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Role and Regulation of Iron-Sulfur Cluster Biosynthesis Genes in *Shigella flexneri* Virulence[▽]

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Shigella flexneri*, a causative agent of bacterial dysentery, possesses two predicted iron-sulfur cluster biosynthesis systems called Suf and Isc. *S. flexneri* strains containing deletion mutations in the entire *suf* operon (UR011) or the *iscSUA* genes (UR022) were constructed. Both mutants were defective in surviving exposure to oxidative stress. The *suf* mutant showed growth that was comparable to that of the parental strain in both iron-replete and iron-limiting media; however, the *isc* mutant showed reduced growth, relative to the parental strain, in both media. Although the *suf* mutant formed wild-type plaques on Henle cell monolayers, the *isc* mutant was unable to form plaques on Henle cell monolayers because the strain was noninvasive. Expression from both the *suf* and *isc* promoters increased in iron-limiting media and in the presence of hydrogen peroxide. Iron repression of the *suf* promoter was mediated by Fur, and increased *suf* expression in iron-limiting media was enhanced by the presence of IscR. Iron repression of the *isc* promoter was mediated by IscR. Hydrogen peroxide-dependent induction of *suf* expression, but not *isc* expression, was mediated by OxyR. Furthermore, IscR was a positive regulator of *suf* expression in the presence of hydrogen peroxide and a negative regulator of *isc* expression in the absence of hydrogen peroxide. Expression from the *S. flexneri suf* and *isc* promoters increased when *Shigella* was within Henle cells, and our data suggest that the intracellular signal mediating this increased expression is reduced iron levels.

As a facultative intracellular pathogen, *Shigella flexneri* spends a significant portion of its life cycle within the epithelial cells lining the human colon. Invasion of and intracellular survival/replication of the bacteria within these epithelial cells requires the ability to sense the environment and initiate an appropriate metabolic strategy during infection. Global analysis of *Shigella* transcription during epithelial cell infection indicated that a variety of metabolic genes, including the *suf* genes, are precisely regulated when *Shigella* is intracellular (16, 27).

The *Shigella flexneri suf* and *isc* loci encode predicted iron-sulfur (Fe-S) cluster biosynthesis systems. Iron-sulfur clusters are essential for a wide variety of biological processes, including redox reactions, substrate binding and activation, iron storage, protein structure, and regulation of gene expression (11). The *S. flexneri isc* locus contains *iscR*, *iscS*, *iscU*, *iscA*, *hscB*, *hscA*, and *fdx* and is highly conserved with the *Escherichia coli isc* locus. *E. coli* IscS catalyzes the desulfurization of L-cysteine for the recruitment of S for Fe-S cluster formation (4). IscU and IscA are predicted to form scaffolds for Fe-S cluster assembly based on similarities with *Azobacter vinelandii* NifU and IscA[Nif] (1, 11, 13). The chaperones HscB and HscA aid in Isc-mediated Fe-S protein maturation, although the specifics are not entirely clear (for a review, see reference 11). Deletion of the *isc* locus in *E. coli* reduced the growth rate due to the pleotropic effect on Fe-S cluster synthesis (11, 34).

The *S. flexneri suf* locus includes *sufA*, *sufB*, *sufC*, *sufD*, *sufS*, and *sufE*. The *suf* genes have been identified in a wide variety of bacterial species and have been studied in *E. coli* and the plant pathogen *Erwinia chrysanthemi*. The *sufA* and *sufS* genes are homologous to the *iscA* and *iscS* genes and are predicted to have similar functions. SufE enhances SufS activity (15, 22). SufBCD have similarities to ABC transporter proteins but do not contain transmembrane segments and are cytoplasmically located in *E. chrysanthemi* (19). The SufBCD complex has been shown to increase SufS activity by an unknown mechanism (15, 22).

In *E. coli*, deletion of the entire *suf* locus had no significant effect on normal growth in vitro or on activity of the Fe-S cluster containing protein succinate dehydrogenase (31); however, *E. coli sufD* mutants showed decreased stability of the Fe-S cluster containing protein FhuF (23). Additionally, an *E. coli suf* mutant was more sensitive to iron starvation when grown with gluconate as the sole carbon source as a result of decreased activity of the Fe-S cluster containing enzyme gluconate dehydratase (21). In the plant pathogen *E. chrysanthemi*, the *suf* genes enhance oxidative-stress survival and virulence (18).

The regulatory stimuli (iron limitation, oxidative stress) that induce transcription of the *suf* and *isc* operons in *E. coli* are similar, although the mechanisms by which the regulatory proteins alter gene expression at each promoter in response to each signal are distinct (14, 21, 37, 38). Specifically, both OxyR and IscR mediate increased expression of the *suf* operon in the presence of oxidative stress, while IscR represses expression of the *isc* operon unless oxidative stress is present (14, 21, 37, 38). Furthermore, iron repression of *suf*, but not *isc*, is mediated by Fe-Fur.

The existence of these two Fe-S cluster assembly systems suggests that although the genes encode proteins with similar biochemical activities, each system is fine-tuned for optimal activity under a certain set of environmental conditions. The Isc system has been predicted to be a housekeeping Fe-S clus-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristic(s)	Reference or source
E. coli strains		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* [ϕ*80dlac*Δ(*lacZ*)*M15*]	29
SM10λ*pir*	*pirR6K*	32
MM294/pRK2013	Kan^r; helper strain for matings	3
S. flexneri strains		
SA100	*S. flexneri* wild-type serotype 2a	24
SM100	SA100 Str^r	S. Seliger
UR011	SM100 Δ*suf*::*cam*	This study
UR022	SM100 Δ*iscSUA*::*cam*	This study
UR021	SM100 Δ*oxyR*::*cam*	This study
UR027	SM100 Δ*iscR*::*cam*	This study
UR028	SM100 Δ*oxyR* Δ*iscR*::*cam*	This study
UR010	SM100 *fur*::Tn*5*	25
Plasmids		
pKD3	Contains *cam* resistance gene	2
pKM208	Phage lambda Red recombinase genes under the control of an inducible promoter on temperature-sensitive plasmid	17
pCP20	FLP recombinase on temperature-sensitive plasmid	2
pWKS30	Low-copy-number cloning vector	37
pSUF	*S. flexneri sufABCDES* in pWKS30	This study
pPK4194	*E. coli iscSUA* in pET11a	30
pLR29	Promoterless GFP vector	27
pLR67	*suf-gfp* fusion on pLR29	27
pAD1	*isc-gfp* fusion on pLR29	This study

ter assembly system, while the Suf system has been postulated to be adapted to synthesize Fe-S clusters under stress conditions (2). Clearly, there is some redundant function, as mutants in each single system are viable, but in *E. coli*, deletion of both the chromosomal *suf* and *isc* loci is lethal unless the *suf* or *isc* gene is provided on a plasmid (31, 34).

