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Characterization of Intracellular Growth Regulator *icgR* by Utilizing Transcriptomics To Identify Mediators of Pathogenesis in *Shigella flexneri*

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Shigella **species Gram-negative bacteria which cause a diarrheal disease, known as shigellosis, by invading and destroying the colonic mucosa and inducing a robust inflammatory response. With no vaccine available, shigellosis annually kills over 600,000 children in developing countries. This study demonstrates the utility of combining high-throughput bioinformatic methods with *in vitro* and *in vivo* assays to provide new insights into pathogenesis. Comparisons of *in vivo* and *in vitro* gene expression identified genes associated with intracellular growth. Additional bioinformatics analyses identified genes that are present in *S. flexneri* isolates but not in the three other *Shigella* species. Comparison of these two analyses revealed nine genes that are differentially expressed during invasion and that are specific to *S. flexneri*. One gene, a DeoR family transcriptional regulator with decreased expression during invasion, was further characterized and is now designated *icgR*, for intracellular growth regulator. Deletion of *icgR* caused no difference in growth *in vitro* but resulted in increased intracellular replication in HCT-8 cells. Further *in vitro* and *in vivo* studies using high-throughput sequencing of RNA transcripts (RNA-seq) of an isogenic Δ*icgR* mutant identified 34 genes that were upregulated under both growth conditions. This combined informatics and functional approach has allowed the characterization of a gene and pathway previously unknown in *Shigella* pathogenesis and provides a framework for further identification of novel virulence factors and regulatory pathways.**

Shigella species are Gram-negative, facultative intracellular pathogens that invade the human colonic epithelium (1). Invasion is dependent on a 220-kb virulence plasmid, which encodes a type three secretion system (T3SS) (2, 3). Symptoms of infection can vary from self-limiting, watery diarrhea to severe dysentery characterized by fever, cramps, and bloody diarrhea, potentially resulting in death (4). *Shigella* bacteria are easily transmitted in contaminated food and water and have a low infectious dose (10 to 100 bacteria) (5). Human populations with deficient sanitation are at increased risk of infection, including those in developing countries. There are approximately 163 million episodes of shigellosis and 1.1 million deaths annually in developing countries (6). Children under five years of age in developing countries are especially vulnerable to *Shigella* infection, with >600,000 deaths each year (6). There are four species of *Shigella*: *S. flexneri*, *S. sonnei*, *S. boydii*, and *S. dysenteriae* (7). *S. flexneri* is the most common cause of shigellosis in developing countries and consists of 14 serotypes. The specific serogroup antigens appear to be a driving force in the development of the protective immune response (8). To date, there is no commercially available vaccine for any of the *Shigella* species or serotypes (9).

Shigella infections begin with *Shigella* entry and transit through M cells to the basolateral side of the epithelium (10). Bacteria are subsequently engulfed by macrophages and use the T3SS and other plasmid-encoded factors to escape from the phagosome (11). From within the macrophage cytosol, the T3SS effector, IpaB, binds to caspase-1 and triggers apoptosis in the macrophages (12). As the infected macrophages undergo apoptosis, they release immune modulators, which cause a massive inflammatory response, characteristic of *Shigella* infection (13). Once free from the macrophage, *Shigella* again utilizes the T3SS to invade colonic epithelial cells from the basolateral side (14). From within the epithelial cells, *Shigella* secretes IcsA/VirG, which induces actin polymerization, facilitating inter- and intracellular spread (15). The presence of bacteria in the cytosol of the epithelial cells triggers the production of interleukin-8 (IL-8), which attracts polymorphonuclear cells (PMNs) (16). While the PMNs are ultimately able to clear the infection, they first contribute to destruction of the epithelial barrier and allow access of more bacteria to the basolateral side of the epithelial cells (17).

The majority of transcriptional studies in *Shigella* have focused on virulence plasmid gene expression and regulation of the T3SS (18). VirF is a transcriptional activator located on the virulence plasmid, which regulates expression of plasmids harboring the genes *virB* and *icsA* (*virG*) (19). Upon increased temperature (host entry), VirF activates transcription of *virB* (18). VirB is an additional transcriptional regulator which activates transcription of multiple genes in a 30-kb region of the plasmid, known as the "entry region" (19, 20). The entry region encodes the structural

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TABLE 1 *Shigella flexneri* 2a strains and plasmids used in this study

