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Exploring the role of *iscSUA* and the *suf* operon in *Shigella flexneri* invasion and resistance to oxidative stress

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Exploring the roles of *iscSUA* and *suf* operons in *Shigella flexneri* invasion and resistance to oxidative stress

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Spring 2008

***Shigella flexneri* is a facultative intracellular pathogen capable of causing dysentery, a condition that afflicts many around the world, especially in developing countries. There are several aspects of *Shigella* pathogenesis that are not well understood, including a number of genetic and cellular changes that allow *Shigella* to adapt to stresses encountered while invading and replicating within the eukaryotic cytosol. It was the goal of this research to examine the roles of *iscSUA* and *suf*, gene systems predicted to encode for iron-sulfur cluster biosynthesis proteins, in *Shigella* surviving exposure to oxidative stress agents and during *Shigella* invasion and plaque formation in a human colon cell line. An *S. flexneri* strain containing a deletion mutation in the *iscSUA* genes (UR022) was created and an earlier constructed *S. flexneri* strain containing a deletion mutation in the entire *suf* operon (UR011) was verified. Both mutant strains were less resistant to hydrogen peroxide and the superoxide-generator phenazine methosulfate than the wild-type strain. Although UR011 was able to grow at a comparable rate to the wild-type strain in both iron-replete and iron-limited media, UR022 showed reduced growth relative to the wild-type in both media. Furthermore, we found that although UR011 formed wild-type plaques in Henle cell monolayers, UR022 could not form plaques because the strain was noninvasive. Our data suggest that while *suf* deletion does not hinder growth or invasiveness, *iscSUA* is critical for *S. flexneri* invasion and normal growth.**

Shigella flexneri is a member of the genus *Shigella* which are composed of gram-negative, nonmotile, invasive bacteria that cause bacillary dysentery. It invades the intestinal mucosa, growing and replicating within the epithelial cells of the colon and rectum.

Shigella is a disease that primarily afflicts developing nations that have poor sanitation infrastructures and hygiene practices. *Shigella* passes from host to host via fecal-oral transmission through untreated, tainted water and food supplies. Young children have the highest risk of succumbing to the disease because of their undeveloped immune systems. Since medical care is also extremely poor in developing nations, *Shigella* can have a high mortality rate, causing over half-a-million deaths a year (24).

Shigella pathogenesis is a complex process that requires the bacteria to rapidly adapt to a number of different environments (24, 27). After reaching the large intestines and penetrating the mucosal lining of the epithelium, *Shigellae* are taken up by M-cells that routinely monitor luminal contents and translocated to the basolateral side of the epithelium where the bacteria are phagocytosed by macrophages. *Shigellae* are able to escape the phagocytic vacuole where they are exposed to antimicrobial reactive oxygen intermediates produced by the macrophage lysosome and proceed to induce apoptosis in the macrophages. The *Shigellae* then adhere to the epithelial cell membrane and induce macropinocytosis through the use of a Type-III secretion system. *Shigellae* lyse the vacuole and replicate within the eukaryotic cytosol where they must survive in an iron-limited environment. It was earlier demonstrated that the ability to invade the colon epithelium and replicate within the epithelial cytosol was essential for *Shigella* to cause disease in man (4, 12). Following replication, *Shigellae* are able to recruit and polymerize host-cell actin for motility and move to adjacent cells without ever returning to the extracellular environment.

It has been shown that a number of *S. flexneri* genes are up-regulated in the intracellular environment including the *suf* system (21). Expression of the *suf* system is suppressed in *Shigella* by high-iron levels (13, 21) and induced by hydrogen peroxide in *E. coli* (5) and *Shigella* (20). The *suf* system is regulated by OxyR (9), IscR (5), and Fur (22). The *suf* system in *S.*

flexneri contains the genes *sufA*, *sufB*, *sufC*, *sufD*, *sufS*, and *sufE* which are predicted to encode for iron-sulfur (Fe-S) cluster biosynthesis proteins in *Shigella* (22). The proteins encoded by the *suf* operon have been implicated in a number of functions in *E. coli* (9, 18) and the plant pathogen *Erwinia chrysanthemi* (16). SufS is a cysteine desulfurase (9), and SufE is believed to be a sulfur-transfer protein that enhances SufS activity (18). SufA is an iron-binding protein believed to transfer iron to the iron-sulfur forming complex (9). SufC is an ABC/ATPase that complexes with SufB and SufD and has been implicated in iron acquisition under conditions of oxidative stress and iron limitation (16). The SufBCD complex is predicted to function in *Erwinia* pathogenicity because of its role in maintaining iron-sulfur clusters under stressful conditions associated with cellular invasion (16). Iron-sulfur clusters carry out a large number of biological functions including redox reactions, iron acquisition and storage, substrate binding and activation, and gene regulation (9, 11) and are abundant in *E. coli* (14), which is phylogenetically indistinguishable from *S. flexneri* (27). We predict that *suf* may play a role in *Shigella* pathogenesis by encoding proteins that increase resistance to reactive oxygen species like those encountered in the macrophage, facilitating the invasion of epithelial cells, and allowing *Shigella* to replicate in the eukaryotic cytosol, which is an iron-limiting environment.

