) or 12 μM DTPA (low iron). Loss-of-function *fur* mutants were isolated as red colonies in excess iron, whereas superrepressor mutants were isolated as white colonies even on low-iron DTPA media. Mutants are listed in Table 1.

Cloning of the *S. typhimurium fur* gene and sequence analysis of *fur* mutants. Two primers that would amplify the *S. typhimurium fur* gene were designed from

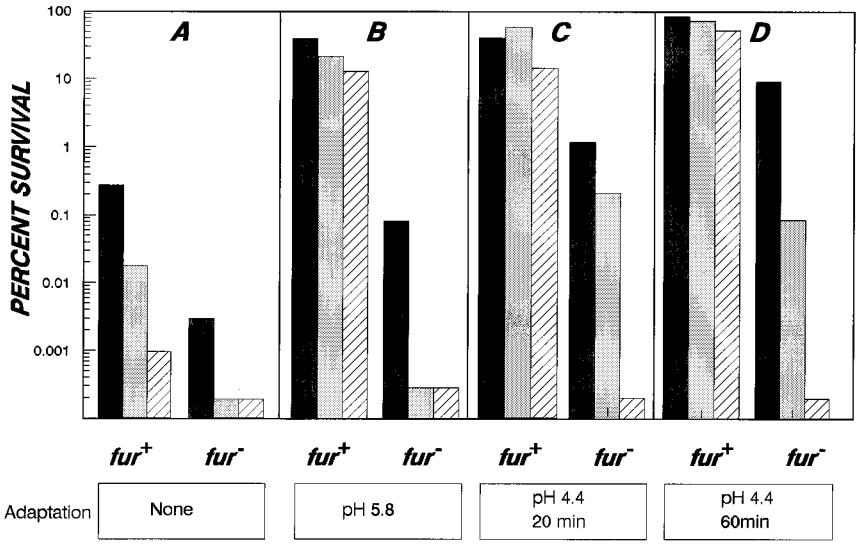

FIG. 1. Effect of *fur* on acid tolerance. Values represent percent survival in minimal EG after 30 (solid bars), 60 (stippled bars), or 90 (cross-hatched bars) min of exposure to a pH 3.0 environment. (A) Cells were grown at pH 7.7 to mid-log phase and challenged to pH 3.0 by the addition of HCl. (B) Cells were grown at pH 7.7 to mid-log phase, adapted at pH 5.8 for one generation, and then acid challenged. (C) Cells were grown at pH 7.7, adapted at pH 4.4 for 20 min, and then acid challenged. (D) Cells were grown at pH 7.7, adapted at pH 4.4 for 60 min, and then acid challenged. Strains illustrated are UK1 (*fur*$^+$) and SF588 (*fur-1*). All ATR assays were performed in triplicate.

the *E. coli fur* sequence. Primer 23 began at position 19 (GCCCTAAAGAAA GCTGGCCTG), and primer 22 began at position 424 (CATCTTCGCGGCAA TCGCCTTC). A 405-bp fragment amplified from *S. typhimurium* LT2 was cloned into the TA cloning vector (version 1.3; Invitrogen) and confirmed to be *fur* by Southern hybridization to a PCR-amplified *E. coli fur* probe. The *E. coli* radiolabeled probe used for hybridization was prepared from pMH15 by using primers 22 and 23 (Prime-a-Gene [Promega], [^{32}P]ATP). Two plasmids were constructed with *S. typhimurium fur* in opposite orientations relative to the *lac* promoter: pHF202 (forward orientation) and pHF201 (reverse orientation). The gene was sequenced by using Sequenase version 2.0 (United States Biochemical) and primers to the TA vector, 44(SP6) (GATTTAGGTGACACTATAG; bases 239 to 255) and 45(T75′) (TAATACGACTCACTATAGG; bases 388 to 407).

Primers 23 and 22 were used to PCR amplify mutant *fur* genes from boiled whole cell preparations (five to six colonies were transferred to 50 μl of H_2O and boiled for 5 min, and then 5 μl was used in a 100-μl PCR). Two independently generated PCR fragments were sequenced for each mutant *fur* gene. Fragments were sequenced by cycle sequencing using an *fmol* kit (Promega) as follows: 95°C for 3 min and then 30 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 1 min.

Two-dimensional SDS-PAGE analysis. Two-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described by Spector et al. (46) with cells labeled for 2 min with ^{35}S-Translabel (ICN Biomedical, Inc.). Approximately 5 μg of protein from sonicated cell extracts was analyzed for each sample. The first dimension was a pH 5 to 7 isoelectric focusing gel containing 1.6% (pH 5 to 7) and 0.4% (pH 3 to 10) ampholytes (Bio-Rad), while the second dimension was an SDS–11.5% polyacrylamide gel.