Strain or plasmid	Gentotype or description	Reference or source
Strains		
2457T	Wild type	52
Δ*icgR* strain	2457T Δ*icgR*::*cat*	This study
Δ*icgR*(pSECicgR) strain	Δ*icgR* mutant transformed with pSEC*icgR* (*icgR* complement)	This study
Plasmids		
pSEC10	Stable, nonantibiotic, low-copy-number plasmid	32
pSECicgR	pSEC10 expressing *icgR* from native promoter	This study
pKD46	Red recombinase, *repA101*(Ts), Ap^r	31
pKD3	Cm^r cassette flanked by FRT sites[a]	31

[a] FRT, FLP recombinase target.

components of the T3SS as well as some of the secreted effector proteins, their chaperones, and regulatory proteins. Secretion of these effectors allows entry of the bacteria into the host cell and activation of another transcriptional regulator, MxiE. From within the host cell, MxiE activates transcription of another set of effectors encoded outside the entry region including several outer *Shigella* protein (Osp) family members (21). These effectors are important for postinvasion processes such as modulating the host immune response (22).

The present study identifies genes that are transcriptionally regulated during invasion and conserved among *S. flexneri* by using a combination of bioinformatics, *in vitro*, and *in vivo* methodologies. Transcriptional analysis was performed on *S. flexneri* grown either in LB medium or within HCT-8 cells (human colonic epithelial cells). Genes that were transcriptionally regulated during *in vivo* growth in HCT-8 cells compared to *in vitro* growth were considered to be relevant for the infection process. Using the publically available *Shigella* genomes, comparative analyses identified genes that were common to *S. flexneri* but were absent in the other *Shigella* species. An understanding of *S. flexneri* pathogenesis and transcriptional networks using these data may lead to a broadly protective *S. flexneri* vaccine.

One of the genes identified to be both differentially expressed during intracellular growth and specific to *S. flexneri* was further characterized. This gene, named *icgR* for intracellular growth regulator, is a putative DeoR family transcriptional regulator. This regulator was present under both growth conditions, but its expression was decreased more than 5.5-fold *in vivo*. Deletion of this regulator resulted in the identification of 176 genes that were upregulated during *in vitro* growth and 500 genes that were upregulated during *in vivo* growth. Functional studies determined that the *icgR* deletion mutant had increased intracellular replication within HCT-8 cells compared to growth of wild-type *S. flexneri*. This approach has identified a gene and pathway previously unknown in *Shigella* pathogenesis and provides a framework for the continued identification and characterization of novel virulence factors and regulatory pathways based on genomic and transcriptomic data sets.

MATERIALS AND METHODS

Strains used in this study. Strains utilized in this study are listed in Table 1. Isolates were grown in Luria broth or tryptic soy agar with Congo red (TSA/CR) medium supplemented with the appropriate antibiotics (15 μg/ml chloramphenicol, 50 μg/ml kanamycin, and 100 μg/ml ampicillin).

Invasion assay. HCT-8 cells were grown in RPMI medium plus 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. Cells were seeded near confluence (7×10^5 cells per well) in a 12-well plate and allowed to adhere overnight. The next day, approximately 1×10^8 CFU of *Shigella* was added to each well, and samples were centrifuged for 5 min. Bacteria were allowed to invade for 90 min at 37 °C with 5% CO_2 before being washed with Dulbecco's phosphate-buffered saline (DPBS; Invitrogen) and treated with 100 μg/ml gentamicin for 30 min to kill extracellular bacteria. HCT-8 cells were then washed and lysed immediately or 3 h after gentamicin treatment with 1% Triton X-100. Serial dilutions were plated on tryptic soy agar plus Congo red to determine CFU counts. The number of CFU recovered was divided by the number of CFU in the inoculum, and the result was multiplied by 100 to calculate the percent recovery.