Shigella also contains *isc*, a second system predicted to encode for Fe-S cluster biosynthesis proteins. In *E. coli*, *isc* is used for housekeeping Fe-S cluster assembly and repair when the bacterium is not undergoing stress (18). The *isc* operon is only known to be regulated by IscR and is upregulated in conditions of oxidative stress in *E. coli* (5) and *Shigella* (22). The *isc* operon is made up of *iscS*, *iscU*, *iscA*, and several other genes in *Shigella* (22). IscS is a cysteine desulferase homologous to SufS that was shown to play a major role in *in vivo* Fe-S cluster formation in *E. coli* by providing sulfur for the Fe-S clusters (25). IscA is homologous to SufA and binds iron before transporting it to IscU, a scaffold protein that assists in the assembling of the Fe-S clusters (3, 9).

The homologous function of the isc system and the suf systems suggests to us that *isc* may also play a role in *Shigella* survival in conditions of low iron and oxidative stress. We also believe that *isc* may play a role in *Shigella* virulence because of the requirement for Fe-S cluster biosynthesis systems in the virulence of other pathogens such as *E. chrysanthemi* (16) and *Mycobacterium tuberculosis* (8). The findings that *suf* and *isc* had two distinct roles in *E. coli* (18) also lead us to believe that *suf* and *isc* may have divergent roles in *S. flexneri*, despite similar biochemical activities.

We designed several experiments to help elucidate the roles of *suf* and *isc* in *S. flexneri*. First, we constructed a strain of *S. flexneri* lacking *iscSUA* and verified that a second strain of S. flexneri lacked the entire *suf* operon. We exposed these mutant strains to several sources of oxidative stress to determine if *isc* or *suf* contributed to oxidative-stress resistance. We then surveyed the ability of the mutants to invade and form plaques in Henle cell monolayers, a human colon epithelial cell line to determine if either *suf* or *isc* was a requirement for *Shigella* virulence.

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 cultured in Luria broth (L-broth) or Luria agar (L-agar). *S. flexneri* strains were grown in L-broth or on Trypticase soy broth agar plus 0.01% Congo red dye at 37°C. For the growth curve experiments, strains were grown in M9 medium containing 0.2% gluconate, 0.05% casein hydrosylate, and 2 μg/ml nicotinic acid. Iron sulfate (100 μM) was added to the M9 media for high iron experiments. Antibiotics were used at the following concentrations: 125 μg/ml carbenicillin and 5 μg/ml chloramphenicol.

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), respectfully. Isolation of DNA fragments from agarose gels was performed using the QIAquick gel extraction kits (Qiagen). All standard PCRs were carried out using *Taq* (Qiagen) polymerase as per the manufacturer's instructions.

Construction of the *Shigella iscSUA::cam* mutant. UR022 was constructed using a modification of Datsenko and Wanner's procedure for inactivating chromosomal genes in *E. coli* (2). A PCR product for allelic exchange was created that contained about 50 bp of the beginning of *iscS*, a chloramphenicol resistance gene (*cam*), and about 50 bp of the end of *iscA*. The plasmid pKD3 (2), containing the *cam* gene, acted as the template for the PCR (2). Primer UR150 contained a 50-nucleotide overhang homologous to the *iscS* end and primer UR151 contained a 50-nucleotide overhang homologous to the *iscA* end; both primers contained priming sites for the pKD3 *cam* gene. The PCR conditions were a 50°C annealing temperature and two-minute extension.

The newly created *iscSUA::cam* PCR fragment was then electroporated into *S. flexneri* SM100 containing the plasmid pKM208 (15). The plasmid pKM208 contains the phage lambda Red recombinase genes under the control of a promoter induced by IPTG. In preparation for the electroporation, SM100/pKM208 was first grown to an optical density of 0.6 to 1.0 at 30°C. Recombinase expression was induced with a 30 min treatment of 1 mM IPTG, followed by a 15-min heat shock at 42°C. The *iscSUA::cam* PCR fragment was electroporated into SM100/pKM208, and transformants were selected on Congo red Trypticase soy broth agar containing 5 μg chloramphenicol per ml. pKM208 was eliminated from the

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* [φ80*dlac*Δ*(lacZ)M15*]	23
S. flexneri strains		
SA100	*S. flexneri* wild-type serotype 2a	19
SM100	SA100 Str[r]	S. Selinger
UR011	SM100 Δ*suf*::*cam*	22
UR022	SM100 Δ*iscSUA*::*cam*	This study
Plasmids		
pKD3	Contains *cam* resistance gene	2
pKM208	Phage lambda Red recombinase genes under the control of an inducible promoter on temperature-sensitive plasmid	15
pWKS30	Low-copy-number cloning vector	27
pSUF	*S. flexneri sufABCDSE* in pWKS30	22
pRJ2	Contains *suf*::*cam* in pWKS30	22
pPK4194	*E. coli iscSUA* in pET11a	25
pBL3	*S. flexneri dnaY* in pPK4194	This study

