Anaerobic Regulation of *Shigella flexneri* Virulence: ArcA Regulates *fur* and Iron Acquisition Genes^{▽†}

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Invasion and plaque formation in epithelial monolayers are routinely used to assess the virulence of *Shigella flexneri*, a causative agent of dysentery. A modified plaque assay was developed to identify factors contributing to the virulence of *S. flexneri* under the anaerobic conditions present in the colon. This assay demonstrated the importance of the ferrous iron transport system Feo, as well as the global transcription factors Fur, ArcA, and Fnr, for *Shigella* plaque formation in anoxic environments. Transcriptional analyses of *S. flexneri* iron transport genes indicated that anaerobic conditions activated *feoABC* while repressing genes encoding two other iron transport systems, the ABC transporter Sit and the Iuc/Iut aerobactin siderophore synthesis and transport system. The anaerobic transcription factors ArcA and Fnr activated expression of *feoABC*, while ArcA repressed *iucABCD iutA*. Transcription of *fur*, encoding the iron-responsive transcriptional repressor of bacterial iron acquisition, was also repressed anaerobically in an ArcA-dependent manner.

Shigella flexneri is a pathogenic, gram-negative bacterium that causes dysentery in humans. During infection of the colon, shigellae gain access to the basolateral surface of the epithelium via M cells. Upon ingestion by macrophages, shigellae promote apoptosis of these cells and are released to subsequently invade intestinal epithelial cells. *S. flexneri* multiplies within the cytoplasm of the epithelial cells and spreads directly to adjacent cells, thus delaying detection by immune cells (reviewed in references 22, 44, and 46). Because shigellosis is restricted to higher primates, *Shigella* pathogenesis is routinely investigated with cultured human epithelial cells. *S. flexneri* forms plaques in confluent monolayers, and the size and number of these plaques correlate with virulence, as plaque formation requires that the bacteria invade, grow intracellularly, and spread directly to adjacent epithelial cells (43).

Shigella, like other pathogenic bacteria, has multiple mechanisms for coping with the iron-restricted environment of the host. The genome of *S. flexneri* 2a encodes at least four iron acquisition systems (23, 65). The aerobactin operon encodes the biosynthesis (*iucABCD*) and transport (*iutA*) of the hydroxamate siderophore aerobactin. This siderophore transports ferric iron (Fe³⁺), the predominant form of iron under aerobic conditions at neutral pH. Additionally, *S. flexneri* expresses the *fhu* genes for transport of the fungal siderophore ferrichrome. *S. flexneri* also encodes two iron acquisition systems, Feo and Sit, which are predicted to transport ferrous iron (Fe²⁺), the more abundant form of iron in anaerobic environments. The *Shigella* SitABCD system has similarity to the *Salmonella enterica* serovar Typhimurium Sit system, which primarily transports manganese (3). The *S. flexneri* Sit system has been shown

to function in iron transport (50, 52), and the *S. flexneri* *sit* genes were up-regulated in the intracellular environment (31, 51). A *sit feo iuc* mutant did not grow in the absence of exogenously supplied siderophore or form plaques in epithelial cell monolayers (52), indicating that there are no other iron transport systems in strain SA100.

Iron is essential for growth, yet free iron can be toxic to cellular components. Therefore, the expression of iron acquisition genes is regulated in response to the intracellular iron concentration. Under iron-replete conditions, the transcription factor Fur binds iron and Fe-Fur represses the expression of iron transport genes (11). Fe-Fur also represses *ryhB*, which encodes a small RNA that promotes degradation of transcripts for iron storage, oxidative metabolism, and stress proteins (35–38). Iron availability influences the transcription of *fur* as well as the activity of the Fur protein. Fe-Fur is an autorepressor, reducing *fur* expression in response to iron (9, 10, 19, 53). *fur* expression is also reduced in strains with mutations in *cya*, encoding adenylate cyclase, and *crp*, encoding the cyclic AMP receptor protein, suggesting that the source of cellular carbon impacts iron uptake (10). *fur* transcription is also activated by OxyR and SoxS, which are redox regulators activated by oxidative stress (68). The increased level of Fur scavenges unbound iron to prevent cell-damaging radical formation as well as turning off iron acquisition. Bacteria also regulate specific iron transporters in response to the oxygen availability. Fnr and ArcA are the primary redox regulators responsible for the activation or repression of genes associated with the transition to anaerobiosis (17), and Fnr has been shown to stimulate transcription of *feoABC* in *Escherichia coli* under anoxic conditions (24). The expression of genes encoding the Sit system in *S. enterica* serovar Typhimurium decreases anaerobically (20). This anaerobic repression was not due to ArcA and Fnr but rather to the availability of the redox metals iron and manganese, which bind to the transcription factors Fur and MntR (20).

Anaerobiosis has been shown to influence the persistence and virulence of enteric pathogens such as *E. coli* (12, 21),

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TABLE 1. Strains and plasmids