***In vitro* RNA extraction.** For broth cultures, several red colonies of *Shigella* bacteria were picked from a TSA/CR plate and used to in inoculate 50 ml of tryptic soy broth (TSB). Cultures were grown at 37 °C with shaking to an optical density at 600 nm (OD_{600}) of 0.2, 0.5, or 1.0. The bacterial cultures were concentrated by centrifugation, and the resulting pellets were resuspended in 10 ml of RNAlater (Ambion) and stored at −20°C. Pellets were resuspended in 10 ml of extraction buffer (50 mM Tris, pH 7.5, 100 mM LiCl, 5 mM EDTA, pH 8, 1% SDS, 1% β-mercaptoethanol) and 10 ml of 5:1 acid phenol-chloroform (55°C). This resuspended pellet was added to lysing matrix B beads (MP Biomedicals), and a FastPrep 120 instrument (MP Biomedicals) was used to lyse the bacterial cells (6 m/s, 40 s). The lysate was then centrifuged at 2,500 × *g* for 10 min, the aqueous layer was transferred to a new tube, and an equal volume of chloroform was added. Tubes were mixed by vortexing vigorously and then separated by centrifugation. The aqueous phase was transferred to a new tube, mixed with a one-third volume of 8 M LiCl, and incubated overnight at −20°C. The following day, samples were thawed and centrifuged, and the RNA pellets were resuspended in 3 ml of TE (Tris-EDTA) buffer and 1 ml of 8 M LiCl. Samples were mixed by vortexing and incubated overnight at −20°C. The following day, samples were thawed and centrifuged. The resulting pellets were washed twice with 5 volumes of 2 M LiCl at 4°C and twice more with 80% ethanol chilled to −20°C. Ethanol was allowed to evaporate, and pellets were resuspended in 200 μl of diethyl pyrocarbonate (DEPC)-treated H_2O. To degrade DNA, 10× DNase buffer and rDNase I (Invitrogen) were added to the samples; they were incubated at 37°C for 1 h, and DNase inactivation reagent was added.

***In vivo* RNA extraction.** For bacteria grown in HCT-8 cells, an invasion assay was performed. HCT-8 cells were seeded in a T-75 flask and allowed to grow to near confluence, after which 1×10^{11} CFU of *S. flexneri* 2457T was added and allowed to incubate for 90 min. Cells were washed with DPBS, and then gentamicin (100 μg/ml) was added; samples were incubated for 3 h, after which 6 ml of TRIzol reagent (Invitrogen) was added. Samples were then scraped from the flask and snap-frozen at −80°C. RNA extraction was performed according to the manufacturer's instructions. Briefly, samples were lysed using a FastPrep 120 instrument (MP Biomedicals), and 0.2 volumes of chloroform was added to the aqueous layer. RNA was then precipitated from the aqueous phase with 3 ml of isopropanol and centrifugation (2,500 × *g*). The resulting pellets were washed with 75% ethanol and resupended in DEPC-treated water. DNA was removed by addition of 10× buffer and rDNase I (Invitrogen) for 1 h at 37°C. Samples were then passed twice over an RNeasy column (Qiagen), washed according to the manufacturer's instructions, and eluted in 50 μl of DEPC-treated water. Eukaryotic and prokaryotic rRNAs were depleted prior to cDNA synthesis using a Ribo-Zero rRNA Removal Kit (Epicentre).

cDNA synthesis. cDNA was generated with SuperScript III (Invitrogen). Briefly, 10 μl of total RNA was incubated with 1 μl (50 ng) of random hexamers (Applied Biosystems) and 1 μl of 10 mM each deoxynucleoside triphosphate (dNTP; Denville) for 5 min at 65°C. Samples

were then reverse transcribed with 4 μl of 5× buffer, 2 μl of 0.1 M dithiothreitol (DTT), 1 μl of RNaseOUT, and 1 μl of SuperScript III (Invitrogen) and incubated for 10 min at 25°C, 50 min at 50°C, and 5 min at 85°C. Remaining RNA was degraded with 1 μl of RNase H at 37°C for 20 min.

Microarray. Approximately 0.8 μg of digested, labeled cDNA was hybridized per array, as described previously (23). Briefly, cDNA was digested with DNA-free DNase to a size range of between 20 and 200 bp. Digested cDNA was labeled with biotin. Labeling, hybridization, washing, and staining were completed according to Affymetrix standard protocols for prokaryotic expression samples (Affymetrix, Santa Clara, CA). Data were normalized and analyzed with the Affymetrix GCOS software, using *in vitro* or wild-type samples as the baseline comparator for corresponding *in vivo* or Δ*icgR* strain samples. Robust multichip average (RMA) analysis was performed using the simpleaffy package for R. Array features were considered to be regulated if there was a greater than 4-fold change in the expression level for *in vivo* samples or a greater than 2-fold change for Δ*icgR* strain samples. Change calls and signal log ratio data were used to select candidates for further analysis using quantitative real-time PCR (qRT-PCR).