TABLE 2. Primers used in this study

Primer	Sequence
C1	5'TTATACGCAAGGCGACAAGG
Suf1	5'CGAGTCTACTGGCATTGTCGC
URsufrev	5'CGGGATCCGTAATTTAATGCCACGCTCCA
UR099	5'CGGAATTCTGGAGCGTTTTTATGGTCATC
UR102	5'CGGGATCCAGGTCATAAACAGCGATCACG
UR150	5'CATTGAGTGATGTACGGAGTTTATAGAGCAATGAAATTACCGATTTATCTCATATGAATATCCTCCTTAG
UR151	5'GCGACCACGGTGGGGTTATCGGTATGCGCATCAAACGTGGAAGCTTTCGCCGTGTAGGCTGGAGCTGCTTCG
UR169	5'TCCCCCGGGTTCGGAGATAATCG
UR170	5'TGCTCTAGACGCGTGAATTATACGGTCAACC

mutants by culture at 42°C because it contains a temperature-sensitive origin of replication (15).

Verification of the *Shigella* mutants. PCR analysis was used to confirm that the correct genes were disrupted in strains UR011 and UR022. Chromosomal DNA isolated from UR011 (22) and from the SA100 wild-type underwent PCR with primer suf1, which contained a priming site for *sufA*, and primer URsufrev, which contained a priming site for *sufE*. The PCR conditions were a 50°C annealing temperature and a six-minute extension.

Chromosomal DNA isolated from UR022 and from SA100 underwent PCR with primer UR099, which contained a priming site for *iscS*, and primer UR102, which contained a priming site for *iscA*. The PCR conditions were a 55°C annealing temperature and a 90-second extension. To complete the verification, PCR fragments were run on an agarose gel and fragment sizes were compared.

Complementation of the *Shigella* mutants. Electroporation was used to insert pSuf (22) into UR011 and pBL3 (see below) into UR022 to insert functional *suf* and *iscSUA*, respectively. Each plasmid was also electroporated into SM100 to provide a control. pBL3 was created to clone the *iscSUA* genes under the control of a constitutive *Shigella* promoter. The *Shigella dnaY* promoter was amplified from

SM100 by PCR with primers UR169 and UR170 with an annealing temperature of 55°C and a one-minute extension. The PCR fragment was digested with XbaI. The plasmid pPK4194 (23), which contains the *E. coli iscSUA* genes, was digested with XbaI and NruI, and the resulting 5.7-kb fragment was ligated with the *dnaY* promoter to form pBL3.

Comparative growth analysis. An aliquot equal to 2 x 10^8 bacteria, as determined by optical density at 650 nm, was pelleted and resuspended in 200 μl saline. From this, 20 μl was added to 2 ml of either M9 or M9 plus iron liquid media (described above) for a final concentration of 10^7 bacteria/ml. Cultures were incubated at 37°C with aeration and optical densities at 650 nm were recorded over a period of fifteen hours.

Oxidative-stress assays. Overnight cultures were diluted 1:50 in saline, and 100 μl of the resulting preparation was spread on L agar plates. A BBL 6-mm-diameter disk (Becton, Dickinson, and Company, Franklin Lakes, NJ) was placed in the center of each plate, and 10 ml 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 across their diameter. Statistical analyses of the data were performed using the single-factor analysis of
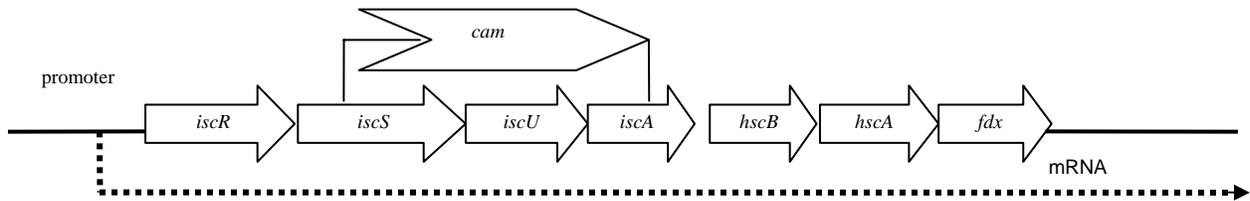
FIG. 1. Structure of the *isc* operon and location of the *cam* insertion. The *isc* locus is depicted with vertical lines showing where the operon is interrupted by the addition of the *cam* gene in UR022.
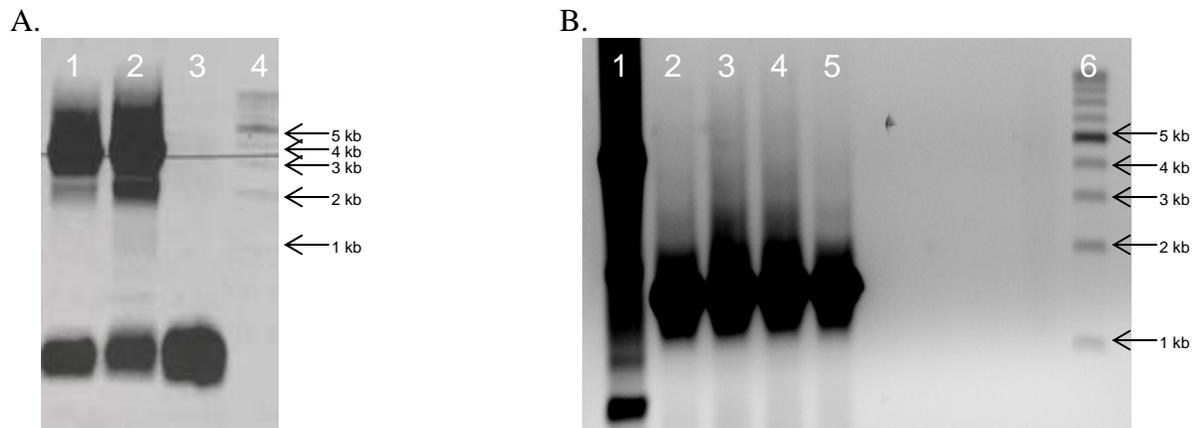
A.