Since the *suf* genes are induced when *Shigella* is intracellular and since intracellular growth is required for virulence, we hypothesized that assembly of Fe-S clusters may be required for *Shigella* virulence. In the work presented here, we test this hypothesis using deletion mutations of the *iscSUA* and *sufABCDSE* genes. Our studies also examined the regulation of the expression of these genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* strains were routinely grown in Luria broth (L broth) or Luria agar (L agar). *S. flexneri* strains were grown in L broth, low-salt L broth (27), or on Trypticase soy broth agar plus 0.01% Congo red dye at 37°C. To grow strains in reduced metal conditions for regulation studies, EZ rich defined medium (EZ-RDM; http://www.genome.wisc.edu/functional/protocols.htm) made without added iron was used as the base medium and was supplemented with 0.2% glucose, 2 μg/ml nicotinic acid, and added iron as indicated in the figure legends. For minimal medium growth assays, strains were grown in M9 medium containing 0.2% gluconate, 0.05% casein hydrosylate, and 2 μg/ml nicotinic acid. Dipyridyl (50 to 400 μM) was added to further limit iron. Antibiotics were used at the following concentrations: 125 μg/ml carbenicillin, 25 μg/ml kanamycin, 10 μg/ml chloramphenicol, 12.5 μg/ml tetracycline, and 200 μg/ml streptomycin.

General DNA methods. All primers used in this study are listed in Table 2. Plasmid and chromosomal DNA were isolated using the QIAprep spin miniprep kit and the DNeasy tissue kit (Qiagen, Santa Clarita, CA), respectively. Isolation of DNA fragments from agarose gels was performed using the QIAquick gel extraction kit (Qiagen). All standard PCRs were carried out using either *Taq* (Qiagen) or *Pfu* polymerase (Stratagene Cloning Systems, La Jolla, CA) in accordance with the manufacturer's instructions. To clone the *suf* operon, the genes were amplified from SM100 by PCR with primers URsufFOR and URsufREV. The *suf* fragment was digested with EcoRI and BamHI and ligated with pWKS30 (35) digested with EcoRI and BamHI to generate pSUF. To clone the *iscSUA* genes under the control of a constitutive promoter, the *dnaY* promoter was amplified from SM100 by PCR with primers UR169 and UR170, digested with XbaI, and ligated with the 5.7-kb XbaI/NruI fragment from pPK4194 (which contains the promoterless *iscSUA* genes) to generate pBL3.

Construction of *Shigella* mutants. The *suf* operon mutant was constructed by splice overlap PCR (7) and classical allelic exchange. Primer set URsufFOR and UR020 was used to generate a PCR DNA fragment containing approximately 1,000 bp upstream of *sufA*, the 5′ end of *sufA*, and a SmaI restriction enzyme site. Primer set URsufREV and UR019 was used to generate a PCR DNA fragment containing a SmaI restriction enzyme site, the 3′ end of *sufE*, and approximately 1,000 bp downstream of *sufE*. These two PCR fragments were then used as primers to one another to create a full-length PCR product containing approx-

TABLE 2. Primers used in this study

Primer	Sequence
URsufFOR	5′CGGAATTCCAAAAAGTGGTTGGTCTGGAA
URsufREV	5′CGGGATCCGTAATTTAATGCCACGCTCCA
UR019	5′CTGCCCGGGCTGCAGAATTACGCGCCGAAGACG
UR020	5′ATTCTGCAGCCCGGGCAGCCCGTTTGCTTCACACC
UR040	5′ATGCATTCAGGAACCTTTAACC
UR041	5′TGAATATCTGATTAAGTCCTTCACG
UR043	5′TATTTCTCTTCCCAGTTGCG
UR115	5′TTTGGCTATGTGCTC GACAG
UR116	5′CCACATTCATTCTGGGCTTT
UR117	5′CACGATTACTAGCGATTCCGACTT
UR118	5′CGTCGTAGTCCGGATTGGA
UR119	5′ACTGTCGCTCAGTGTAATCG
UR121	5′AAAAATGGTCTGGTTTCCAGC
UR122	5′ACCAGACACATCCAGCACTTC
UR123	5′TGGAGCGTTTTTATGGTCATC
UR124	5′GGTCTAGAGCGCCCTTTAGATGTCAGTC
UR169	5′TCCCCCGGGTTCGGAGATAATCG
UR170	5′TGCTCTAGACGCGTGAATTATACGGTCAACC
UR171	5′ATGGACGGAACCTTTGGTAAC
UR172	5′TGACGGCAAGTATCCAGTACC

imately 1,000 bp upstream of *sufA*, the 5′ end of *sufA*, a SmaI restriction enzyme site, the 3′ end of *sufE*, and approximately 1,000 bp downstream of *sufE*. The full-length PCR product was digested with EcoRI and BamHI and cloned into pBSK− digested with the same enzymes to generate pRJ1. pRJ1 digested with SmaI was ligated with a 1.6-kb HincII fragment containing a chloramphenicol resistance gene (*cam*) from pMA9 (9). The *suf* fragment with the *cam* resistance cassette was then excised as a SalI-XbaI fragment and ligated into pHM5 digested with SalI-XbaI to generate pRJ2. Allelic exchange using pRJ2 was then done in SM100 as described previously (26).

The *iscSUA*, *oxyR*, and *iscR* mutants were constructed using a modification of the procedure for one-step inactivation of chromosomal genes (2). Briefly, a PCR product for allelic exchange that contains approximately 50 bp of the beginning of the region of interest, a chloramphenicol resistance gene (*cam*), and approximately 50 bp at the end of the region of interest was generated. The template for this PCR was the plasmid pKD3 (which contains the *cam* gene), and each PCR primer contained a 50-nucleotide overhang homologous to one of the ends of the region of interest and the priming sites for the pKD3 *cam* gene (2). *S. flexneri* SM100 containing the plasmid pKM208 (17), which harbors the phage lambda Red recombinase genes under the control of an inducible promoter, was grown to an optical density of 0.6 to 1 at 30°C. Recombinase expression was induced with 1 mM IPTG for 30 min, followed by a 15-min heat shock at 42°C. The *Shigella* gene::*cam* PCR fragment was electroporated into SM100/pKM208, and transformants were selected on Congo red agar containing 5 to 10 μg chloramphenicol per ml. pKM208, which has a temperature-sensitive origin of replication, was eliminated from the mutants by culture at 42°C. Disruption of the appropriate genes was confirmed by PCR analysis using a *Shigella* primer set flanking the original *Shigella* PCR fragment.