RNA-seq. RNA isolated from *in vitro* samples was pooled from three biological replicates of wild-type and *icgR* mutant bacteria. RNA isolated from *in vivo* samples was taken from two independent replicates of wild-type and *icgR* mutant bacteria. *In vivo* RNA samples were depleted for eukaryotic mRNA using a GenElute mRNA Miniprep kit (Sigma), and rRNA was depleted using a Ribo-Zero rRNA Removal Kit (Epicentre). An Ovation Prokaryotic RNA-Seq System (NuGEN) (where RNA-seq refers to high-throughput sequencing of RNA transcripts) was used to generate cDNA from both *in vitro* and *in vivo* RNA samples, followed by library preparation with the Encore NGS Library Multiplex System I (NuGEN). Sequencing and analysis were performed as described previously (23). Briefly, libraries were sequenced with the Illumina GA-II platform (Illumina) at the Institute for Genome Sciences Genomics Resource Center (http://www.igs.umaryland.edu/grc). Reads were then mapped to the *S. flexneri* 2a strain 2457T chromosome (24) and the *S. flexneri* 2a strain 301 plasmid (25) with the Bowtie aligner (26). Counts for each annotated genomic feature were determined by HTSeq-count (http://www-huber.embl.de/users/anders/HTSeq/doc/index.html). Differential expression between counts for each feature was then calculated with DESeq (27).

BSR analysis. BLAST score ratio (BSR) analysis (28) was conducted using the genomes of *Shigella* species that were previously available in GenBank (see Table S1 in the supplemental material). The BSR analysis divides the protein query BLAST score (29) by the reference blast score to determine relatedness between peptide sequences. Genes were considered to be conserved if they had a BSR of >0.8, unique if they had a BSR of <0.4, and divergent if they had a BSR between these values.

qRT-PCR. Quantitative real-time PCR was performed using a 7900HT Real-Time PCR Machine (Applied Biosystems) (primers utilized are listed in Table S2 in the supplemental material). The cDNA samples were diluted to 10 ng in 2.5 μl and added to 5 μl of Power SYBR green PCR Master Mix (Applied Biosystems) and 2.5 μl of a 0.8 μM primer mix. Cycling parameters were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C, followed by a dissociation stage. The $\log_2$ fold change was calculated as previously described (23).

PCR screening. Genomic DNA was isolated from 78 *Escherichia coli* and 108 *Shigella* strains (66 *S. flexneri*, 14 *S. sonnei*, 21 *S. boydii*, and 7 *S. dysenteriae* isolates) and used as a template for screening by PCR. Primers deoR_F/deoR_R, rfbG_F/rfbG_R, and sfsA_F/sfsA_R (see Table S2 in the supplemental material) were designed for *icgR*, *sfsA*, and *rfbG*, respectively, using Primer3 (30). Touchdown PCR conditions were used as follows: 4 cycles of 94°C for 30 s and 68°C for 45 s, 4 cycles of 94°C for 30 s and 70°C for 45 s, 30 cycles of 94°C for 30 s and 72°C for 45 s, and a final extension at 72°C for 5 min. Amplicons were visualized by gel electrophoresis on 1% agarose gels stained with ethidium bromide on a Bio-Rad GelDoc system.

Lambda red mutagenesis. Gene deletions were performed as described by Datsenko and Wanner (31). Primers listed in Table S2 in the supplemental material were used to amplify the *cat* gene from pKD3, resulting in a product containing *cat* with 40-bp overhangs homologous to the regions flanking *icgR*. The purified PCR product was electroporated into *S. flexneri* 2457T(pKD46), and chloramphenicol-resistant colonies were screened for ampicillin sensitivity to confirm loss of pKD46 and by PCR for loss of *icgR*.

Complementation of *icgR*. Using primers picgR_F/picgR_R (see Table S2 in the supplemental material) *icgR* and 117 bp of upstream DNA were amplified and ligated into TOPO TA vector pCR2.1 (Invitrogen). This was digested with EcoRI and ligated into pSEC10. This plasmid carries the *hok-sok* genes for stable maintenance in the absence of antibiotics and is related to pSEC10S-83 (32).

***Shigella* plaque assay.** L2 mouse fibroblast cells were seeded at 1.5×10^5 cells per 60-mm dish, and 5 days later, 1×10^4 to 2×10^4 CFU of wild-type, Δ*icgR*, or *icgR* complemented bacteria were added to two dishes and incubated for 2 h at 37°C with 5% CO_2 with rocking. Bacteria were aspirated, and an agarose overlay containing 20 μg/ml of gentamicin was applied. Cells were incubated for two more days at 37°C with 5% CO_2 and then stained for 2 h with 0.5% neutral red dye. Overlays were then removed, and five plaques from each strain were measured in four directions using a Zeiss Primo Star microscope at ×10 magnification with Axiovision software.

Microarray data accession number. All microarray data for this project have been deposited in the Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/projects/geo/) under accession number GSE40851.