B.

FIG. 2. Verification of the *S. flexneri suf* and *iscSUA* mutants. PCR was used to confirm the creation of the *suf::cam* mutant (A). UR011 chromosomal (1), pRJ2 (2), and SA100 chromosomal (3) DNA underwent PCR with primers URsufrev and Suf1 and the product was run on a gel with a 1-kb ladder (4). PCR was also used to confirm the creation of the *iscSUA::cam* mutant (B). Chromosomal DNA from potential UR022 candidates underwent PCR with primers UR099 and UR102 and the resulting products were run on a gel (2-5) along with similarly treated SA100 chromosomal DNA (1). Lane 6 contains the 1-kb ladder. The size of the 1-kb ladder bands are indicated.

variance statistics package in Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA).

Plaque assays on Henle cells. 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, 1x minimal essential medium nonessential amino acid solution (Invitrogen), and 10% fetal bovine serum (Invitrogen) and were grown in 5% CO_2 atmosphere at 37°C. Assays were carried out as described previously (17), using the modifications described by Hong et al. (7). Briefly, Henle cell monolayers were grown in Falcon Multiwell[TM] 6-Well Tissue Culture Plates (Becton Dickinson Labware, Franklin Lakes, NJ) until confluent. Bacteria were cultured overnight and subcultured until growth had reached an optical density of 0.6. To each Henle cell monolayer, an aliquot equivalent to either 10^3 or 10^4 bacteria was applied. After a period of 1.5 h, gentamicin was added to each well without an agar overlay. After 48 h, the monolayers were stained with Giemsa-Wright stain, photographed, and plaques were scored.

Invasion assays of Henle cells. Invasion assays of Henle cells were done as described previously (6, 7), with the addition of gentamicin at 45 min postinvasion. Briefly, Henle cells were grown in a 6-well plate to semi-confluence.

An aliquot equal to 10^8 bacteria was added to each well. After the addition of the gentamicin, assays were incubated for an additional 3 h. After this time, each well was stained with Geimsa-Wright stain and invasion was scored by observing 100 random Henle cells for the presence of bacteria under 1000x oil immersion microscopy.

RESULTS

Verification of the *Shigella* iron-sulfur cluster biosynthesis mutants. In order to study the importance of genes predicted to be iron-sulfur cluster assembly proteins in *Shigella*, the *S. flexneri suf* operon and the *iscSUA* genes were deleted. The mutant UR011 (*Δsuf::cam*) was constructed previously (22), and the mutant UR022 (*ΔiscSUA::cam*) was constructed for this study. The *isc* operon with the location of the *cam* insertion is described in Figure 1.

PCR analysis of chromosomal DNA was used to verify the successful creation of UR011 and UR022. Using primers that encoded for regions just outside the deleted segment, wildtype (SA100) *Shigella* PCR fragments were compared to mutant PCR fragments.

UR011 was analyzed with primers URsufrev and Suf1 which bind within *sufE* and *sufA*, respectively. The PCR of pRJ2 provided a positive control as this plasmid encodes the *suf::cam* sequence anticipated in UR011, which has an expected size of 3.5 kb. Electrophoresis of the PCR products on an agarose gel revealed that both UR011 and pRJ2 PCR products migrated the same distance and that both were approximately 3.5 kb when compared to the 1 kb ladder standard (Fig. 2A). There were no bands at 3.5 kb for the SA100 chromosomal DNA. Taken together, these data suggest that UR011 chromosomal DNA contains an altered DNA sequence for the *suf* operon.

UR022 was analyzed using primers UR099 and UR102 which bind within *iscS* and *iscA*, respectively. Four potential UR022 candidates were analyzed this way and compared to SA100 chromosomal DNA by electrophoresis of the PCR products on an agarose gel (Fig. 2B). The wild-type *iscSUA* DNA was approximately 4.3 kb in length and the expected size of the *iscSUA::cam* mutant sequence was approximately 1.4 kb in length. Although the band of the SA100 DNA PCR product was smeared due to overloading, there was a prominent concentration of DNA approximately 4.3 kb in size when compared to the 1 kb ladder. The four UR022 candidates had PCR products that had an approximate size of 1.4 kb, suggesting that UR022 was successfully created and contained a mutated *iscSUA* region in comparison to the wild-type.