To construct the *oxyR iscR* double mutant, the FLP recombination target (FRT)-flanked *cam* resistance gene in UR021 (Δ*oxyR*::*cam*) was removed using pCP20, which contains the FLP recombinase that catalyzes the deletion of the FRT-flanked *cam* resistance gene (2). UR021 was transformed with pCP20, incubated at 30°C for 2 hours, and then plated on ampicillin plates and incubated at 37°C overnight. Single-colony purified transformants were then streaked on Congo red plates lacking antibiotics and incubated at 42°C overnight to select for the loss of pCP20. The deletion of the FRT-flanked *cam* resistance gene was verified by PCR. This new strain was then used to construct the *oxyR iscR*::*cam* strain as described above.

Oxidative-stress assays. Overnight cultures were diluted 1:50 in saline, and then 100 μl was spread on L agar plates. A BBL 6-mm-diameter blank paper disk (Becton, Dickinson and Company, Franklin Lakes, NJ) was placed in the center of each plate, and 10 μl of either hydrogen peroxide (1 M) or phenazine methosulfate (PMS; 0.1 M) was spotted onto the disk. The plates were incubated for 24 to 48 h at 37°C, and zones of growth inhibition were measured. Statistical analyses of the data were performed using the single-factor analysis of variance statistics package in Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA).

Cell culture assays. Monolayers of Henle cells (intestine 407 cells; American Type Culture Collection, Manassas, VA) were maintained in minimum essential medium (Invitrogen) supplemented with 2 mM glutamine, 1× minimal essential medium nonessential amino acid solution (Invitrogen), and 10% fetal bovine serum (Invitrogen) and were grown in a 5% CO_2 atmosphere at 37°C. Invasion assays of Henle cells were done as described previously (6, 8), with the addition of gentamicin at 45 min postinvasion. Plaque assays on Henle cells were done as described previously (20), using the modifications described by Hong et al. (8), except that agar was eliminated from the gentamicin overlays. Plaques were scored after 2 to 3 days.

RNA isolation. Before RNA isolation, samples were stabilized by the addition of stabilizing buffer (95% ethanol-5% phenol [pH 4.3]) for 5 min. Total RNA was isolated from bacteria using the RNeasy mini kit (Qiagen), which included a DNase I treatment step to degrade DNA. Isolated RNA was treated again with DNase I (Qiagen) to remove any residual contaminating DNA.

Nonquantitative RT-PCR for operon structure analyses. Reverse transcription (RT) reactions were performed using total RNA from *S. flexneri* SA101, ThermoScript Plus (Invitrogen, Carlsbad, CA), and either primer UR043 within the *sufE* gene or primer UR119 within the *iscA* gene. The *sufE* RT product was then amplified by PCR using Platinum *Taq* (Invitrogen) and the primer set UR040 and UR041, located within the *sufA* gene. Likewise, the *iscA* RT product was amplified with primer set UR121 and UR122, located within the *iscR* gene.

***Shigella suf* and *isc* expression studies.** To construct the reporter fusion between the *isc* gene and the green fluorescent protein gene (*gfp*), *isc* primers UR123 and UR124 were used to amplify the *Shigella isc* promoter from SM100. The PCR product was digested with XbaI and cloned into pLR29 (27) digested with XbaI and SmaI to generate pAD1. *suf* expression was measured using the plasmid-borne *suf-gfp* fusion pLR67 (27). After growth under the appropriate conditions, samples were fixed in 2% paraformaldehyde as described previously (27) and fluorescence was quantitated using a FACSCalibur (Becton, Dickinson and Company) fluorescence-activated cell sorter (FACS) with excitation at 488 nm to measure single-cell fluorescence. FACSCalibur settings were forward scatter, E01; side scatter, 505; and relative fluorescence between 515 and 545 nm, 798.

For quantitative RT-PCR, cDNA was made from 200 ng total RNA using Superscript III (Invitrogen). Quantitative real-time PCR was performed on the cDNA samples using the Platinum Sybr green quantitative PCR kit (Invitrogen) and the Chromo4 continuous fluorescence detector with an alpha unit DNA Engine thermocycler (Bio-Rad, Hercules, CA). Primers for the PCRs were as follows: for *sufA*, UR115 and UR116; for *iscS*, UR171 and UR172; and for *rrsA*, UR117 and UR118. Data analysis was done using the Opticon monitor software package (Bio-Rad). A standard curve was generated for each gene by using 10-fold dilutions of SM100 chromosomal DNA, and the amount of the cDNA for *suf* or *isc* in each cDNA sample was extrapolated from the standard curve. Finally, the level of *suf* or *isc* gene expression was normalized to that of the housekeeping gene *rrsA* by dividing the relative amounts of *suf* or *isc* cDNA by the relative amounts of *rrsA* cDNA in each sample.

TABLE 3. Contribution of the Suf and Isc systems to oxidative-stress survival in *S. flexneri*

Strain[b]	Zone of growth inhibition (mm)[a]	
	H_2O_2[c]	PMS[d]
SM100 (wild type)	26 ± 3	31 ± 4
UR011 (Δ*suf*::*cam*)	37 ± 3[e]	37 ± 4[e]
UR022 (Δ*iscSUA*::*cam*)	38 ± 1[e]	43 ± 1[e]
SM100/pSUF	30 ± 3	31 ± 0
UR011/pSUF	31 ± 1[f]	31 ± 0[g]
SM100/pBL3	27 ± 2	32 ± 2
UR022/pBL3	27 ± 3[f]	31 ± 1[f]

[a] The data presented are the means of at least three experiments, and the standard deviations of the means are indicated.
[b] pSUF carries the *suf* genes, and pBL3 carries the *iscSUA* genes under the control of the *S. flexneri* constitutive *dnaY* promoter.
[c] Exposure to 10 μl of 1 M H_2O_2.
[d] Exposure to 10 μl of 0.1 M PMS.
[e] The zone of growth inhibition for the indicated mutant strain is significantly larger than that for the wild-type strain SM100 treated with the same oxidative-stress agent ($P < 0.01$).
[f] The zone of growth inhibition for the indicated mutant strain with the complementation plasmid is significantly smaller than that for the mutant strain without the complementation plasmid treated with the same oxidative-stress agent ($P < 0.01$).
[g] The zone of growth inhibition for the indicated mutant strain with the complementation plasmid is significantly smaller than that for the mutant strain without the complementation plasmid treated with the same oxidative-stress agent ($P < 0.04$).