RESULTS

Fur is essential for the ATR. We reported previously that when acid challenged at pH 3.3, *fur* mutations have only a minor effect on the acid tolerance of *rpoS*$^+$ cells but will eliminate the transient response that remains in an *rpoS* mutant (28, 38). However, when acid challenge was conducted at a more severe pH of 3.0 to 3.1, Fur proved critical for acid tolerance, even in an *rpoS*$^+$ cell (Fig. 1). Cells lacking Fur were extremely sensitive to pH 3 when adapted for one generation at pH 5.8 (Fig. 1B). They were also more acid sensitive than the wild type when adapted by acid shock at pH 4.4 for 20 min (Fig. 1C) or 60 min (Fig. 1D), although they did regain some inducible tolerance. We attribute the partial tolerance in Fig. 1C and D to the induction of RpoS-dependent, and perhaps other, systems that contribute to acid tolerance. However, these systems were not sufficient to afford maximal tolerance. This finding illustrates that multiple pathways of acid survival exist. The more severe an acid stress, the more systems must be engaged to combat that stress. For the purposes of this study, we have removed the σ^s-dependent system from the acid tolerance equation by using an *rpoS* mutant (SF1) defective in σ^s production (28).

Figure 2 illustrates that the acid-sensitive phenotype of *fur* mutants is not due to a polar effect on potential downstream genes. Plasmid pMON2064 contains a gene fusion between the *E. coli fur* gene and the promoter/operator region of *recA*. This construct expresses Fur protein at a low level unless induced by DNA damage (56) and does not contain any chromosomal DNA upstream or downstream of *fur*. A comparison of iron-regulated *iroA-lacZ* expression by a *fur* mutant with and without pMON2064 revealed that a normal iron-repressible regulatory pattern was reestablished by pMON2064 (Fig. 2A). In addition, pMON2064 restored normal control over enterochelin expression in a *fur* mutant (Fig. 3B and F). Thus, the *E. coli fur* locus will complement the iron-regulatory aspects of a *S. typhimurium fur* mutant. Figure 2B shows that pMON2064 also restored the ATR in an *S. typhimurium fur* mutant to near the wild-type adapted level. These studies confirm previous findings demonstrating an important role for Fur in the ATR.

Iron solubility is not the signal for acid tolerance. Because Fur protein is required for the ATR and metallated Fur is considered the active form that represses iron-regulated genes, we examined whether intracellular iron concentrations might be critical for adaptation to acid stress. Iron is less soluble at alkaline pH than it is in acid (57). Consequently, the increased solubility of $Fe(OH)_3$ at pH 5.8 or 4.4 could translate into higher intracellular Fe(II) concentrations. If iron availability was the sole signal for acid tolerance, then cells grown at pH 7.7 replete with iron should mimic the increased intracellular iron concentration and acid tolerance of pH 5.8-adapted cells. Before testing this premise, we needed to assess whether intracellular iron levels could be modulated under alkaline conditions. To do this, we used the iron-regulated gene fusion, *iroA-lacZ*, as a reporter of intracellular iron status. At pH 7.7