TABLE 3. Contribution of the Suf and Isc System 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 (*Δsuf::cam*)	37 ± 3[e]	37 ± 4[e]
UR022 (*ΔiscSUA::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 H2O2.

[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).

Contributions of the Isc and Suf systems to oxidative stress survival in *S. flexneri*. The newly created *Shigella* mutants were examined to determine the contributions of Suf and Isc to oxidative-stress survival. UR011 and UR022 were exposed to either hydrogen peroxide or PMS, a superoxide generator, in a disk diffusion assay and the resulting zone of growth inhibition was measured. These zones were compared to those generated by the parental strain SM100 (Table 3). Analyses of the resulting data show that both UR011 and UR022 had significantly larger zones of inhibition from both hydrogen peroxide and PMS when compared with the wild-type. Complementing UR011 with pSuf, which contains a fully functional *Shigella suf* operon, and UR022 with pBL3, which contains constitutively expressed *iscSUA* genes, resulted in zones of inhibition that were similar in size to wild-type SM100 containing the same plasmids (Table 3). The complemented strains also had significantly smaller zones of inhibition than the noncomplemented strains suggesting that *sufABCDSE* and *iscSUA* gene products contribute to oxidative-stress survival of *Shigella*.

Contributions of the Isc and Suf systems to the growth of *S. flexneri* in minimal media. We observed that UR022 grew much slower than both UR011 and SM100 on L agar. Since the *suf* operon was induced in *Shigella* when iron availability was limited (21), the growth of UR011 and UR022 was assessed in minimal media with low iron and minimal media supplemented with iron sulfate by measuring optical density of liquid cultures over a 15-hour period. The resulting growth curves showed that UR011 grew as well as wild-type strain SM100 in conditions of low iron (Fig. 3A) and high iron (Fig. 3B). By contrast, UR022 grew significantly slower in both types of media. This slow growth phenotype was restored to wild-type growth levels by complementation with pBL3 (Fig. 3). These results suggest that proteins encoded by *iscSUA* are necessary for wild-type *S. flexneri* growth, and that proteins encoded by *sufABCDSE* are not necessary for maintaining normal growth levels in low-iron media.

Contribution of the Suf and Isc systems to plaque formation by *Shigella* in Henle cells. Since the *suf* operon was induced when *Shigella* invaded Henle cells (13, 21), the plaque forming abilities of the *S. flexneri iscSUA* and *suf* mutants were evaluated in a plaque assay with Henle cell monolayers. Although the *suf* mutant UR011 was able to form plaques that were comparable to those formed by the wild-type strain SM100 with regard to size and number, the *iscSUA* mutant UR022 was unable to form any plaques, even with a 10-fold increase in the inoculum size (Fig. 4). The plaque forming phenotype was restored by complementing UR022 with pBL3, which contained
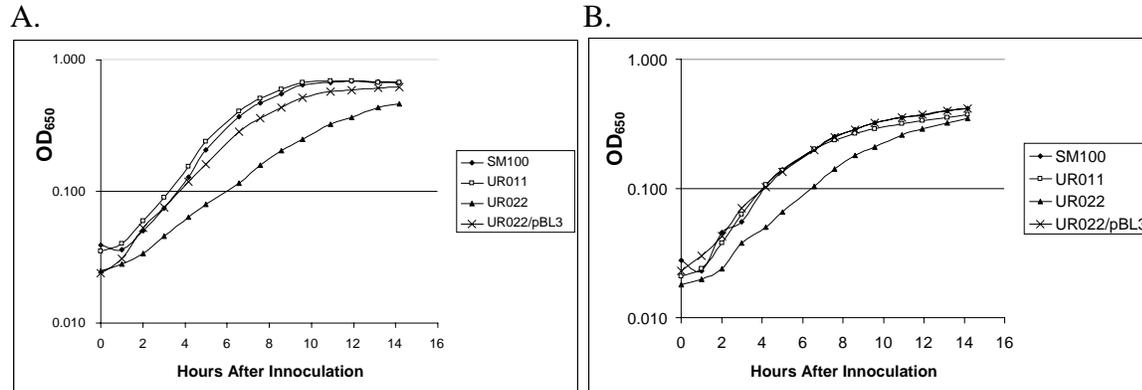
FIG. 3. Growth patterns of the *S. flexneri isc* and *suf* mutants. *Shigella* strains were grown in either M9 media (A) or M9 media supplemented with 100 μM FeSO$_4$ (B) and optical densities were measured over a period of 15 hours at a wavelength of 650 nm.