RESULTS

Contribution of the Suf and Isc systems to oxidative-stress survival in *S. flexneri*. To begin to elucidate the importance of genes that are predicted to encode proteins that make Fe-S clusters in *Shigella*, the *S. flexneri suf* operon and the *iscSUA* genes were deleted. These newly constructed mutants were designated UR011 (Δ*suf*::*cam*) and UR022 (Δ*iscSUA*::*cam*). To examine the contributions of Suf and Isc to oxidative-stress survival, we compared the zones of growth inhibition of the *S. flexneri suf* and *iscSUA* mutants in a disk diffusion assay with either hydrogen peroxide or PMS, a superoxide generator. Both the *suf* and *isc* mutants UR011 and UR022 showed zones of growth inhibition by hydrogen peroxide and PMS that were significantly larger than that of the parental strain SM100 (Table 3). Complementation analysis showed that addition of either the *sufABCDSE* genes on pSUF to UR011 or addition of

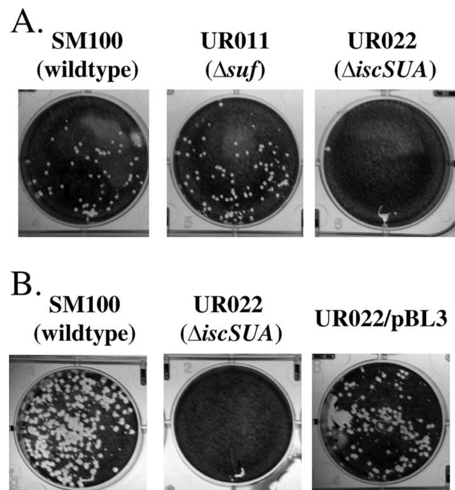

FIG. 1. *S. flexneri* iron-sulfur cluster biosynthesis mutants in Henle cell plaque assays. Confluent Henle cell monolayers were infected with 10^3 (A) or 10^4 (B) bacteria per 35-mm-diameter plate, and the plaques were photographed after 2 days. pBL3 carries the *iscSUA* genes under the control of the constitutive *dnaY* promoter. The experiments were performed three times, and results of a representative experiment are shown.

the *iscSUA* genes on pBL3 to UR022 restored zones of inhibition to the same size as the wild-type strain SM100 containing these plasmids (Table 3). Furthermore, the complemented strains had significantly smaller zones of growth inhibition than the noncomplemented strains. These results suggest that the *sufABCDSE* and the *iscSUA* gene products contribute to the oxidative-stress survival of *Shigella*.

Contribution of the Suf and Isc systems to growth of *S. flexneri* in iron-limiting media. Since the *suf* operon was induced when *Shigella* experienced iron limitation (27), we assessed the growth of the *S. flexneri suf* and *iscSUA* mutants in minimal media containing various levels of iron by measuring the optical densities of the cultures over a 24-hour period. The *suf* mutant UR011 was able to grow as well as the wild-type strain SM100 in all levels of iron availability (data not shown). The *iscSUA* mutant UR022, however, grew significantly slower in both the high-iron and low-iron media, and this slower growth could be complemented by the addition of the *iscSUA* genes on pBL3 (data not shown).

Contribution of the Suf and Isc systems to growth of *S. flexneri* within epithelial cells. Since the *suf* operon was induced when *Shigella* was within Henle cells (16, 27), we tested the *S. flexneri suf* and *iscSUA* mutants for growth in the intracellular environment by examining the plaque formation of these strains on Henle cell monolayers. Although the *suf* mutant UR011 formed plaques in the same number and size as the wild-type strain SM100, the *iscSUA* mutant was unable to form plaques (Fig. 1).

Since plaque formation is the result of several sequential events, including Henle cell invasion, lysis of the endocytic vacuole, bacterial multiplication within the host cell, and intercellular spread via actin polymerization, we investigated whether the *iscSUA* mutant UR022 was defective in any of

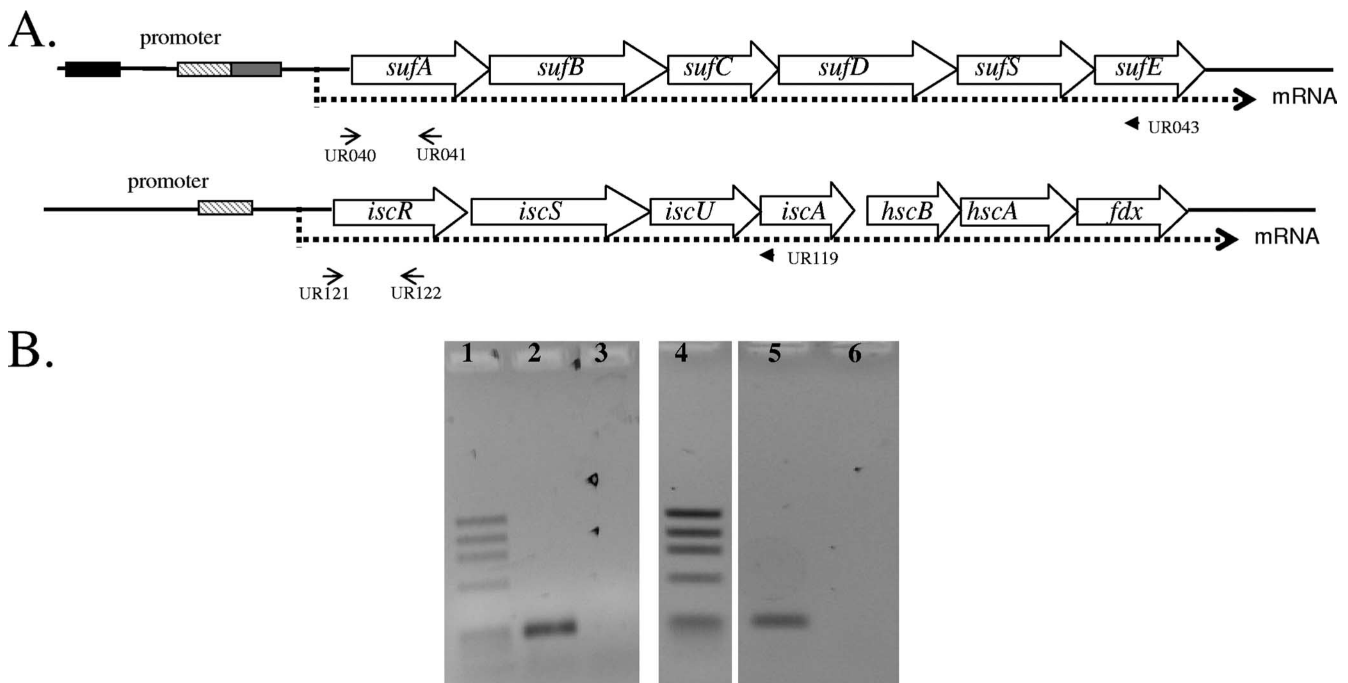

FIG. 2. Structure of the *S. flexneri suf* and *isc* operons. (A) The *suf* and *isc* loci are depicted. The black, hatched, and gray boxes represent putative OxyR, IscR, and Fur binding sites, respectively. (B) RT reactions were performed on total RNA from *S. flexneri* SA101, using either a primer within the *sufE* gene or a primer within the *iscA* gene. The *sufE* and *iscA* RT products were then amplified using PCR primers located within *sufA* or PCR primers located within *iscR*, respectively. Lane 1, ϕX174 HaeIII DNA standard; lane 2, RT-PCR product for *suf*; lane 3, control experiment for the absence of contaminating DNA (no-RT step) for *suf*; lane 4, ϕX174 HaeIII DNA standard; lane 5, RT-PCR product for *isc*; lane 6, control experiment for the absence of contaminating DNA (no-RT step) for *isc*.