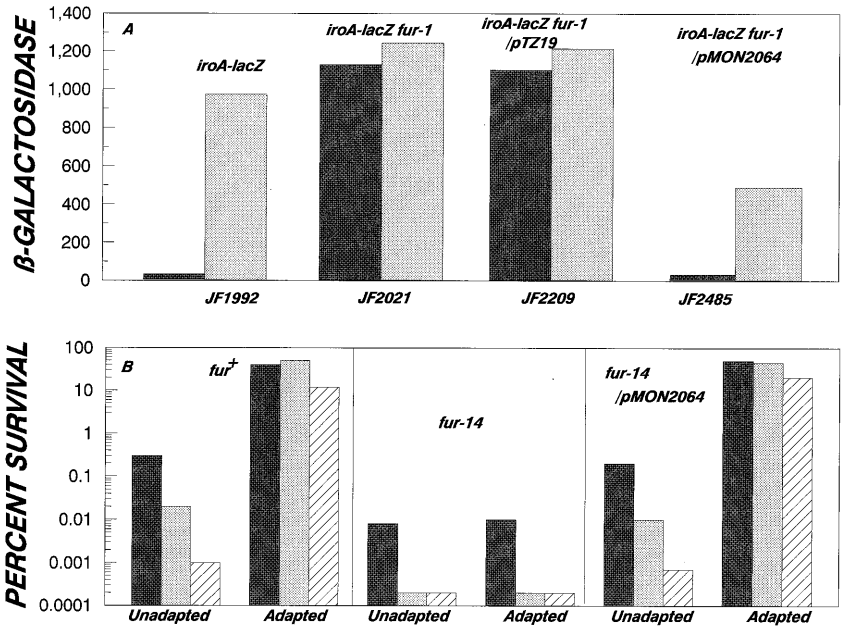

FIG. 2. The iron-regulatory and ATR phenotypes of a *fur* mutant are complemented by an *E. coli fur*$^+$ clone. (A) Expression of *iroA-lacZ*. β-Galactosidase activity is expressed in Miller units. Cells were grown in LB-MOPS medium (pH 8.0) to an OD_{600} of 0.2, at which point the medium was supplemented with 60 μM $FeCl_3$ (iron replete; solid bars) or 200 μM DTPA (iron depleted; stippled bars) and cells were grown to an OD_{600} of 0.4. Strains used were JF1992 (*iroA1*::MudJ), JF2021 (*iroA1*::MudJ *fur-1*), JF2209 (*iroA1*::MudJ *fur-1*/pTZ19R), and JF2485 (*iroA1*::MudJ *fur-1*/pMON2064 *fur*$^+$). (B) ATR assays. Unadapted cells were grown in EG (pH 7.7) to 2×10^8 CFU/ml and challenged at pH 3.3. Cultures marked for adaptation were grown to 10^8 CFU/ml, the culture pH was adjusted with HCl to pH 5.8, and the culture was allowed to continue growing for one doubling. Values represent average survival at pH 3.3 (30 min [solid bars], 60 min [stippled bars], and 120 min [cross-hatched bars]). Strains used were SF1 (*fur*$^+$), JF2391 (*fur-14*), and JF2486 (*fur-14*/pMON2064 *fur*$^+$). All assays were performed in triplicate.

(EG), expression of this locus was fully repressed by 100 μM $FeCl_3$. In the same medium but without added iron, *iroA-lacZ* was partially induced (100-fold). Full induction (300-fold) at pH 7.7 was accomplished with the addition of 50 μM DTPA (iron-deficient conditions). We could not repress this gene any further by shifting cells to pH 5.8 (data not shown). This response pattern confirmed that intracellular iron levels could be effectively manipulated in a pH 7.7 environment. We then examined whether increasing intracellular iron levels at pH 7.7 would allow induction of the ATR. The results depicted in Fig. 4A demonstrate that unadapted SF1 cells (pH 7.7) treated with 20 and 100 μM $FeCl_3$ were as acid intolerant as cells grown under standard unadapted conditions. Thus, an increased iron concentration cannot be the sole adaptation signal needed to survive acid stress. Acid adaptation requires more than Fur protein and iron. Acidic pH is clearly required.

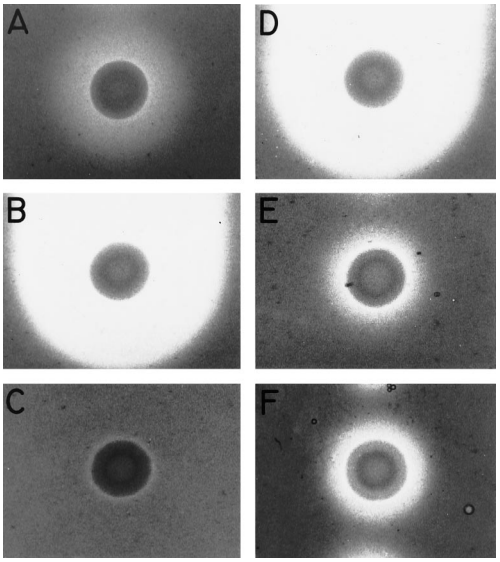

FIG. 3. Enterochelin production. Enterochelin production is observed in chrome azurol S blue agar medium as a zone of clearing around representative colonies. (A) LT2; (B) JF2391 (*fur-14*); (C) SF381 (*ent*); (D) JF2423 (*fur-9*); (E) JF2851 (*fur*^{s}*-15*); (F) JF2486 (*fur-14*/pMON2064).

Potential iron toxicity in *fur* mutants does not mask the ATR. Because *fur* mutations derepress iron acquisition genes, it was considered possible that unregulated delivery of iron into the *fur* mutant cell could enhance the lethal effects of low pH by accelerating macromolecular damage. However, excess iron added to *fur*$^+$ cells did not prevent or diminish adaptation (Fig. 4B). The data in Fig. 4C further reveal that ATR competence in the *fur* mutant could not be restored by lowering iron levels through the addition of DTPA or by raising iron levels through the addition of $FeCl_3$. We also tested whether overproduction of enterochelin by *fur* mutants could explain the acid-sensitive (Atr$^-$) *fur* phenotype. If overproduction of enterochelin and, thus, excessive delivery of iron to the cell is lethal in the ATR protocol, then a *fur* mutant unable to synthesize enterochelin should exhibit a normal ATR. However, a *fur ent* double mutant was as exquisitely acid sensitive as the *fur ent*$^+$ parent (Fig. 4D). We conclude that the level of enterochelin itself does not significantly alter the adaptive process. It is important to note that a *fur*$^+$ *ent* mutant did exhibit a normal ATR under these conditions (data not shown). Hence, the effect of Fur on acid tolerance appears to go beyond its role in iron acquisition. Accordingly, we conclude that a component of the *fur* regulon has a vital function in the cellular response to stressful acid environments.