constitutively expressed *E. coli iscSUA* genes (Fig. 4B). These data indicate that while *iscSUA* is necessary for plaque formation of *S. flexneri* in Henle cells, the *suf* operon is expendable.
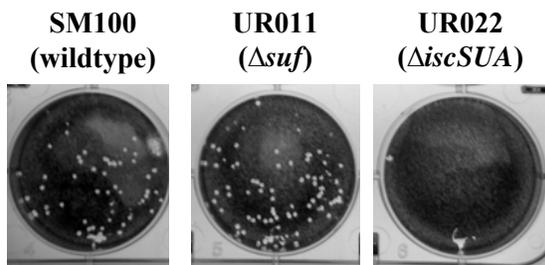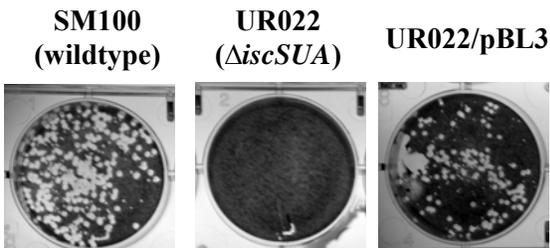

FIG. 4. *S. flexneri* iron-sulfur cluster biosynthesis mutants in Henle cell plaque assays. Confluent Henle cell monolayers were infected with 10^3 (A) or 10^4 (B) bacteria per 35-mm-diamter plate, and the plaques were photographed after 2 days. pBL3 carries the *iscSUA* genes under the control of the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

Contributions of the Suf and Isc systems to invasion of Henle cells by *Shigella*. We wanted to determine what part of plaque formation was disrupted in the *iscSUA* mutant UR022. The first event in plaque formation is Henle cell invasion. Thus, we decided to evaluate the ability of the mutant strains to invade Henle cells with an invasion assay. We used oil immersion light microscopy to determine the percentage of Henle cells that were invaded by each strain. Both wild-type *S. flexneri* and UR011 invaded 90-100% of the Henle cells without having any remarkable differences in morphology or number within the eukaryotic cells when observed under the microscope. The *iscSUA* mutant strain UR022, however, was unable to invade Henle cells at any detectable levels. Complementation of UR022 with pBL3, which contains constitutively expressed E. coli *iscSUA*, was able to partially restore the invasive phenotype to levels approximately half that of the wild-type strain SM100 (54% Henle cell invasion). These results suggest that proteins encoded by *iscSUA* are necessary for *Shigella* invasion of Henle cells, and that an inability to invade was what prevented UR022 from forming plaques in Henle cells.

DISCUSSION

Surviving within and rapidly adapting to the eukaryotic cytosol is an important feature of *Shigella* virulence. In order to reach their target epithelial cells, *Shigellae* must first survive within macrophages where they are exposed to high levels of oxidative stress delivered to destroy the invading pathogen. Following escape from the macrophage, *Shigellae* are able to invade colonic epithelial cells. Once in the cytosol, *Shigellae* are exposed to a nutrient limiting environment where necessary nutrients like iron are less available than in the extracellular environment

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(24). Previous research has shown that these new environments and stresses bring about rapid changes in gene expression within *Shigella* (13, 20, and 21), including the up-regulation of the *suf* system (21). This work sought to expand on this previous research by examining the effect of removing the Suf system or the related *iscSUA* system from *S. flexneri* to further elucidate their importance for eukaryotic invasion and survival in conditions of oxidative stress and iron starvation.

The first step of this work was removing the genes of interest. This was successfully accomplished by the creation of UR011 (*Δsuf::cam*, 22) and UR022 (*ΔiscSUA::cam*). Although these mutations were verified by PCR analysis, there was one discrepancy in the data concerning the verification of UR011. The expected size of the UR011 product was 3.5 kb, which we observed for UR011 and pRJ2. Since SA100 contains a fully intact *suf* operon, we expected PCR analysis to produce a 5.5 kb product for SA100 chromosomal DNA; however, we were unable to produce this 5.5 kb fragment after several attempts. It is important to note that a 3.5 kb fragment was also not produced, indicating that UR011 contained mutated DNA. We believe that our failure to produce a 5.5 kb fragment was due to its large size which caused incomplete extension by *Taq* polymerase. To remedy this, we lengthened the extension time to ten minutes to provide more time for the polymerase to carry out the reaction. We also attempted using *Pfu* polymerase with the ten minute extension time since *Pfu* replicates DNA more accurately than *Taq*. Neither modification to the PCR procedure was successful. The SA100 result did not negate the UR011 results which showed that UR011 had the correct mutation as its PCR profile matched that of the pRJ2 control which contained the *suf::cam* sequence.