Strain or plasmid	Characteristics	Reference or source ^a
<i>E. coli</i> strains		
DH5 α	Cloning strain	56
DH5 α (λ pir)	Donor strain for triparental conjugation	56
ECL5331	MC4100 <i>arcA::kan</i>	30
MM294/pRK2013	Helper strain for triparental conjugation	R. Meyer, UTA
<i>S. flexneri</i> strains		
SA100	Wild-type <i>S. flexneri</i> serotype 2a	47
SM100	SA100 Str ^r	S. Seliger, UTA
SA101	SA100 Crb ⁻	8
MBF100	SA100 <i>fnr::cam</i>	This study
MBF100W	SA100 Crb ⁻ <i>fnr::cam</i>	This study
MBF200	SA100 <i>arcA::kan</i>	This study
MBF200W	SA100 Crb ⁻ <i>arcA::kan</i>	This study
MBF300	MBF200 <i>fnr::cam</i>	This study
MBF300W	SA100 Crb ⁻ <i>arcA::kan fnr::cam</i>	This study
SA192	SA100 <i>feoB::dhfr iucD::Tn5</i>	52
SM191	SM100 <i>feoB::dhfr sitA::cam</i>	52
SA167	SA100 <i>iucD::Tn5 sitA::cam</i>	52
SM193	SM100 <i>feoB::dhfr iucD::Tn5 sitA::cam</i>	52
SM1301	SM100 <i>fur::cam</i>	45
Plasmids for mutant construction and gene expression		
pCC1	Single- and inducible-copy vector	Epicenter Biotechnologies
pCVD442N	Sucrose-selectable suicide vector for allelic exchange	67
pDRIVE	PCR cloning vector	QIAGEN
pMTLcam	Source of <i>cam</i> cassette	66
pQE-2	IPTG-inducible gene expression vector	QIAGEN
pDRIVE <i>fnr</i>	pDRIVE with SA100 <i>fnr</i> region	This study
pDRIVE <i>fnr::cam</i>	pDRIVE <i>fnr</i> with <i>cam</i> cassette disruption	This study
pCVD442 <i>fnr::cam</i>	pCVD442N with <i>fnr::cam</i> of pDRIVE <i>fnr::cam</i>	This study
pMBarcAQE	pQE-2 with <i>arcA</i> coding region inserted into BseRI and HindIII sites	This study
pMBarcAccQE	pCC1 with IPTG-inducible <i>arcA</i> and <i>lacI</i> regions from pMBarcAQE	This study
pMB <i>fur</i> QE	pQE-2 with <i>fur</i> coding region inserted into BseRI and HindIII sites	This study
Plasmids for GFP reporter assays		
pLR29	<i>gfp</i> reporter vector	51
pEG2	pLR29 with <i>sitA</i> promoter	51
pEG6	pLR29 with <i>iutA</i> promoter	L. Runyen-Janecky and E. Gonzales, UTA
pMB <i>feo</i> _{Alt}	pLR29 with altered <i>feoA</i> promoter	This study
pMB <i>feo</i>	pLR29 with <i>feoA</i> promoter	This study
pMB <i>fur</i> _{Alt}	pLR29 with altered <i>fur</i> promoter	This study
pMB <i>fur</i>	pLR29 with <i>fur</i> promoter	This study
pMB <i>lldP</i>	pLR29 with <i>lldP</i> promoter	This study
pMB <i>minC</i>	pLR29 with <i>minC</i> promoter	This study
pMB <i>narG</i>	pLR29 with <i>narG</i> promoter	This study

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Salmonella spp. (6, 27, 60), *Vibrio cholerae* (2, 58), and *Yersinia enterocolitica* (57). Because *S. flexneri* infects the colon, which is an oxygen-limited environment, studies were undertaken to determine whether anaerobiosis and the anaerobic transcription factors ArcA and Fnr affect *S. flexneri* iron metabolism and virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are described in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar (16). *S. flexneri* strains were grown in RPMI (RPMI 1640 [Invitrogen]) with L-glutamine, without phenol red [Gibco; Invitrogen], and buff-

ered with 100 mM HEPES) supplemented with 2.5 μ M FeSO₄ where indicated or on tryptic soy broth agar plus 0.01% Congo red dye (Congo red agar) at 37°C. Antibiotics were used at the following concentrations (per milliliter): 125 μ g of carbenicillin (Car), 200 μ g of streptomycin (Str), 20 μ g of kanamycin (Kan), and 7.5 μ g of chloramphenicol (Cam).

For green fluorescent protein (GFP) reporter and real-time reverse transcription (RT)-PCR assays, *S. flexneri* strains were plated on Congo red agar with appropriate antibiotics and grown overnight at 37°C. Isolated colonies were used to inoculate 2 ml RPMI with 2.5 μ M FeSO₄ and appropriate antibiotics for plasmid maintenance. After overnight growth at 37°C, cultures were diluted to an optical density at 600 nm (OD₆₀₀) of 0.05 into 2 ml RPMI containing antibiotics and grown aerobically at 37°C for 2 h. These were subcultured to an OD₆₀₀ of 0.03 into the same medium and grown to exponential phase either aerobically with vigorous shaking or anaerobically in Oxoid AnaeroJars with AnaeroGen

sachets and Anaerobic Indicators (Oxoid Ltd., Hampshire, England), as indicated. All data are the average of at least three independent experiments.

Tissue culture and plaque assays. Henle cells (Intestine 407 cells; American Type Culture Collection, Manassas, VA) were grown in minimal essential medium (MEM) or RPMI 1640 (Invitrogen) supplemented with 10% Bacto tryptose phosphate broth (Difco; Becton Dickinson Company), 2 mM glutamine, MEM nonessential amino acid solution (Invitrogen Corporation), and 10% fetal bovine serum (Invitrogen) in a 5% CO₂ atmosphere at 37°C. Plaque assays were performed as described previously (52), except that the medium was supplemented with 100 mM HEPES (pH 7.5) and agarose was omitted from the overlay. For the anaerobic plaque assays, the plates were incubated in the BD BBL GasPack pouch anaerobic system (Becton, Dickinson, and Company) for the duration of the experiment.

Construction of mutants. The *S. flexneri* *arcA::kan* mutant was constructed by bacteriophage P1 transduction (56) from the *E. coli* *arcA::kan* mutant ECL5331 (30). Insertional inactivation of the *arcA* gene in MBF200 was confirmed by PCR using primers flanking the *arcA* gene, IWDarcAOut1 and IWDarcAOut2.

The *S. flexneri* *fur* strain was made by allelic exchange. The *fur* region was amplified from SA100 by PCR using primers MWDFnrUS1 and MWDFnrDS1, digested with PstI and SalI, and ligated with the pDRIVE vector. A *cam* cassette was excised from pMTLcam using SmaI and was ligated into pDRIVEfur digested with BclI and made blunt with the Klenow fragment of DNA polymerase I (New England Biolabs, Ipswich, MA). *fur::cam* was then excised from pDRIVEfur:cam using SmaI and ligated into SmaI-digested pCVD442N. The resulting plasmid, pCVD442fur:cam, was then mated into SM100 by triparental conjugation. Primary integrants were selected by growth in the presence of Car, Str, and Cam and verified with primers MWFFnrDS1 and FNRintFor. The *fur::cam* mutant was isolated by growth in the presence of sucrose and Cam and was confirmed by PCR using primer pair FNRintFor and FNRintRev and primer pair MWFFnrUS1 and MWFFnrDS1. MBF100 (*SA100 fur::cam*) and MBF300 (*SA100 arcA::kan fur::cam*) were obtained by P1 transduction of *fur::cam* from SM100 *fur::cam* to SA100 and MBF200, respectively, and verified by PCR. All primer sequences are listed in Table S1 in the supplemental material.