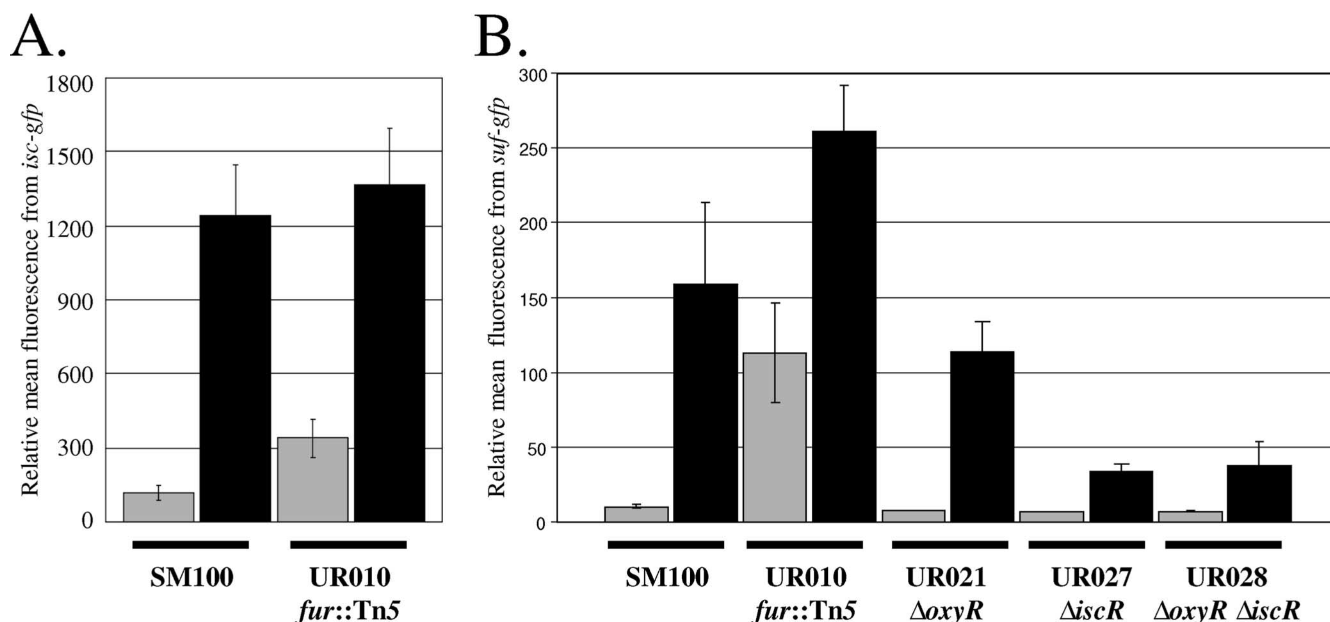

FIG. 3. Iron regulation of the *S. flexneri isc* and *suf* promoters. *Shigella* strains carrying either *isc-gfp* on pAD1 (A) or *suf-gfp* on pLR67 (B) were grown for 5 hours in EZ-RDM containing carbenicillin with (gray bars) or without (black bars) 40 μM ferrous sulfate, and the fluorescence was quantitated by FACS after 5 h. A total of 10^4 bacterial cells were assayed for each experimental condition. The data presented are the means of at least three experiments, and the standard deviations of the means are indicated (error bars).

these processes, beginning with cellular invasion. The ability of SM100 and UR022 to invade Henle cells was tested by using light microscopy to determine the percentage of the Henle cells that were invaded by each strain. Wild-type *S. flexneri* SM100 invaded 90 to 100% of the Henle cells; however, UR022 was unable to invade Henle cells at detectable levels.

Structure of the *S. flexneri suf* and *isc* operons. The *suf* and *isc* genes are located in distinct clusters on the *Shigella* chromosome, suggesting that each set of genes forms an operon. To test this hypothesis, we examined whether the *S. flexneri sufABCDSE* genes were cotranscribed and whether the *iscRSUA* genes were cotranscribed by using RT-PCR. *sufA* is the first gene and *sufE* is the last gene in the putative *suf* operon. *iscR* is the first gene and *iscA* is the fourth gene in the putative *isc* operon. An RT product generated using a *sufE* primer could be PCR amplified by *sufA* primers, indicating that all the *suf* genes are transcribed on one mRNA from the promoter located upstream of *sufA* (Fig. 2). An RT product generated using an *iscA* primer could be PCR amplified by *iscR* primers, indicating that the *iscRSUA* genes are all transcribed on one mRNA from the promoter located upstream of *iscR* (Fig. 2).

Regulation of *S. flexneri suf* and *isc* expression by iron. Previous work showed that transcription from the *Shigella suf* promoter was repressed by iron (16, 27). We tested whether the *isc* promoter was regulated similarly by measuring the expression levels of the *isc-gfp* fusion in *S. flexneri* grown in EZ-RDM with and without added iron. There was a 10-fold increase in expression of the *isc-gfp* fusion after growth in the iron-limiting EZ-RDM, in comparison to growth in iron-replete EZ-RDM (Fig. 3A). Likewise, expression of the *suf-gfp* fusion increased 15-fold in the iron-limiting EZ-RDM (Fig. 3B).

The *S. flexneri suf* promoter contains a putative binding site for the iron-responsive transcriptional repressor Fur. However, there is not an obvious Fur binding site in the *iscR* promoter region (Fig. 2). To examine the contribution of Fur to *suf* and *isc* gene regulation, we measured the expression of these genes in the *S. flexneri* strain UR010 containing a mutation in the *fur* regulator gene. In iron-replete medium, expression of the *suf-gfp* fusion was 16-fold higher in the *fur* mutant UR010 than in the parent strain SM100 containing the *suf-gfp* fusion (Fig. 3B); however, expression of the *iscR-gfp* fusion was only 3-fold higher in the *fur* mutant UR010 than in the parent strain SM100 when the strains were grown in iron-replete medium (Fig. 3A), and there was still significant repression of the *iscR* promoter in this medium. This suggests that although the *suf* promoter is repressed by Fur in iron-replete conditions, the majority of the regulation of the *iscR* promoter by iron occurs independently of Fur. Quantitative RT-PCR analysis showed that the addition of the *S. flexneri fur* gene on pMS1 restored wild-type gene regulation to the Fur mutant (data not shown).