Since expression of both *iscSUA* and *suf* was increased by exposure to either hydrogen peroxide or superoxide in *E. coli* (1, 5, and 28), we wanted to examine the role of Isc and Suf in *Shigella* oxidative stress survival. Using a disk diffusion assay with either hydrogen peroxide or the superoxide generator PMS, we showed that both UR011 and UR022 were less resistant to both types of oxidative stress in comparison to the wild-type SM100 strain. Resistance was restored by complementing UR011 and UR022 with pSuf and pBL3, respectively. The fact that both strains were similarly affected by hydrogen peroxide was a bit surprising based on earlier research that indicated that in *E. coli*, *iscS*, *iscU*, and *iscA* were only moderately up-regulated in the presence of hydrogen peroxide compared to members of the *suf* operon (*sufA*, *sufB*, and *sufC*) that were among the genes that were most highly induced by hydrogen peroxide (28). However, our findings support recent data showing that *isc* and

suf have similar increases in transcription after exposure to hydrogen peroxide in *Shigella* (22). The data suggest that both *iscSUA* and *suf* are equally necessary for *Shigella* resistance to hydrogen peroxide and PMS. This is important because *Shigellae* must be able to survive exposure to reactive oxygen species when they are within macrophages. If resistance to oxidative stress was compromised enough by the removal of *suf* or *isc*, it is possible that macrophages would be able to prevent *Shigellae* infection by destroying the bacteria before they could induce apoptosis, thus preventing widespread infection of colon epithelial cells.

Since *suf* expression is up-regulated in response to iron-limiting conditions in *E. coli* (9) and *Shigella* (22), we wanted to examine the ability of UR011 and UR022 to grow in minimal M9 media, which contains low levels of iron. We found that the *suf::cam* mutant UR011 was able to grow as well as the wild-type strain SM100 in low-iron media, suggesting that although *suf* is induced in low-iron conditions, its removal did not alter the growth phenotype of the strain *in vitro*. By contrast, the *iscSUA::cam* mutant did have substantially retarded growth in both low-iron and high-iron minimal media. Complementation with pBL3 restored growth levels to that of the wild-type strain SM100 showing that the *iscSUA* deficiency was responsible for the slowed growth. This result suggests that *iscSUA* is necessary for normal growth-phenotype in M9 minimal media regardless of iron levels. In *Azotobacter vinelandii*, a species of cyanobacteria that has an *iscSUA* locus similar to that of *E. coli*, the *iscA* deficient mutants had slower growth in conditions of elevated oxygen and that *iscS* and *iscU* deficient mutants could not grow well where glucose was the primary carbon source (10). Since gluconate, not glucose, was the carbon source used for our experiments and since our cultures were aerated, it is possible that the *iscSUA* mutant grew poorly because of elevated oxygen, and that it might grow better in conditions of lower oxygen. Alternatively, *iscSUA* may encode for proteins that are necessary for normal *S. flexneri* growth under a wide variety of environmental conditions. *E. coli iscS* deficient mutants also exhibited a number of defects including a slow growth phenotype (18).

Shigella suf was up-regulated in the eukaryotic cellular environment (21) indicating that the operon might be necessary for *Shigellae* to survive within eukaryotic cells. Furthermore, *suf* was necessary for the virulence of the plant pathogen *Erwinia chrysanthemi* (16) leading us to predict that it might also be necessary for *Shigella* virulence. Our plaque assay experiments with Henle cell monolayers, however, revealed that this was not the case. The *suf::cam* mutant UR011 had a similar plaque-forming

phenotype to the wild-type SM100 strain. By contrast, the *iscSUA::cam* mutant UR022 completely lacked the ability to form plaques in Henle cells even at an inoculation level 10-fold larger than that used for the wild-type. These data indicate that while *suf* is not essential for plaque formation in Henle cell monolayers, *iscSUA* is absolutely critical.

In an attempt to understand what stage of plaque formation was deficient in the UR022 strain, we conducted an invasion assay to determine if the plaque defect was a result of an inability of UR022 to invade Henle cells. As expected, the wild-type SM100 strain was able to consistently invade between 90 and 100% of the cells in the monolayer, as was UR011, which had already demonstrated similar plaque formation to SM100. UR022 was unable to invade Henle cells at any detectable levels over several trials and with extended incubation periods. In order to invade colonic cells, *Shigellae* must bind to the host cell membrane and induce uptake by macropinocytosis (24). Binding is accomplished by adhesins and other membrane components that have not been completely characterized in *Shigella*, while a Type-III secretion system encoded on the *Shigella* virulence plasmid induces macropinocytosis (27). Proteins containing Fe-S clusters, who biosynthesis requires *iscSUA*, may be necessary for either or both of these processes. It is possible that a yet unidentified membrane protein required for proper adhesion is not correctly formed in the *iscSUA* mutant so that the bacteria do not adhere to the eukaryotic cells. Alternatively, part of the Type-III secretion machinery may be defective in the *iscSUA* mutant causing UR022 to harmlessly adhere to the Henle cells but not invade; thus, the mutant would be killed during the gentamicin application step of the invasion assay. Since there is only a partial list of *Shigella* products known to be involved in these two stages of pathogenesis (27), further research will need to be conducted to determine the exact reason that UR022 cannot invade Henle cells.

We verified that the *iscSUA* mutation was the cause of the invasion deficiency when we found that UR022 was able to invade Henle cells and form plaques in confluent Henle cell monolayers after complementation with pBL3, which contained constitutively expressed *E. coli iscSUA* under the control of the *Shigella dnaY* promoter. Invasion and plaquing for UR022/pBL3 was approximately 50% of the wild-type strain SM100. It is possible that constitutive expression of *iscSUA*-encoded proteins might impair the ability of the cell to invade and replicate within the eukaryotic environment. Over-expression might cause UR022 to expend already limited resources making extra proteins not necessary for survival, while tying up translational machinery and preventing the expression of proteins necessary for invasion and intracellular spread.