Construction of plasmids for gene expression. A single-copy, IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *arcA* vector was engineered by first cloning the *arcA* gene under the inducible T5 promoter of plasmid pQE-2 (QIAGEN). SA100 genomic DNA was used as a template for PCR using the primers MBarcAforQE and MBarcArevQE (see Table S1 in the supplemental material), and the fragment was cloned into pQE-2 digested with BseRI and HindIII to generate pMBarcAQE. The *FspI* fragment, including the *lacI* and *T5-arcA* genes, was excised from pMBarcAQE and ligated into the blunt-cloning-ready pCC1 vector (Epicenter Biotechnologies, Madison, WI), resulting in pMBarcAccQE, which was verified by DNA sequencing.

A plasmid for IPTG-inducible expression of *fur* was constructed by PCR amplification of the *fur* gene from SA100 DNA using primers MBfurForQE and MBfurRevQE (see Table S1 in the supplemental material). The PCR product and pQE-2 vector were digested with BseRI and HindIII and ligated, and the resulting pMBfurQE plasmid was confirmed by DNA sequence analysis.

Microarray analysis. Microarrays were printed and postprocessed as described previously (45). Wild-type *S. flexneri* batch cultures were grown aerobically in RPMI medium without added iron to mid-logarithmic phase in a BIOFLO 110 Fermentor/Bioreactor (New Brunswick Scientific, Edison, NJ) to maintain constant pH, dissolved oxygen concentration, temperature, and agitation. A portion of the culture was removed to obtain the aerobically grown bacteria. The dissolved oxygen concentration was then depleted in the remaining culture by the addition of nitrogen gas, and 15 min after depletion, the anaerobic bacteria were isolated. RNA was purified with RNeasy Midi kits (QIAGEN). Reverse transcription of RNA to generate amino allyl-dUTP-incorporated cDNA, Cy3 and Cy5 coupling, probe generation, and array hybridization were performed as described previously (45), with RNA derived from aerobically grown bacteria labeled with Cy3 and from anaerobically grown strains labeled with Cy5. Microarrays were scanned by the Genepix array scanner 4000A (Axon Instruments, Union City, CA). Preliminary analysis of microarrays was performed with Genepix 5.0 software, and normalization of microarray data was carried out by the Longhorn Array Database, an open-source, MIAME-compliant implementation of the Stanford Microarray Database (28). Normalized data were filtered so that spots with a regression correlation of lower than 0.6 and those that were in areas of high background were excluded. Additionally, genes that did not exhibit greater than a twofold difference in expression in at least two arrays and those that showed inconsistent patterns of induction or repression were excluded from further analysis.

GFP reporter assays. *gfp* transcriptional fusions (Table 1) were constructed by PCR amplification of the promoter of each gene (primers are listed in Table S1

in the supplemental material) and ligation into the promoterless *gfp* vector pLR29. To measure GFP, overnight cultures were diluted to an OD₆₀₀ of 0.05 into RPMI with Car and 100 mM HEPES and grown aerobically at 37°C for 2 h. These were subcultured to an OD₆₀₀ of 0.03 into the same medium and grown for 2 h either aerobically with vigorous shaking or anaerobically in Oxoid AnaeroJars with AnaeroGen and Anaerobic Indicators (Oxoid Ltd., Hampshire, England). The cultures were diluted to an OD₆₀₀ of 0.08, and fluorescence was measured in a VersaFluor fluorometer (Bio-Rad Laboratories, Hercules, CA). The instrument was blanked using the parent strains with the pLR29 control plasmid, and the range was set to 15,000 relative fluorescence units (RFU) with the constitutive pMBminC vector, which expresses *gfp* constitutively. The data are shown as relative expression levels, with the culture giving maximal expression set at 100%. The average RFU/OD₆₀₀ of each transcriptional fusion was normalized to the RFU/OD₆₀₀ of the pMBminC vector in the same strain. Results are the average of three independent experiments. The plasmids pMBnarG and pMBldP served as positive controls for Fnr and ArcA regulation, respectively.

Real-time RT-PCR. *S. flexneri* strains SA100/pCC1, MBF200/pCC1, and MBF200/pMBarcAccQE were grown as for the GFP reporter assays, except that 1 μM IPTG was added to the final subculture medium to induce ArcA expression; anti-ArcA (generously provided by P. Silverman [59]) immunoblots showed that this amount of IPTG induced wild-type levels of ArcA. RNA was isolated on RNeasy Mini columns (QIAGEN) following the addition of 1/5 volume of 95% ethanol–5% phenol (vol/vol) to logarithmically growing, anaerobic cultures. RNA was DNase treated (DNase I; QIAGEN) on the RNeasy column and again after elution with amplification-grade DNase I (Invitrogen) according to the manufacturers' instructions. cDNA was generated from approximately 5 μg of each RNA sample with the High Capacity cDNA Archive kit (Applied Biosystems). Real-time RT-PCR mixtures in a total volume of 25 μl contained 1× Power SYBR green PCR Master Mix (Applied Biosystems), 800 nM concentrations of the indicated primers, and 1/200 of the cDNA reaction mixture. *fur* cDNA was detected with primers MBfurRT1 and MBfurRT2. *rrsA* cDNA was detected with primers RrsA.for and RrsA.rev. Real-time RT-PCR and analyses were carried out with an Applied Biosystems 7300 Real Time PCR System and software. Standard curves for each primer set were generated by using cDNA obtained from 10-fold dilutions of SA100 RNA, and the amount of cDNA in each sample was extrapolated from the standard curve. The relative amounts of *fur* cDNA were normalized by dividing the values by the relative amounts of *rrsA* control cDNA in each sample.