The *Shigella suf* promoter contains putative binding sites for OxyR, which activates gene expression in the presence of hydrogen peroxide, and for the transcriptional regulator IscR (Fig. 2). Thus, we also measured expression of the *suf-gfp* fusion in *S. flexneri* strains UR021, UR027, and UR028, which contained a single mutation in *oxyR* or *iscR* or double mutations in both genes, respectively. Expression of the *suf-gfp* fusion in the strains lacking *iscR* (UR027 and UR028) was fivefold lower than that in strain SM100 in the iron-limiting EZ-RDM, while expression of the *suf-gfp* fusion in the strain lacking just *oxyR* (UR021) was not significantly different from that of the parent strain SM100 in the iron-limiting EZ-RDM (Fig. 3B). These results suggest that IscR is required for maximal expression of *suf* in iron-limiting conditions.

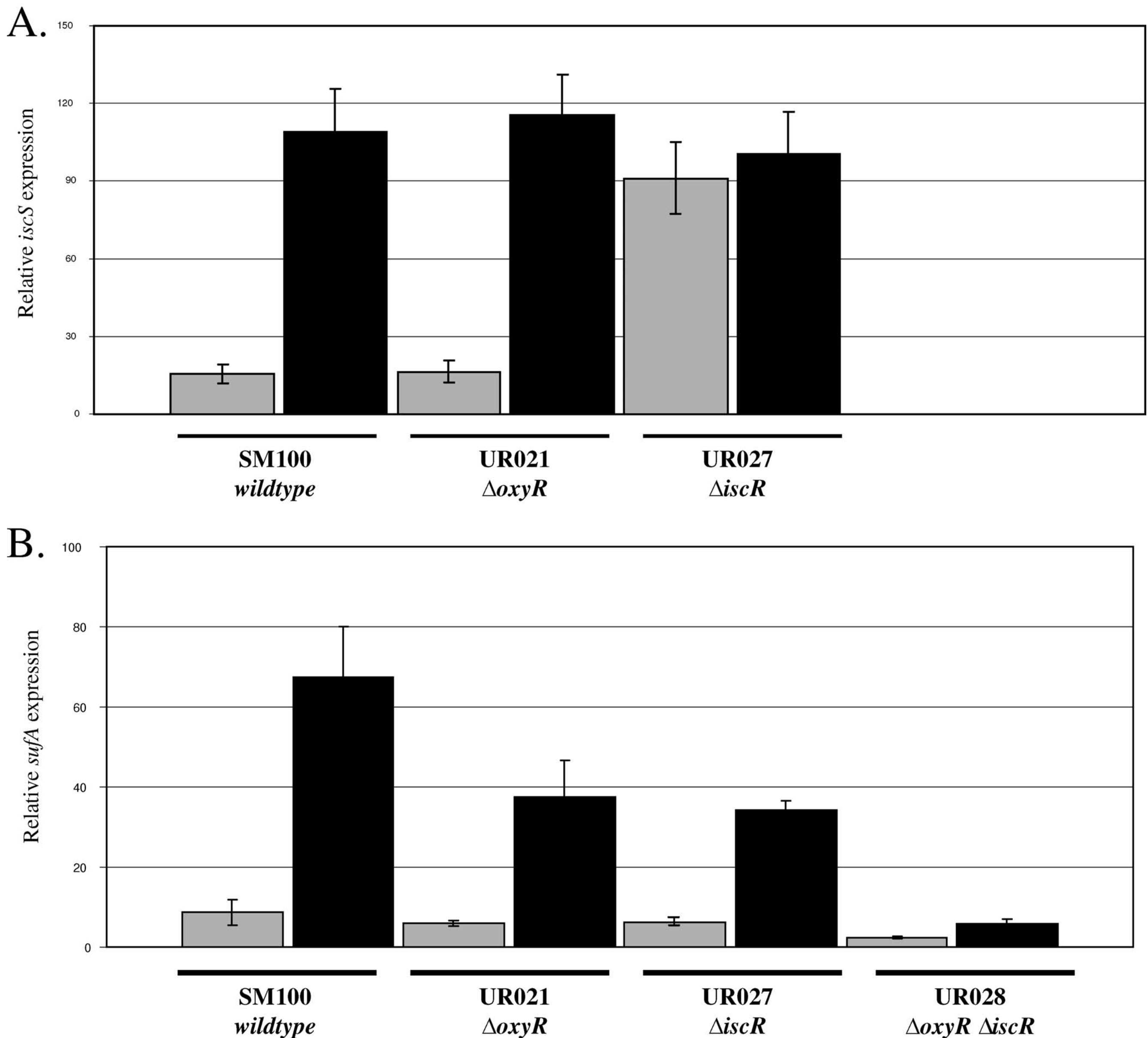

FIG. 4. Oxidative-stress regulation of the *S. flexneri isc* and *suf* promoters. *Shigella* strains were grown for 2 h in L broth. Hydrogen peroxide was added at a final concentration of 1 mM for 10 min (black bars). RNA was isolated from each sample and used to generate cDNAs which were amplified using real-time PCR. The level of *iscS* (A) or *sufA* (B) gene expression was normalized to that of the housekeeping gene *rrsA* by dividing the relative amounts of *iscS* or *sufA* cDNA by the relative amounts of *rrsA* cDNA in each sample. The data presented are the means of at least three experiments, and the standard deviations of the means are indicated (error bars).

Regulation of *S. flexneri suf* and *isc* expression by oxidative stress. Since both the Suf and Isc systems enhance oxidative-stress survival, we measured the expression of the *suf* and *isc* genes in *Shigella* after exposure to hydrogen peroxide using promoter-*gfp* fusions and/or quantitative RT-PCR. The activity of the *suf* and *isc* promoters increased significantly after exposure to 1 mM hydrogen peroxide (Fig. 4 and 5). In the *fur* mutant UR010, *suf* expression still increased in response to oxidative stress (Fig. 5), suggesting that hydrogen peroxide-dependent induction of *suf* expression is distinct from Fe-Fur regulation of *suf* expression.

To examine the contribution of the OxyR and IscR proteins to the regulation of *suf* and *isc*, we measured the expression of the genes in *S. flexneri* strains containing single deletions of either the *oxyR* or *iscR* gene and in an *S. flexneri* strain containing deletions in both regulator genes. Induction of *suf* expression by hydrogen peroxide decreased slightly in the *oxyR* mutant UR021 and very slightly in the *iscR* mutant UR027; however, in the double mutant lacking both OxyR and IscR (UR028), there was no significant induction of *suf* expression in response to hydrogen peroxide (Fig. 4B and 5). In contrast, hydrogen peroxide-dependent induction of *isc* expression was unaffected by the *oxyR* mutation in UR021 (Fig. 4A). Furthermore, expression of *isc* in the absence of hydrogen peroxide