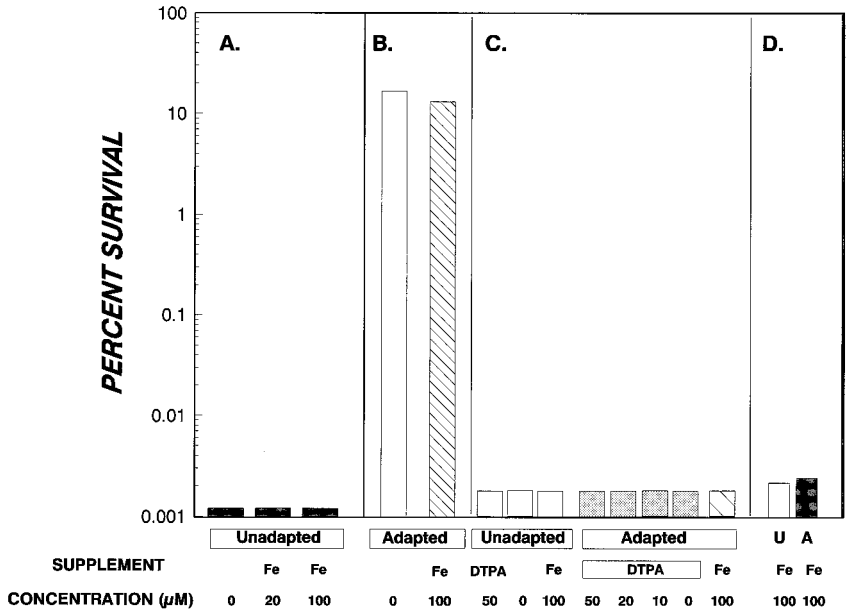

FIG. 4. Iron levels and the ATR of *fur*$^+$, *fur*, and *fur ent* mutants. ATR profiles are given as percent survival in minimal EG following 120 min of exposure to pH 3.3. Shifts to pH 3.3 were made at a cell density of 2×10^8/ml. (A) Unadapted cultures of LT2 grown at pH 7.7 with 0, 20, and 100 μM $FeCl_3$. (B) Adapted (pH 5.8) cultures of LT2 grown with no supplement (open bar) or 100 μM $FeCl_3$ (cross-hatched bar) added during growth. (C) Cultures of JF2023 (*fur-1*) were treated as indicated below each bar with DTPA or $FeCl_3$. Supplements were added at a cell density of 10^8/ml. Cells were unadapted or adapted at pH 5.8 as indicated below the bars. (D) JF2709 (*fur-1 ent-1*) was unadapted or adapted (pH 5.8) in the presence of 100 μM $FeCl_3$.

The acid tolerance properties of Fur are genetically separable from iron regulation. Previous two-dimensional SDS-PAGE analysis revealed that Fur has a very broad regulatory role in the cell (18). In addition to 19 iron starvation-inducible proteins negatively controlled by a Fe(II)-Fur repressor complex, we discovered that the expressions of 26 proteins were positively controlled by Fur. Nine iron-inducible proteins required Fe(II)-Fur for positive control, whereas six iron starvation-inducible proteins required demetallated Fur as a positive regulator. Twelve proteins were positively regulated by Fur seemingly irrespective of iron status. We have also uncovered nine ASPs regulated by Fur, several of which are regulated in an apparently iron-independent manner (14, 15, 18). In an effort to separate the iron-regulatory features of Fur from those involved with acid, we generated a series of *fur* mutants that were defective in the regulation of *iroA-lacZ* and enterochelin production. We then screened those mutants for defects in acid tolerance. Figure 5A presents the effects of the various *fur* mutations on *iroA-lacZ* expression. Most of the mutations caused derepression of this iron-regulated gene (Fig. 5A) and also increased the production of enterochelin (Fig. 3). Three of the mutants (*fur*^{s}*-15*, *-18*, and *-19*) were isolated as superrepressors in which *iroA-lacZ* expression was repressed even in an iron-poor environment.

Figure 5B shows the effects of the various *fur* mutations on acid tolerance. In most cases, the iron-blind *fur* mutations also obliterated inducible acid tolerance. The one notable exception was *fur-9*, which retained almost normal acid tolerance. In addition, the superrepressors also retained a significant ATR. These results suggested that Fur controls iron- and acid-regulated genes by different mechanisms. Further evidence for this is found in Table 2, which illustrates the effects of loss-of-function *fur* (*fur-2*), superrepressor *fur* (*fur*^{s}*-15*), and iron-blind *fur-9* mutations on the expression of a *hyd-lacZ* fusion. We have previously shown that the *hyd* locus is coinduced by acid and anaerobiosis and that Fur is required for *hyd* induction at pH 5.8 (19). This finding is confirmed in Table 2 (JF2392). However, both the *fur*^{s}*-15* superrepressor mutant (JF2856) and the *fur-9* iron-blind mutant (JF3158) proved capable of mediating acid induction of *hyd* even though the two mutations had opposite effects on iron-regulated expression of *iroA-lacZ* (Fig. 5A). These results indicate that acid-inducible regulation of *hyd* by Fur is not dependent on the iron-sensing properties of Fur.