Antibody supershift assays. The promoter-*gfp* fusion plasmids served as templates for PCRs to generate probes for the promoter regions, using primers listed in Table S1 in the supplemental material. After gel extraction of the PCR fragments, the DNA was digested with XmaI for probes generated with the pLR29EMSAfor 5' primer, which cuts just upstream of the BamHI restriction enzyme site in the pLR29 vector, while the *iuc* probe was digested with XbaI. The probes were gel purified, and the ends were filled in using Klenow fragment (New England Biolabs, Ipswich, MA) and a mixture of nucleotides for cold probes. dCTP was replaced with [³²P]dCTP (Perkin Elmer, Boston, MA) for radiolabeled probes. Unincorporated nucleotides were removed with Micro Bio-Spin P-30 Tris chromatography columns (Bio-Rad Laboratories), and all probes were phenol-chloroform extracted and ethanol precipitated (1). Radioactivity was measured by liquid scintillation counting, and probe concentrations were determined by measuring the absorbance.

Cell extracts from MBF200/pCC1 and MBF200/pMBarcAccQE were prepared by the method of Tardat and Touati (61), with the exception of growth conditions. *S. flexneri* cultures were grown overnight in HEPES-buffered RPMI with 2.5 μM FeSO₄ and antibiotics, subcultured at 1:100 into the same medium without added iron, and grown aerobically at 37°C to mid-logarithmic phase. The cultures were then diluted into the same medium containing 1 μM IPTG to induce *arcA* expression, and the cultures were grown for 2 h under anaerobic conditions.

The antibody supershift assays were performed essentially as described by Ausubel et al. (1). Binding buffer consisted of 10 mM Tris-HCl (pH 7.4), 10% glycerol, 10 mM CaCl₂, 100 mM KCl, 1 mM EDTA, 5 μg/ml bovine serum albumin, 1 mM dithiothreitol, and 1 μg poly(dI-dC) in a reaction volume of 30 μl, also containing 5 μg of crude protein extract, 1 μl of antiserum diluted 1/1,000, and approximately 1 ng of labeled probe. Fragments were separated by electrophoresis in a 5% polyacrylamide–Tris-borate-EDTA gel, and radioactive bands were visualized with a Bio-Rad Molecular Imager FX after overnight exposure of the dried gel to a phosphor screen (Bio-Rad Laboratories). Fluorophore band intensity was analyzed with Quantity One software (Bio-Rad Laboratories).

FIG. 1. Plaque formation by *S. flexneri* under aerobic and anaerobic conditions. Henle cell monolayers were infected with 10^4 wild-type *S. flexneri* (SA100), avirulent *Crb*⁻ mutant (SA101), *feoB iucD* mutant (SA192), *feoB sitA* mutant (SM191), *iucD sitA* mutant (SA167), or *feoB iucD sitA* mutant (SM193) cells. The plates were incubated for 2 days in medium containing gentamicin under either aerobic (top) or anaerobic (bottom) conditions and stained to visualize plaque formation.

RESULTS

Roles of iron transport systems in aerobic and anaerobic plaque formation. The plaque assay, a standard measure of *S. flexneri* virulence, is performed under aerobic conditions, which may not allow assessment of factors influenced by the anaerobic environment of the colon. Therefore, we developed a modified plaque assay in which infected Henle cell monolayers were incubated anaerobically. As shown in Fig. 1, wild-type *S. flexneri* formed plaques under both aerobic and anaerobic conditions, whereas the avirulent *Crb*⁻ strain was unable to form plaques under either condition. *S. flexneri* mutants defective in one or more iron transport systems (29, 52) were also tested in this assay (Fig. 1), since we had previously shown that the *S. flexneri* *iuc*, *feo*, and *sit* iron transport systems contribute to iron acquisition in cultured cells. A mutant lacking all three of these systems, which is defective in aerobic plaque formation (52), also failed to form plaques anaerobically (Fig. 1), while all strains expressing at least two of these three iron transport systems formed plaques in either environment (data not shown). The strain with only the *Sit* transporter was also able to form plaques under both conditions, although under anaerobic conditions the plaque size was reduced compared to that of the wild type (*feo iuc* mutant [Fig. 1]). The *iuc sit* double mutant, in which only the *Feo* system is functional, formed plaques under anaerobic but not aerobic conditions. This is consistent with the ferrous iron ligand for the *Feo* system being enriched under anaerobic conditions. In contrast, the *feo sit* double mutant, which expressed only the aerobactin system, formed plaques solely under aerobic conditions, as might be expected for a ferric iron transporter. This effect of oxygen availability on the function of these iron transport systems suggested that the regulation of their genes could also be influenced by oxygen.

Regulation of iron acquisition genes by oxygen availability. Because plaque formation was influenced by the availability of

oxygen, microarray analysis was used to screen *S. flexneri* iron transport genes or other virulence genes for the effect of oxygen on expression. Wild-type cultures grown aerobically were compared with those subjected to oxygen depletion. Genes previously reported to be influenced in *E. coli* by oxygen availability or by the anaerobic transcription factor ArcA or Fnr (25, 30, 54, 55) showed the expected regulation in *S. flexneri* (Table 2). Representative genes induced aerobically included the TCA cycle loci *acnA*, *mdh*, and *lpd-ace*, the *F₁F₀* ATPase *atpCD GAHF* genes, the *ndk/nrd* aerobic ribonucleotide reductases, and genes encoding Fe-S cluster biogenesis and oxidative stress proteins. Under anaerobic conditions, the genes encoding nitrate reductase (*narGHII*) and fermentation genes (*adhE* and *pflB*) were elevated (Table 2). This indicates that the conditions used for analysis of *S. flexneri* were appropriate for detecting anaerobically induced or repressed genes. The transcription of several iron acquisition genes also responded to oxygen availability. The *feo* genes were induced anaerobically (Table 2). Conversely, the *sit* and *iuc* transcripts were more abundant aerobically (Table 2). *dps*, which has been shown to bind ferrous iron (4), was also elevated anaerobically (Table 2).

The regulatory effects detected by microarray were further analyzed with GFP reporter fusion assays (Fig. 2). Transcriptional *gfp* reporter fusions in the vector pLR29 were made using promoters upstream of the *feoABC*, *sitABCD*, and *iucABCD iutA* operons. Strains of wild-type *S. flexneri* containing these reporter fusions were grown aerobically or anaerobically to compare the relative fluorescence. The reporter data confirmed that the *iuc* and *sit* promoters were more active aerobically than anaerobically, while the *feo* promoter was stronger anaerobically (Fig. 2).