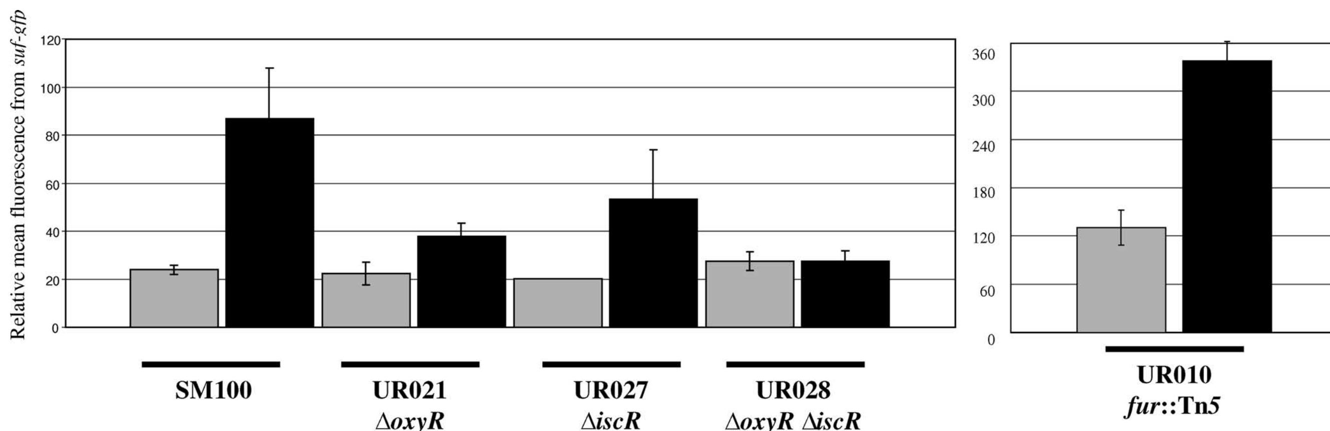

FIG. 5. Oxidative-stress regulation of the *S. flexneri suf* promoter. *Shigella* strains carrying *suf-gfp* on pLR67 were grown for 2 hours in low-salt L broth containing carbenicillin. Samples were treated with hydrogen peroxide (1 mM) for 30 min (black bars), and the relative fluorescence levels were quantitated by FACS. A total of 10^4 bacterial cells were assayed for each experimental condition. The data presented are the means of at least three experiments, and the standard deviations of the means are indicated (error bars).

was aberrantly high in the *iscR* mutant UR027, in comparison to that in the parental strain (Fig. 4A). These data suggest that hydrogen peroxide-dependent induction of *suf* expression, but not *isc* expression, is mediated by OxyR and that IscR is a positive regulator of *suf* expression in the presence of hydrogen peroxide and a negative regulator of *isc* expression in the absence of hydrogen peroxide in *Shigella*.

Regulation of *S. flexneri suf* and *isc* expression within the intracellular environment. Previous work showed that expression of the *S. flexneri suf* gene is induced in the eukaryotic intracellular environment (16, 27). To determine whether the *isc* operon is under similar control, we compared the regulations of the *S. flexneri isc* and *suf* promoters by using *isc-gfp* and *suf-gfp* transcriptional fusions. We infected Henle cells with *S. flexneri* strains carrying these fusions and measured the levels of bacterial gene expression using flow cytometry. The relative amount of GFP per bacterial cell driven by the *isc* and *suf* promoters increased fourfold and sevenfold, respectively, after infection of Henle cells (Fig. 6). Induction of the *Shigella isc* promoter during intracellular growth was confirmed with quantitative RT-PCR on total RNA isolated from infected Henle cells, and *isc* expression increased fivefold postinfection (data not shown).

Since several iron-regulated *Shigella* genes have increased

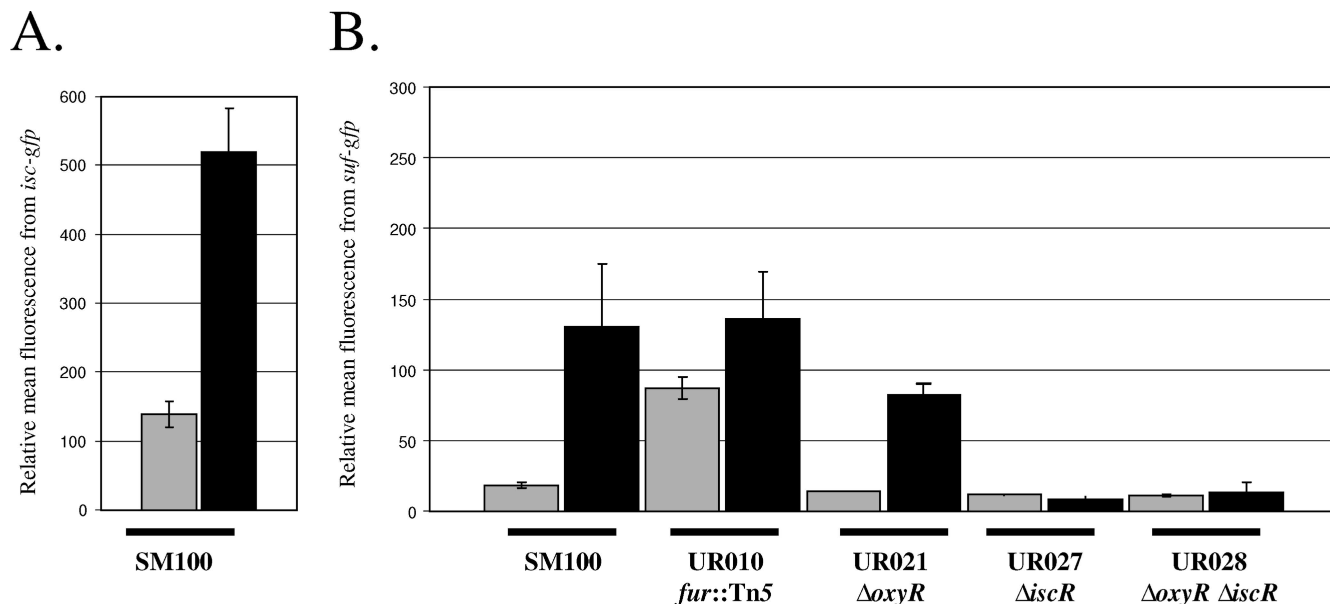

FIG. 6. Intracellular regulation of the *S. flexneri isc* and *suf* promoters. Henle cells were infected for approximately 3.5 h with *Shigella* strains carrying either *isc-gfp* on pAD1 (A) or *suf-gfp* on pLR67 (B). Intracellular bacteria (black bars) were released from Henle cells by deoxycholate treatment, and the relative bacterial fluorescence levels were quantitated by FACS and compared to that of extracellularly grown bacteria (gray bars). A total of 10^4 bacterial cells were assayed for each experimental condition. The data presented are the means of at least three experiments, and the standard deviations of the means are indicated (error bars).

expression when *Shigella* is intracellular (16, 27) and since iron levels regulate *suf* expression, we hypothesized that low iron was the signal mediating increased intracellular expression of *suf*. To test this hypothesis, we examined the expression of the *Shigella suf-gfp* fusion in various regulator mutant backgrounds (*oxyR*, *iscR*, and *fur*) when the strains were grown within Henle cells. We infected Henle cells with *S. flexneri* regulator mutant strains carrying the *suf-gfp* fusion and measured the level of bacterial gene expression using flow cytometry. The relative amount of GFP per bacterial cell driven by the *suf* promoter when UR010 (*fur*::Tn*5*) was intracellular was similar to GFP levels in extracellular UR010 and similar to GFP levels in intracellular SM100 (Fig. 6). Furthermore, the pattern of *suf* expression in Henle cells (Fig. 6) most closely matches that seen with iron limitation in extracellular expression studies, as shown in Fig. 3B. Taken together, these data support the model for iron limitation as the signal that increases the intracellular expression of *suf*.