To confirm that Fur-9 was iron blind, its effect on *iroA-lacZ* expression was tested over a broad range of iron concentrations. Whereas full repression of *iroA* normally occurs at 15 μM $FeCl_3$ (Fig. 5), the Fur-9 product did not mediate iron control of *iroA* over the range of 15 to 200 μM $FeCl_3$ (data not shown). If Fur-9 simply exhibited a lower affinity for Fe(II), one would expect the mutant protein to repress *iroA* at the higher iron concentrations.

Figure 6 presents the sequence of *S. typhimurium fur* and the positions of several of the mutations examined in Fig. 5. Five mutations eliminated acid tolerance and iron control. Two of those were nonsense mutations (*fur-1* and *fur-17*) that introduced stop codons near the N terminus. The other three were missense mutations, G51D (*fur-7*), R57C (*fur-11*), and I67M (*fur-5*), two of which affected amino acid residues (G-51 and R-57) totally conserved among the 10 sequenced Fur genes (3, 25, 26, 30, 31, 39, 43, 48, 52). These residues occur in the amino-terminal half of Fur associated with DNA binding (50). Of particular interest was the H90R (*fur-9*) mutation, which also affected a totally conserved amino acid residue. As indicated above, this mutant lost iron control but retained acid tolerance, suggesting that this residue is important for iron regulation but not for regulating acid tolerance. Thus, the iron-regulatory function of Fur is genetically separable from its role in acid tolerance. Western blot (immunoblot) analysis revealed that none of the *fur* mutations other than *fur-1* and *fur-17* (the nonsense mutations) affected the production or stability of Fur protein (data not shown). Hence, the H90R

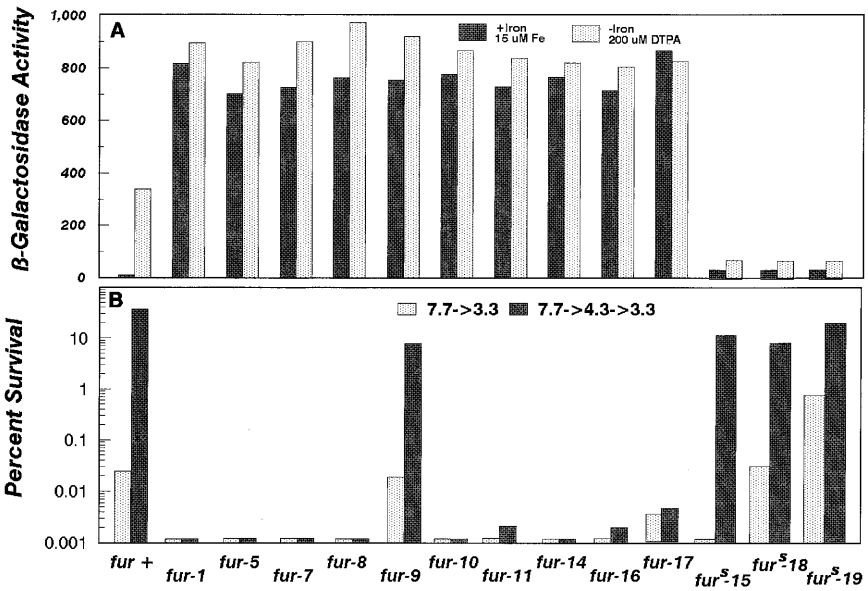

FIG. 5. Effects of *fur* mutations on *iroA-lacZ* expression and acid tolerance. (A) Effects of *fur* mutations on *iroA-lacZ* expression. Cells were grown in LB to an OD_{600} of 0.2, at which point 15 μM $FeCl_3$ or 200 μM DTPA was added; growth then continued for one doubling before assay. β-Galactosidase activity is given as Miller units (36). (B) Effects of *fur* mutations on acid tolerance in the SF1 background. Cells were adapted at pH 4.4 for 20 min and then acid challenged at pH 3.3 for 120 min.

mutant phenotype is not caused by an aberrant level of active Fur.