***feo* is induced by ArcA and Fnr, while ArcA represses *iuc*.** To determine whether the transcription factors ArcA and Fnr have a role in the regulation of iron acquisition genes in response to oxygen availability, *arcA* and *fnr* mutants of *S. flexneri*

TABLE 2. Transcriptional changes of selected genes in response to oxygen availability in *S. flexneri*

Gene	Function	Fold change ^a		
		Range	Avg	SD
Transcripts elevated aerobically				
O ₂ -regulated metabolic genes				
<i>ndk</i>	Aerobic ribonucleotide reductase	1.0–2.8	1.8	0.7
<i>nrdA</i>	Aerobic ribonucleotide reductase	1.6–2.2	2.0	0.3
<i>nrdI</i>	Aerobic ribonucleotide reductase	1.7–2.6	2.1	0.4
<i>atpC</i>	F ₁ F _o ATPase	2.5–3.2	2.8*	
<i>atpD</i>	F ₁ F _o ATPase	2.1–3.1	2.7	0.4
<i>atpG</i>	F ₁ F _o ATPase	1.5–2.4	1.9	0.4
<i>atpA</i>	F ₁ F _o ATPase	1.8–3.6	2.7	0.8
<i>atpH</i>	F ₁ F _o ATPase	2.3–5.3	3.8	1.5
<i>atpF</i>	F ₁ F _o ATPase	2.0–5.3	3.1	1.9
<i>aceE</i>	Pyruvate dehydrogenase	4.4–6.7	5.5*	
<i>lpdA</i>	Pyruvate dehydrogenase	3.0–9.1	5.7	3.1
<i>acnA</i>	Aconitase	2.0–7.5	4.8*	
<i>mdh</i>	Malate dehydrogenase	3.8–8.5	5.8	2.4
O ₂ -regulated stress response genes				
<i>sufE</i>	Fe-S cluster formation	1.4–2.8	2.2	0.7
<i>sufS</i>	Fe-S cluster formation	1.1–3.2	2.2	0.9
<i>sufD</i>	Fe-S cluster formation	1.0–3.0	2.1	0.9
<i>sufC</i>	Fe-S cluster formation	1.4–3.0	2.2	0.6
<i>sufA</i>	Fe-S cluster formation	1.3–2.4	1.8	0.5
<i>iscA</i>	Fe-S cluster formation	1.0–3.6	2.5	1.3
<i>iscU</i>	Fe-S cluster formation	2.6–15.3	9.0*	
<i>iscR</i>	Fe-S cluster formation	1.9–20.4	8.2	10.5
<i>sodA</i>	Superoxide dismutase	3.1–16.3	9.7*	
<i>tpx</i>	Oxidative stress response	3.3–8.9	5.8	2.9
<i>zwf</i>	Oxidative stress response	1.2–2.8	1.9	0.7
O ₂ -regulated iron transport genes				
<i>iucA</i>	Aerobactin synthesis	3.3–7.0	4.8	1.6
<i>iucB</i>	Aerobactin synthesis	2.5–3.9	3.0	0.7
<i>iucC</i>	Aerobactin synthesis	2.3–4.2	3.2	1.0
<i>iucD</i>	Aerobactin synthesis	4.2–6.1	5.3	0.7
<i>iutA</i>	Aerobactin transport	3.8–9.4	6.2	2.5
<i>sitA</i>	Fe and Mn acquisition	2.4–5.7	3.9	1.1
<i>sitB</i>	Fe and Mn acquisition	3.0–7.2	4.5	1.6
<i>sitC</i>	Fe and Mn acquisition	2.6–6.1	4.1	1.5
<i>sitD</i>	Fe and Mn acquisition	2.7–4.5	3.6	0.8
Transcripts elevated anaerobically				
O ₂ -regulated metabolic genes				
<i>adhE</i>	Alcohol dehydrogenase	1.9–3.5	2.6	0.7
<i>pflB</i>	Pyruvate formate lyase	1.7–3.0	2.4	0.5
<i>hypA</i>	Hydrogenase maturation	1.4–3.3	2.5	1.0
<i>hypB</i>	Hydrogenase maturation	1.5–3.6	2.7	1.1
<i>hypC</i>	Hydrogenase maturation	1.9–3.5	2.5	0.9
<i>narK</i>	Nitrate reduction	2.2–30.5	12.2	12.8
<i>narG</i>	Nitrate reduction	2.8–32.1	15.5	14.4
<i>narH</i>	Nitrate reduction	1.9–15.4	8.8	6.1
<i>narJ</i>	Nitrate reduction	2.4–15.7	9.5	6.6
<i>narI</i>	Nitrate reduction	1.6–9.5	4.9	4.1
O ₂ -regulated stress response genes				
<i>hdeB</i>	Acid response	1.3–3.0	2.3	0.7
<i>hdeA</i>	Acid response	2.0–4.1	3.1	0.8
<i>gadA</i>	Acid response	1.5–3.8	2.6	1.0
<i>dps</i>	Oxidative stress, iron-binding protein	2.1–5.7	3.8	1.4
O ₂ -regulated iron transport genes				
<i>feoA</i>	Ferrous iron transport	1.3–4.3	2.7	1.0
<i>feoB</i>	Ferrous iron transport	1.7–3.1	2.2	0.6
<i>feoC</i>	Ferrous iron transport	1.2–3.0	2.2	1.0

^a Microarrays were used to determine the transcriptional profiles of cells switched to anaerobic conditions after aerobic growth. Genes known to be oxygen regulated in *E. coli* and those that may be involved in plaque formation were analyzed. The range, average, and 1 standard deviation (SD) for the indicated gene spot on six arrays are shown. Data are shown as fold change for the anaerobic sample compared to the aerobic sample. *, only two of the arrays had spots that passed the filters; thus, the standard deviation is not shown.