DISCUSSION

Genome-wide analysis of gene expression when bacteria are within the cytoplasm of eukaryotic cells suggests that regulation of basic metabolism and physiology is an important component of adaptation to the intracellular environment (12, 16, 27). Since we previously found that the Suf Fe-S metabolism system was induced when *Shigella* was within Henle cells, we hypothesized that the ability to build and maintain Fe-S clusters might be important for intracellular life. This is especially likely to be true given that Fe-S clusters are found in proteins with diverse functions, including redox reactions, Fe storage, and gene regulation (for a review, see reference 11).

Examination of the phenotypes of mutations in each of the *Shigella* Fe-S cluster biosynthesis systems demonstrated a role for both systems in oxidative-stress survival. Mutations in either system led to a decreased ability to survive exposure to either hydrogen peroxide or superoxide generated from PMS (Table 3). Similar effects of *suf* and *isc* mutations on superoxide survival have been observed in *E. coli*, but there are conflicting reports on whether the Suf system mediates survival to hydrogen peroxide exposure (14, 33). In natural human infections, the presence of the Isc and/or the Suf systems might therefore enhance *Shigella* survival after exposure to reactive oxygen species released by macrophages.

Outten et al. (21) found that growth of an *E. coli suf* mutant was more sensitive to iron starvation than the wild-type strain in media in which glucontate was the sole carbon source. They proposed that the lower growth of the *suf* mutant in iron-limiting gluconate minimal medium was due to decreased de novo synthesis of the Fe-S cluster in gluconate dehydratase, which is required for growth on gluconate via the Entner-Doudoroff pathway. Although *Shigella* contains the genes for this pathway, we saw no difference in growth between the *S. flexneri suf* mutant and the parent strain in iron-limiting gluconate minimal medium. These data suggest that there are some differences in the roles of the Suf system in *Shigella* and *E. coli*.

Although both the *suf* and *isc* systems had increased expression levels when *Shigella* was intracellular (Fig. 6), only the *isc* genes were required for *Shigella* to form plaques on Henle cell monolayers (Fig. 1). The *isc* genes may compensate for the lack of the *suf* genes when *Shigella* is intracellular or proteins containing Suf-dependent Fe-S clusters may not be required for intracellular survival/multiplication. Further analysis of the virulence phenotypes of the *isc* mutant showed that the *isc* mutant could not form plaques because the mutant was noninvasive. There are several reasons why the *isc* mutant may be noninvasive. A protein required for invasion may contain an Isc-dependent Fe-S cluster protein. A role of redox reactive proteins in *Shigella* virulence has been demonstrated by Watarai et al. (36). In this study, the disulfide oxidoreductase activity of DsbA was required for *Shigella* invasion of MK2 cells. Although DsbA does not use an Fe-S cluster for redox reactivity, an Fe-S-containing protein may interact with some of the virulence proteins in *Shigella* in a manner similar to that of DsbA. Finally, it is formally possible that the lower growth rate of the *isc* mutant may indirectly influence expression of *Shigella* invasion proteins and, thus, invasion.

Since the Suf and Isc systems mediate oxidative-stress survival, it is logical that expression of these systems increases in the presence of oxidative stress. Our data, like those from *E. coli*, indicate that hydrogen peroxide-dependent induction of the *S. flexneri suf* operon is mediated by both OxyR and IscR (14, 21, 37, 38) and that the hydrogen peroxide-dependent increase in *isc* expression is mediated by alleviation of IscR repression (21, 30, 37).

Even though our data suggest that the *S. flexneri* Suf system does not have a specialized role in Fe-S cluster metabolism during iron-limiting conditions, we still found that expression from both the *suf* and *isc* promoters increased when iron was limiting (Fig. 3). However, the proteins that mediate the iron regulation are different for each promoter. Iron-dependent repression of *isc* was predominantly independent of *Shigella* Fur. Increased expression of *isc* in iron-limiting media may be a result of alleviation of IscR repression because of the decreased availability of Fe-S clusters for the repressor IscR.

Iron regulation of *suf* expression was more complicated than that of *isc*. There is a Fur box in the *Shigella suf* promoter and, as in *E. coli* (23, 37), Fur mediated repression of *S. flexneri suf* expression in iron-replete media. However, the maximal increase in *suf* expression when iron was limiting also depended on the presence of IscR, which has not been reported before. There are several possibilities for this observation. First, a deletion of *iscR* could increase iron levels in the cell, making Fur-Fur repression more efficient. This seems somewhat unlikely, since an increase in expression of a number of genes under IscR repression, which encode proteins with Fe-S clusters, might actually decrease free Fe in the cell and thus partially relieve Fur repression (5). Furthermore, iron regulation of two other promoters (*iucA* and *sitA*) is normal in *S. flexneri* strains carrying *iscR* mutations (L. Runyen-Janecky, unpublished observations). Alternatively, IscR could regulate an unknown regulator of *suf* expression. At least two genes of unknown function that are regulated by IscR in *E. coli* are transcriptional regulators (5). One of these (*yqjI*) is located adjacent to the *yqjH* gene which has significant homology to siderophore receptors; however, *yqjH* is predicted to be a pseudogene in *S. flexneri*.

The data presented here support the model that a decreased iron level is the main signal to which the *suf* promoter, and possibly the *isc* promoter, is responding. First, when the *Shigella fur* mutant was within Henle cells, there was no significant additional increase in *suf* expression relative to the extracellular expression. Since oxidative stress is able to induce the *suf* operon in the *fur* mutant, a similar induction would be expected in Henle cells if oxidative stress was a relevant environmental signal. Second, since the intracellular environment thought to be reducing because the ratio of reduced glutathione to oxidized glutathione is at least 30:1, a high level of oxidative stress in epithelial cells is not likely (10). Furthermore, the *Shigella oxyR* mutant shows an increased intracellular expression of *suf* that was similar to that of the parental strain. Finally, like *suf* and *isc*, numerous other iron-repressed genes have increased expression levels when *Shigella* is intracellular, suggesting that a decreased iron level is an important environmental signal for intracellular *Shigella* (16, 25, 27, 28).

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