Effect of H90R on ASP synthesis. Several ASPs were previously shown to require Fur for their control (14, 15, 18). Some of these proteins were regulated by acid shock in an apparently iron-independent manner. Consequently, we predicted that these proteins, or a subset of these proteins, should remain regulated by acid shock in the H90R mutant. Figure 7 presents two-dimensional SDS-PAGE analysis of polypeptides produced by *fur*$^+$, *fur* (null), and *fur*^{H90R} mutants before (pH 7.7) and after (pH 4.4) acid shock. A total of 12 proteins are highlighted. Numbered protein spots marked with circles in Fig. 7B are Fur-dependent ASPs as defined by their lack of acid pH induction in the *fur-1* mutant. The one exception to this trend was ASP-33, which is really an iron-regulated protein (IRO-29) that is repressed by Fur-(FeII). ASP-33 expression seems to reflect pH-mediated changes in iron solubility rather than a response to pH itself. In contrast to the other Fur-dependent ASPs, ASP-33 was overexpressed in the *fur-1* mutant even at pH 7.7. It is significant that the H90R *fur-9* mutant maintained acid shock control of the four remaining Fur-dependent ASPs even though the cell produces an iron-blind Fur (Fig. 7D and E). These results support those presented above indicating that although residue H-90 is needed by Fur for iron sensing, it is not required for acid pH control.

In contrast to the ASPs, iron-regulated proteins marked with squares and numbered in Fig. 7C were not ASPs but were overexpressed in both the *fur-1* and iron-blind *fur-9* mutants, confirming that Fur negatively regulates these genes. The one exception was IRO-28, an iron-repressible gene product that requires deferrated Fur for induction in iron-deficient conditions (18). This is the opposite of the classic Fur-Fe(II) repressor model seen with other IRO proteins. The absence of IRO-28 in the *fur-1* mutant (Fig. 7C) and its presence in the *fur*^{H90R} mutant (Fig. 7E) confirm that Fur acts as a positive regulator of this gene and that Fur^{H90R} is iron blind yet active. It appears that the binding of Fe(II) to Fur inactivates Fur induction of IRO-28. The data cannot be explained by a cascade of regulators in which Fur-Fe(II) represses a positive regulator of IRO-28 expression because deferrated Fur is required for IRO-28 expression (Fig. 7C and E). The data presented illustrate that Fur can positively and negatively regulate gene expression and that regulation can occur in the absence of the iron-sensing function of Fur.

TABLE 2. Effects of *fur* mutations on *hyd-lacZ* expression

Strain	Genotype	β-Galactosidase activity[a]		
		pH 8	pH 5	Fold induction
JF1534	*hyd-lacZ*	13	790	61
JF2392	*hyd-lacZ fur-1*	2	10	5
JF2856	*hyd-lacZ fur^s-15*	10	560	56
JF3158	*hyd-lacZ fur-9* (H90R)	2	400	200

[a] Cultures were grown anaerobically (under paraffin oil) in LB-MOPS (pH 8) or LB-MES (pH 5) to mid-log phase. β-Galactosidase activity is given in Miller units (36).

DISCUSSION

Previous work from our laboratory implicated the iron-regulatory gene *fur* in the ATR of *S. typhimurium*. Mutations in *fur* were defective in the induction of several ASPs and imparted an acid-sensitive phenotype (13, 14). We have confirmed this observation and have shown that *fur*, not a downstream gene, is directly responsible for this phenotype. Two additional questions regarding the role of Fur in acid tolerance were addressed during this study: first, is iron solubility in low pH the signal for acid tolerance, and second, is the acid tolerance phenotype of Fur separable from iron regulation?

Since iron is essential for growth, an environmental shift from high- to low-iron concentrations serves as an important regulatory signal to the cell. Because iron solubility is a function of pH, it was reasonable to speculate that Fur may sense

```
1          10                  30                  50                  60
           .         .         .         .         .         .
GCCCTAAAGAAAGCTGGCCTGAAAGTAACGCTTCCTCGTTTAAAAATTCTGGAAGTTCTT
                                                (fur-1)T
.... A  L  K  K  A  G  L  K  V  T  L  P  R  L  K  I  L  E  V  L
        10                                  20
                                                         *

61         70                  90                 110                 120
           .         .         .         .         .         .
CAGGAACCAGATAACCATCACGTCAGTGCGGAAGATTTATACAAACGCCTGATCGACATG
T(fur-17)
Q  E  P  D  N  H  H  V  S  A  E  D  L  Y  K  R  L  I  D  M
        30                                  40
*

121        130                 150                 170                 180
           .         .         .         .         .         .
GGTGAAGAAATCGGTCTGGCAACCGTATACCGTGTGCTGAACCAGTTTGACGATGCCGGT
              A(fur-7)          T(fur-11)
G  E  E  I  G  L  A  T  V  Y  R  V  L  N  Q  F  D  D  A  G
        50                                  60
           D                 C

181        190                 210                 230                 240
           .         .         .         .         .         .
ATCGTGACCCGCCATAATTTTGAAGGCGGTAAATCCGTTTTTGAACTGACGCAACAGCAT
  G(fur-5)
I  V  T  R  H  N  F  E  G  G  K  S  V  F  E  L  T  Q  Q  H
        70                                  80
M

241        250                 270                 290                 300
           .         .         .         .         .         .
CATCACGACCATCTTATCTGCCTTGATTGCGGAAAAGTGATTGAATTTAGTGATGACTCT
            C(fur-9)
H  H  D  H  L  I  C  L  D  C  G  K  V  I  E  F  S  D  D  S
        90                                  100
           R

301        310                 330                 350                 360
           .         .         .         .         .         .
ATTGAAGCGCGCCAGCGTGAAATTGCGGCGAAACACGGTATTCGTTTAACTAATCACAGC
I  E  A  R  Q  R  E  I  A  A  K  H  G  I  R  L  T  N  H  S
        110                                 120

361        370                 390                 406
           .         .         .         .
CTCTATCTTTACGGCCACTGCGCTGAAGGCGATTGCCGCGAAGATG
L  Y  L  Y  G  H  C  A  E  G  D  C  R  E  D   .....
        130                                 141
```