FIG. 2. Effect of O_2 on expression of *gfp* fused to iron transport gene promoters. Wild-type *S. flexneri* containing plasmids carrying iron acquisition gene promoters fused to *gfp* were grown to mid-log phase in the presence or absence of oxygen, and relative fluorescence was measured. For each promoter, the condition with the highest fluorescence value was set at 100%. Experiments were performed in triplicate, and error bars represent 1 standard deviation.

were constructed and the relative expression from the *gfp* reporter fusions was determined in these strains. The activity of *iuc* promoter fusions was the same in wild-type and *fnr* strains (data not shown) but was slightly elevated in the *arcA* mutant (Fig. 3A). This suggests that ArcA represses *iuc*, and a putative ArcA box was found in the promoter sequence (Fig. 3B). In contrast, the activity of the *feo* promoter was approximately half of the wild-type level in the *arcA* and *fnr* single mutants and one-fifth of the wild-type level in the *arcA fnr* double mutant (Fig. 4A), indicating that the anaerobic induction of *feo* transcription in the wild-type strain is due to Fnr and ArcA and that their effects are additive.

The Fnr regulation of *feo* transcription was expected since

FIG. 3. Effect of anaerobiosis on expression of the *iuc* promoter. (A) Cultures were grown anaerobically to mid-log phase, and relative *gfp* expression from the *iuc* promoter of pEG6 in SA101 (WT) and MBF200W (*arcA*) was determined. The highest relative fluorescence value was set at 100%. Experiments were performed in triplicate, and error bars represent 1 standard deviation. (B) The *S. flexneri* *iucABCD iutA* chromosomal region is depicted, showing the relative positions of the Fur box, Shine-Dalgarno sequence (SD), and putative ArcA box. The putative ArcA box sequence in *iuc* is shown below the map, with bases matching the ArcA box consensus sequence in black and bases not matching the consensus in gray. Lowercase letters indicate bases not conserved in the ArcA box weight matrix.

FIG. 4. Anaerobic expression of native and altered *feo* promoters. (A) Cultures were grown anaerobically to mid-log phase, and relative *gfp* expression from the *feo* (pMB*feo*) and *feo*_{Alt} (pMB*feo*_{Alt}) promoter fusions in SA101 (WT), MBF100W (*fnr*), MBF200W (*arcA*), and MBF300W (*arcA fnr*) was determined. The highest relative fluorescence value was set at 100%. Experiments were performed in triplicate, and error bars represent 1 standard deviation. (B) The *S. flexneri* *feoABC* chromosomal region shows the relative positions of the Fur box, Shine-Dalgarno sequence (SD), and putative ArcA box and Fnr box. The sequences resembling the putative ArcA box in the native and altered *feo* promoters are indicated below, with bases matching those of the putative ArcA box consensus sequence in black, bases not matching the consensus in gray, and bases changed in the altered promoter underlined. Lowercase letters indicate bases not conserved in the ArcA box weight matrix.

induction of the *feo* promoter under anaerobiosis was shown previously to be Fnr dependent in *E. coli*. The *S. flexneri* *feo* promoter sequence is identical to that of *E. coli* *feo*, including a site with near identity to the Fnr consensus sequence (TTGAT[n₄]ATCAA) (24). However, ArcA regulation of the *feo* promoter has not been shown previously. Although consensus sequences for the ArcA recognition site have been reported in the literature, the optimal ArcA binding sequence is not known (13, 14, 30, 32, 39, 40). Using parameters from one of these predictions (14) and adding sequences from newly identified ArcA binding sites, we generated an ArcA consensus sequence (Fig. 5) and used this sequence to identify a putative ArcA binding sequence in the *feo* promoter (Fig. 4B). To

FIG. 5. Predicted ArcA regulatory motif. Sequences of ArcA-regulated promoters were entered into the SeSiMCMC interface (<http://favorov.imb.ac.ru/SeSiMCMC/>), and the algorithm reported a conserved weight matrix for ArcA sequence recognition. The sequence logo was obtained by entering the weighted matrix derived from a multiple sequence alignment into the interface at <http://weblogo.berkeley.edu/>.

FIG. 6. ArcA-dependent repression of *fur* transcription under anaerobic conditions. (A) *S. flexneri* SA100 (WT) with pCC1 (vector) and MBF200 (*arcA*) with pCC1 (vector) or pMBarcAccQE (pArcA) were grown to mid-log phase anaerobically with 1 μ M IPTG. The level of *fur* mRNA was determined by RT-PCR. Experiments were performed in triplicate, and error bars represent 1 standard deviation. (B) The relative *gfp* expression levels obtained from *fur* (pMB*fur*) and *furAlt* (pMB*furAlt*) promoters in SA101 (WT) and MBF200W (*arcA*) are shown with the highest relative fluorescence value set at 100%. Experiments were performed in triplicate, and error bars represent 1 standard deviation. (C) The *S. flexneri* *fur* chromosomal region shows the relative positions of promoter elements involved in *fur* regulation, including the putative ArcA boxes, OxyR box, Crp box, and Fur box. The two putative ArcA boxes are indicated by the arrows, and their sequences are shown below. The bases in the native and altered *fur* promoters matching those of the putative ArcA box consensus sequence are shown in black. Bases not matching the consensus are in gray, and bases changed in the altered promoter are underlined. Lowercase letters indicate bases not conserved in the ArcA box weight matrix.

determine whether this site is important for ArcA regulation of *feo*, several bases were changed to the least-common bases found at those positions in the weight matrix of the ArcA box consensus sequence. The activity of the altered *feo* promoter (*feoAlt*) was similar in wild-type and *arcA* strains (Fig. 4A), indicating that ArcA did not stimulate expression from the altered *feo* promoter. This confirms that ArcA stimulates *feo* transcription and that the sequences changed in the *feoAlt* promoter are required for stimulation. The activity of the altered *feo* promoter in the *fur* mutant was reduced to approximately half its level in the wild-type strain (Fig. 4A), which indicates that Fnr binding was not disrupted by the base changes. This is consistent with the sequence prediction that the Arc and Fnr binding sites do not overlap (Fig. 4B).

The *sit* promoter fusion was also examined for ArcA regulation. The average relative *gfp* expression of the reporter fusion in the *arcA* mutant grown under aerobic conditions (0.98 ± 0.01) was the same as under anaerobic conditions (0.98 ± 0.2), indicating that the higher level of expression seen under aerobic conditions in the wild type (Fig. 2) required ArcA. However, no sequence matching the ArcA consensus was identified in the *sit* promoter region, suggesting that an effect of ArcA on *sit* expression might not be direct.