Additionally, IscA was found to be an iron-binding protein in *E. coli* with a high iron association constant (3) suggesting that its over-expression may damage *Shigella* by binding too much intracellular iron.

These results suggest that the roles of *iscSUA* and *suf* in *Shigella* are distinct and different from those observed in *E. coli*. Outten *et al*. showed that *E. coli suf* mutants were more sensitive to iron starvation when gluconate was the sole carbon source and that *suf* was more critical for the maintenance of Fe-S clusters in conditions of iron starvation and oxidative stress (18). In these experiments, however, we observed that the *Shigella suf* mutant grew as well as the wild-type strain in iron-limiting media with gluconate as the sole carbon source. Furthermore, growth within Henle cells, as indicated by plaque size, was no different between UR011 and SM100, despite the fact that the eukaryotic cytosol is iron-limiting. The deletion of *iscSUA*, linked to housekeeping maintenance of Fe-S clusters in *E. coli* (18), was shown to be necessary for *Shigella* virulence in Henle cells, which had not been previously shown.

The results of this study still leave many unanswered questions that will require additional research. For instance, we were unable to complete experiments that tested the survival of the *suf* or *isc* mutants within macrophages. Escape from the phagocytic vesicle and the induction of apoptosis in macrophages is a necessary part of *Shigella* pathogenesis (24). Microarray analysis showed that when *Shigella* infected HeLa epithelial cells or U937 macrophages, *sufA* expression was up-regulated in *Shigella* in the epithelial cells and down-regulated in *Shigella* in the macrophages (13). Although this would suggest that *suf* was more important for *Shigella* survival in epithelial cells than macrophages, our data showed that *suf* deletion had no effect on replication within Henle epithelial cells as measured through the plaque assays. This may be due to Isc functioning to replace the lost Suf system in *Shigella*. Still, both UR011 and UR022 were more sensitive to oxidative stress which would be more prevalent in the macrophage environment. This suggests that both UR011 and UR022 may be less able to survive within macrophages than wild-type *Shigella*. Furthermore, the inability of UR022 to invade Henle cells may be caused by a dysfunctional Type-III secretion system which would prevent the induction of macropinocytosis. The Type-III secretion system in *Shigella* has been linked to phagocytosis by macrophages and the release of pro-inflammatory cytokines (27). Thus, UR022 may not be as prone to phagocytosis as wild-type *Shigella* or may cause a less pronounced cytokine response.

Since UR022 was halted at invasion, we could not determine if *iscSUA* was necessary for other stages of *Shigella* pathogenesis, such as lysis of the vacuole,

replication within the eukaryotic cytosol, the recruitment and polymerization of host-cell actin, or intercellular spread. An experiment to further explore this issue would be to put *iscSUA* under the control of a promoter that would be active in the extracellular environment and inactive in the intracellular environment. Functional *iscSUA* would allow the bacteria to invade Henle cells; after invasion, *iscSUA* would no longer be expressed allowing us to further elucidate the importance of *iscSUA* in *Shigella* pathogenesis. Using a similar methodology to the invasion assays used in this study, we would be able to stain and count bacteria within the Henle cells so that we could compare rates of replication within the cytoplasm to the wild-type strain. Since *iscSUA* deficient *Shigellae* replicated much slower than wild-type *Shigellae* in iron-limited and iron-replete media, replication in Henle cells would likely be slower for the *iscSUA* deficient mutants. A plaque assay with this strain could be used to determine if *iscSUA* is necessary for intercellular spreading. UR022 was unable to invade Henle cells, which could theoretically be the result of a dysfunctional Type-III secretion system. In addition to invasion, the Type-III secretion system is necessary for intercellular spread because of its role in the lysis of the double-membrane that is formed around *Shigellae* when they pass into adjacent cells (27). If we found that *iscSUA* deficient *Shigella* could not form plaques after invasion, it could potentially be the result of failure to escape this double-membrane. This result would not refute the theoretical possibility that intact *iscSUA* is necessary for the proper functioning of the Type-III secretion system in *Shigella*.

In closing, we were able to demonstrate that both *suf* and *iscSUA* are necessary for *Shigella* resistance to oxidative stress. Furthermore, *iscSUA* is necessary for normal growth in both low-iron and high-iron media and for invasion of and, thus, plaque-formation in Henle cells. This is an important development because it contributes to other studies that have shown that the iron-sulfur biosynthesis pathways are potential therapeutic targets for fighting intracellular pathogens. In *Mycobacterium tuberculosis*, disruption of the single iron-sulfur cluster biosynthesis machinery had profound effects: the resulting strain was unable to survive oxidative stress and iron-starvation to the point where it was hypothesized that it might be unable to cause disease (8). In a world affected by antibiotic resistance, the findings of new therapeutic targets for intracellular bacterial pathogens are extremely significant.

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