FIG. 6. *S. typhimurium fur* sequence and positions of *fur* mutations. The numbers under the deduced amino acid sequence reflect positions relative to *E. coli* Fur. Because of the PCR cloning strategy, the sequence presented is missing six amino acids at the N terminus and seven amino acids at the C terminus. Shaded nucleotides and amino acid residues below the main sequences reflect mutant base and resulting amino acid changes in mutant *fur* alleles. The mutant alleles are identified by allele number next to the mutant bases.

acid indirectly in terms of iron availability. However, the results presented show that iron availability alone will not trigger an ATR. The cell must experience low pH. Consequently, the role of Fur in acid tolerance appears to transcend its ability to sense iron. An alternative explanation, that the overproduction of membrane-associated iron transport proteins in a *fur* mutant could weaken the integrity of the cell to acid challenge seems unlikely since the fur^{H90R} mutation, which also increased the levels of these membrane-bound proteins, did not affect acid tolerance (data not shown).

The results also indicate that the role of Fur in acid regulation can be separated genetically from its role in iron regulation. This conclusion is based on the ability of the H90R Fur mutant to mediate acid induction of specific Fur-dependent ASPs and enable induction of acid tolerance in spite of the fact that the mutant Fur cannot mediate iron regulation. We have also provided evidence that Fur can, either directly or indirectly, act as a positive regulator of the pH-regulated locus, *hyd* (19). This acid control also appears to be iron independent in that a Fur superrepressor and the iron-blind Fur^{H90R} still regulated *hyd* in response to pH. Thus, Fur appears to sense acid stress and affect the expression of some genes in an apparent iron-independent fashion.

The positive control of *hyd* and ASP expression by Fur cannot be explained by a simple repressor cascade in which Fur-Fe(II) complex would repress or activate a pH-responsive intermediate regulator of the target genes. Two lines of evidence argue against this model. First, the genes in question appear regulated by acid but not by iron. If Fur-Fe(II) regulated an intermediate regulator, then the target gene should also be regulated by iron. Second, the Fur H90R mutant, defective in iron sensing, will still mediate pH control. Thus, the level of Fur-Fe(II) is irrelevant to the pH control of specific genes but Fur itself is needed. The data support a model in which Fur can mediate pH control of gene expression in an iron-independent manner. There still may be a cascade of regulators leading to the target gene, but it seems the pH control resides with Fur.

Several laboratories have begun to define the functional regions of Fur involved in Fe(II) sensing and in binding DNA as a repressor (4, 6, 40–42, 50). The results of fusion protein studies combining different domains of Fur and the λ repressor cI857 indicate that the N terminus of Fur is involved in DNA binding while the C terminus is required for dimerization (50). Biochemical analysis indicates that repressor activation occurs by two metal ions per dimer binding to the C-terminal domain