ArcA represses transcription of *fur*. Since several iron acquisition genes were regulated in response to oxygen availability (Table 2), the effect of ArcA and Fnr on transcription of *fur*, which encodes the regulator of iron transport genes, was de-

termined. Real-time RT-PCR was performed with RNA isolated from anaerobically grown wild-type *S. flexneri* and the *arcA*, *fnr*, and *arcA fnr* mutants. While there was no difference in the level of *fur* mRNA between the wild type and the *fnr* mutant (data not shown), there was a significant increase of the *fur* transcript in strains lacking *arcA* (Fig. 6A), suggesting that *fur* transcription was repressed in an ArcA-dependent manner. *fur* repression was restored in the *arcA* strain by inducing *arcA* expression from a plasmid (Fig. 6A).

To confirm that ArcA regulates *fur* promoter activity, a *gfp* reporter fusion to the *fur* promoter was constructed and the relative fluorescence of *gfp* was determined in the wild-type and *arcA* mutant strains. Relative to the wild type, the level of GFP was elevated in the *arcA* mutant (Fig. 6B), indicating that this promoter was negatively regulated by ArcA. Several bases matching the ArcA box consensus sequence in the *fur* promoter were then changed to the least-common bases occurring at those positions in the weight matrix derived from ArcA boxes of known ArcA-regulated promoters (*furAlt* [Fig. 5 and Fig. 6C]). The activity of the altered *fur* promoter was higher than that of the native *fur* promoter in the wild-type ArcA⁺ strain, and the difference between the amount of GFP in the *arcA* strain relative to the wild type was significantly decreased (Fig. 6B). These results were consistent with ArcA repression of *fur* transcription and implicated the altered bases in contributing to ArcA binding at this promoter.

FIG. 7. ArcA binds *feo*, *fur*, and *iuc* promoters. *S. flexneri* protein extracts prepared from strain MBF200 (*arcA*) containing either pCC1 vector (*ArcA*⁻) or pMBarcAccQE (*ArcA*⁺) were incubated with the indicated radiolabeled probes and anti-ArcA antibody. Samples were electrophoresed on a 5% polyacrylamide gel. Positions of probes (*) and supershifted bands (brackets) are indicated.

ArcA binds the *iuc*, *feo*, and *fur* promoters. The presence of putative ArcA binding sites in the *fur*, *iuc*, and *feo* promoters suggested that the observed regulation of these genes by oxygen availability was mediated directly by ArcA. To detect binding of ArcA to the *feo*, *iuc*, and *fur* promoters, anti-ArcA antibody supershift assays were performed (Fig. 7). The *lld* (*lct*) promoter, which contains a known ArcA binding site (32), was used as a positive control. Incubation of the *lld*, *iuc*, *feo*, or *fur* promoter with an extract containing ArcA and anti-ArcA antibody slowed electrophoretic mobility (Fig. 7). Incubation of the probes with extract from the *arcA* mutant did not affect mobility of the radiolabeled probe (Fig. 7). The altered *feo* and *fur* probes were also tested, and as predicted from the *gfp* expression results, the altered probes showed reduced anti-ArcA-dependent supershifting (Fig. 7; compare *feo* with *feo*_{Alt} and *fur* with *fur*_{Alt}). Some binding of ArcA to the altered probes was still observed, particularly with *fur*_{Alt}. The amount of probe in the supershifted bands was quantified by measuring the radioactivity; the mean counts in the supershifted region

were 3,910 for the native *feo*, compared with 1,511 for *feo*_{Alt} and 1,918 for *fur*, compared with 1,607 for *fur*_{Alt}. The relatively small effect of changing the *fur* promoter may reflect the fact that there are regions with homology to the consensus ArcA box on both DNA strands of the *fur* probe. The base changes introduced alter each site differently, and there may be residual ArcA binding to one or both sequences on the altered *fur* probe. ArcA did not bind the *sit* probe, as indicated by the lack of supershifting (Fig. 7), which was in agreement with our failure to find an ArcA box sequence in the promoter. These data demonstrate that ArcA directly binds the *feo*, *iuc*, and *fur* promoters and that the altered bases of *feo* are within the region required for DNA recognition by ArcA.

***S. flexneri* transcription factors ArcA, Fnr, and Fur impact plaque formation in Henle cell monolayers.** The expression of iron acquisition genes is regulated by ArcA, Fnr, and Fur, and since iron uptake is required for plaque formation by *S. flexneri*, we assessed the importance of these regulators in aerobic and anaerobic plaque assays. The *arcA* and *fnr* single mutants formed plaques under anaerobic conditions (Fig. 8A). In the absence of oxygen, these mutants also formed plaques but there was a slight reduction in plaque size (Fig. 8A). These data indicate that ArcA and Fnr exhibit a partial redundancy in the ability to regulate one or more of the steps required for plaque formation. In contrast, the *arcA fnr* double mutant was able to form plaques only in the presence of oxygen (Fig. 8A). Additionally, the double mutant formed plaques when the infected Henle cells were incubated aerobically for 2 days following the usual period of anaerobic incubation (data not shown). This indicates that under anaerobic conditions, the double mutant could invade and remain viable inside epithelial cells but was defective in intracellular growth or cell-to-cell spread. Since these transcription factors influence iron acquisition, the defective anaerobic plaque formation by *arcA fnr* mutants may reflect reduced *feo* expression and aberrant expression of *fur*.

FIG. 8. Fur and the anaerobic regulators ArcA and Fnr are important for plaque formation by *S. flexneri*. Henle cell monolayers were infected with 10^4 CFU of the indicated *S. flexneri* strain. The plates were incubated for 2 days in medium containing gentamicin under either aerobic (A) or anaerobic (B) conditions and stained to visualize plaque formation. (A) SA100 (WT) and mutants MBF100 (*fnr*), MBF200 (*arcA*), and MBF300 (*arcA fnr*). (B) SM100 (WT), SM1301 (*fur*), or SM100/pMBfurQE (WT/*pfur*) incubated with or without 50 μ M IPTG to induce *fur* expression.

The effect of altered *fur* expression on the ability of *S. flexneri* to form plaques was then investigated. An *S. flexneri* *fur* mutant and the wild-type strain expressing *fur* from an IPTG-inducible plasmid were assayed for aerobic and anaerobic plaque formation in the Henle cell model of infection. Overexpression of *fur* prevented plaque formation, while a mutation in *fur* reduced the number of plaques formed (Fig. 8B). The effects of altered expression of *fur* on plaque formation occurred in both aerobic and anaerobic environments, indicating that proper regulation of *fur* is critical for the virulence of *S. flexneri* regardless of oxygen availability.