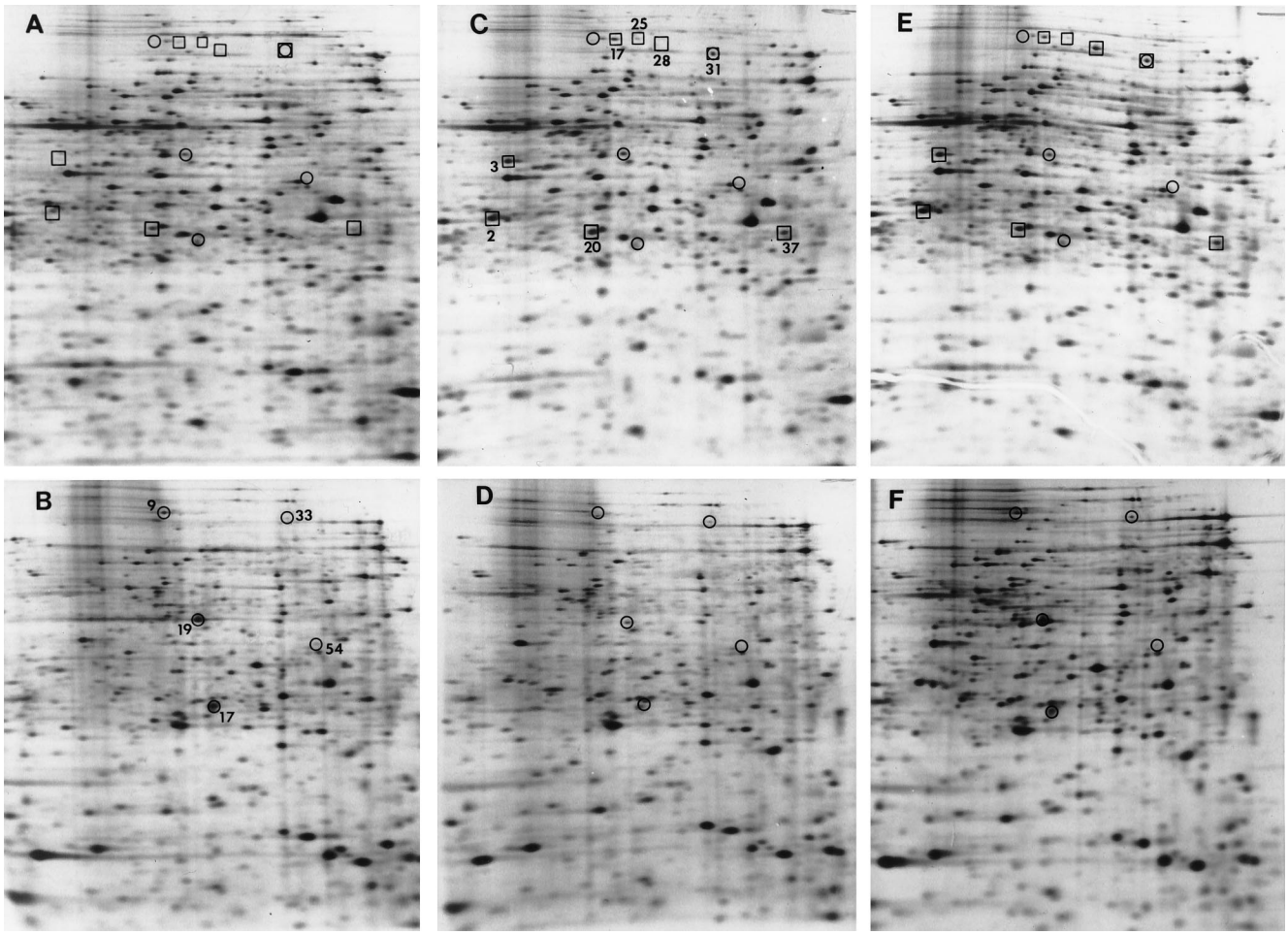

FIG. 7. Two-dimensional analysis of Fur-regulated ASPs. SF1 (A and B), JF2023 (*fur-1*; C and D), and JF2423 (fur^{H90R}; E and F) were grown to mid-log phase in EG (pH 7.7) (A, C, and E), and samples were acid shocked for 15 min at pH 4.4 (B, D, and F). Cells were radiolabeled for 2 min with 40 μCi of ^{35}S-Translabel (ICN) per ml and processed for two-dimensional SDS-PAGE as described in Materials and Methods. Acidic proteins are on the right, and basic proteins are on the left of each autoradiograph. Circled spots (numbered proteins in panel B) indicate Fur-dependent ASPs. Spots indicated with a square (numbered proteins in panel C) are iron-regulated (IRO) proteins.

(20). Fe(II) binding induces a conformational change in the N-terminal domain which subsequently permits operator binding (50).

The most likely ligands for ferrous iron binding are histidines and cysteines. Fur contains 12 histidines and 4 cysteines, 2 of which are found in the CysXYCysGly motif commonly used as a ligand by iron-sulfur proteins (1). Site-directed mutagenesis of the histidine and cysteine residues of *E. coli* Fur argue that cysteines 92 and 95 are the most important for iron binding (6). Of the histidines, only H-89 (equivalent to our H-90) partially reduced iron regulation of an aerobactin operon-*lacZ* fusion. This contrasts with our study of *S. typhimurium* Fur, in which H-90 proved essential for iron regulation in vivo. A separate study of *Vibrio cholerae fur* also supports a role for H-90 in iron binding. Lam et al. (27) generated an H90L Fur mutant that has no iron-regulatory function in vivo. The reason for the somewhat disparate results between the *E. coli* and *V. cholerae* studies involving identical H90L substitutions is not apparent. However, all three studies do suggest a role for H-90 in iron regulation.

It is interesting that nuclear magnetic resonance studies examining ionization of the histidine residues suggest that Fur is a monitor of iron concentration and pH (40). Our results support this prediction. Clearly, the H90R modification creating an iron-blind Fur did not perceptibly alter the ability of Fur to participate in acid tolerance or to control the induction of several ASPs. How Fur might function to regulate acid shock genes independently of iron remains a mystery. It will be interesting and informative to identify the residues within Fur that serve as pH sensors and ultimately identify the acid tolerance proteins regulated by Fur.

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