DISCUSSION

Studies on the growth of *Shigella* spp. in response to conditions encountered within the host or in model systems have shed light on genes required for initiating and sustaining infection. *S. flexneri* virulence is typically assayed in epithelial cell monolayers to determine its proficiency in adherence, invasion, intracellular growth, and intercellular spread (43). Because tissue culture models are typically performed under aerobic conditions, while the lumen of the colon is considered to be anaerobic, we developed a plaque assay model of *S. flexneri* pathogenesis to measure *S. flexneri* plaque formation in an anaerobic environment. This model of infection may be of value in assessing mutants of *S. flexneri* and other enteric pathogens, since it measures aspects of virulence under conditions more like those encountered in vivo.

The anaerobic plaque assay demonstrated that the Feo system is an important iron transporter for anaerobic iron acquisition by *S. flexneri* in epithelial cells. The lack of ability of the Feo system to sustain aerobic plaque formation likely reflects its reduced expression when oxygen is abundant. The Feo iron transport system has recently been shown to be important for intracellular survival or virulence of a number of pathogenic bacteria, including *Legionella pneumophila* (5, 48, 54), *Campylobacter jejuni* (41), *Helicobacter pylori* (63), and *S. enterica* serovar Typhimurium (3, 62).

Not all environments encountered by *S. flexneri* have limited oxygen availability; therefore, aerobically expressed iron transport systems may also be important for the virulence of *S. flexneri*. Under aerobic conditions, the Sit system was sufficient for wild-type plaque formation. The genes of the Sit system are widespread among enteroinvasive pathogens (52). Further, the *S. flexneri* *sit* genes have been shown to be derepressed upon entry into epithelial cells (51). Thus, this system may be important during intracellular replication. In addition to its role in iron acquisition, the Sit system is able to transport manganese (26), which correlates with increased survival of *S. flexneri* in macrophages and under oxidative stress conditions (50). Thus, the prevalence of the *sit* genes among enteroinvasive pathogens may reflect its role in both iron and manganese acquisition.

The strain that expressed only the aerobactin system, which transports Fe^{3+} , formed small plaques aerobically and failed to form plaques anaerobically. This is in agreement with our previous findings demonstrating that the aerobactin locus is repressed intracellularly (18). This suggests that the siderophore is less critical than other mechanisms of iron transport for the intracellular growth of *S. flexneri*. The aerobactin

genes have been shown to be important for the growth of *S. flexneri* within extracellular tissues in a ligated ileal loop model of infection, however (29, 42). Thus, expression of siderophore iron acquisition systems is likely related to their role during certain aerobic, extracellular stages of pathogenesis.

The availability of oxygen is expected to influence iron acquisition in several ways. The amount of available ferric and ferrous iron is affected by oxygen, with the ferric form predominating when oxygen is present and ferrous iron more abundant in anaerobic environments. Additionally, the activity of enzymes such as the oxygen-requiring lysine/ornithine *N*-mono-oxygenase (IucD), which is necessary for aerobactin biosynthesis, would also be reduced in the absence of molecular oxygen (64). Consistent with these expectations, the promoters of the *iucABCD iutA* operon and *fur* were repressed anaerobically by ArcA, while the *feoABC* promoter, previously shown to be induced by Fnr in *E. coli*, was induced by both Fnr and ArcA in *S. flexneri*. Although expression of the *sit* operon was elevated aerobically, its promoter did not appear to be directly regulated by ArcA or Fnr. A study of transcriptional regulation of the *Salmonella* *sit* locus also demonstrated that its expression increased under aerobic conditions independently of ArcA and Fnr; both Fur and MntR contributed to the reduction in *sit* transcription under anaerobic conditions (20).

Our data agree with studies indicating regulation of *fur* and iron acquisition genes by anaerobiosis and ArcA in *E. coli* (7, 25, 30, 54, 55). Liu and De Wulf (30) identified putative ArcA boxes upstream of the *E. coli* *feo* and *fur* operons, and the *fur* transcript, but not the *feo* transcript, was derepressed in the *arcA* mutant. Our studies provide direct evidence that ArcA represses *fur* and confirm ArcA involvement in the regulation of *feo* and siderophore biosynthesis and transport genes. These data support a role for oxygen availability as a general signal for regulation of iron acquisition.

Since anoxic conditions and the anaerobic regulators ArcA and Fnr impact the virulence of several enteric pathogens, it was not surprising that mutations in these genes affected anaerobic plaque formation. However, the transition to anaerobiosis did not appear to regulate the expression of the virulence genes found on the *Shigella* virulence plasmid (Table 2 and data not shown). Because ArcA and Fnr are pleiotropic transcription factors with overlapping functions, it is impossible to attribute the lack of plaque formation by the *arcA fnr* double mutant to one specific pathway. ArcA and Fnr, however, both regulate iron acquisition on more than one level, and given the importance of iron acquisition in *S. flexneri* virulence, aberrant iron uptake in the *arcA fnr* double mutant is likely a contributing factor in the loss of plaque formation.

Factors in addition to oxygen deprivation activate ArcA (15, 33, 34, 49). Reduced quinones accumulate in the membrane when the respiratory dehydrogenases, reductases, or electron carriers are inactive due to an inadequate supply of cofactors, including iron, or a lack of substrate availability. These quinols lead to autophosphorylation of the ArcB sensor, which activates ArcA. ArcA helps the cell conserve energy and acquire ATP through substrate-level phosphorylation by down-regulating TCA cycle and aerobic respiratory enzymes and inducing fermentation genes and anaerobic respiratory complexes. ArcA may also help the cell conserve energy by derepressing the Feo ferrous iron transporter while repressing synthesis of

the more energetically expensive siderophore system. It is also likely that ArcA repression of *fur* leads to an increase in RyhB, which helps regulate iron storage and cellular metabolism (36). Interestingly, many of the targets of RyhB overlap those of ArcA and Fnr, and so an increase in RyhB may be a mechanism for additional fine-tuning of metabolic pathways during the depletion of oxygen as well as of iron.

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