RESULTS

Microarray identification of transcriptionally altered genes. To examine the gene expression of *S. flexneri* 2457T *in vivo* and *in vitro*, an *E. coli/Shigella* pan-genome microarray was utilized (33). The transcriptome of *S. flexneri* 2457T grown *in vivo* within HCT-8 cells for 3 h was compared to that grown *in vitro* in LB medium to mid-log-phase growth. When the data were examined, with the criteria of having a feature on the array called present under both growth conditions, a *P* value of less than 0.05, and a fold change of greater than 4, 344 features were increased (see Table S3 in the supplemental material), and 1,062 features were decreased under the *in vivo* condition compared to *in vitro* growth (see Table S4). Genes of interest that were upregulated *in vivo* include iron storage and transport genes such as *bfr* and *sitA*. Iron acquisition is known to be important for bacterial growth (34), and *sitA* has been reported by other investigators to be upregulated during intracellular growth of *Shigella* (35, 36). Downregulated genes include O-antigen synthesis genes of the *rfb* gene cluster (37). Decreased expression of O-antigen genes has been observed during intracellular growth of both *Shigella* (36) and the related bacteria *Salmonella* (38).

Identification of *S. flexneri*-specific genes. The pan-genome microarray contains genes from representatives of each of the four *Shigella* species; however, the goal of this study was to determine those genes with changes that were restricted to *S. flexneri* in an effort to focus functional work on the most abundant disease-causing species. BLAST score ratio (28) analysis was used to identify 81 predicted genes (see Table S5 in the supplemental material) that were present in *S. flexneri* but absent in the genomes of the other *Shigella* species examined. Of these 81 *S. flexneri*-specific genes, 9 were also identified as transcriptionally altered *in vivo*, as determined by microarray (Table 2). The three genes with the greatest change in expression by microarray were selected for fur-

TABLE 2 *S. flexneri*-specific genes with altered expression during invasion

Strain 2457T gene identifier	Affymetrix identifier	Fold change (log2)	Annotation
S2558	A0384_s_at	−2.00	Hypothetical protein
S2219	S2219_s_at	−2.02	*rfc*; O-antigen polymerase
S4048	SB5_2279_s_at	−2.32	lpfC; putative long polar fimbriae
S2215	S2215_s_at	−2.63	Hypothetical protein
S4837	S4837_s_at	−2.63	Hypothetical protein
S2221	SFV_2093_s_at	−2.83	*rfbF*; dTDP-rhamnosyl transferase
S2220	SFV_2092_at	−4.43	*rfbG*; dTDP-rhamnosyl transferase
S4473	S4473_s_at	−4.73	Putative *deoR* family transcriptional regulator
S4810	SF3265_s_at	−6.49	Hypothetical protein

ther characterization. These genes included the following: *S. flexneri* 2457T gene S4810, encoding a hypothetical protein now designated *Shigella flexneri* specific gene A (*sfsA*); S4473, a putative DeoR family transcriptional regulator now designated intracellular growth regulator (*icgR*); and S2220, a gene with predicted dTDP-rhamnosyl transferase function, known as *rfbG*. The *in vivo* decreased expression, relative to *in vitro* expression, observed in the microarray was confirmed by quantitative real-time PCR (qRT-PCR) for all three genes (Fig. 1A).

Confirmation of gene prevalence in *S. flexneri* species. To confirm the specificity of these three genes in *S. flexneri*, PCR assays were developed for each gene. This additional screen was required to confirm that the identification of these genes was not a result of the limited number of genomes (eight) that were used in the BSR analysis. Primers deoR_F/deoR_R, rfbG_F/rfbG_R, and sfsA_F/sfsA_R (see Table S2 in the supplemental material) were used to screen for the presence of *icgR*, *rfbG*, and *sfsA*, respectively, using genomic DNA from 108 diverse isolates of *Shigella* (66 *S. flexneri*, 14 *S. sonnei*, 21 *S. boydii*, and 7 *S. dysenteriae*). Additionally, to determine the presence of these three genes in closely related *E. coli* isolates, the DECA collection containing 77 isolates of diarrheagenic *E. coli* was utilized (http://shigatox.net/new/reference-strains/deca.html) (39). The PCR assays identified that each of the three genes was significantly more prevalent in *S. flexneri* than in other *Shigella* species or *E. coli* (Student's *t* test, $P < 0.0005$) (Fig. 1B). While not of statistical significance, it is interesting that *icgR* was present in 32% of the *E. coli* strains screened. It is possible that the regulation by IcgR may be different in *S. flexneri* than it is in extracellular *E. coli*.

The combination of the microarray expression data and the prevalence data indicates that the methods have identified genes that are transcriptionally altered during *in vivo* growth and are specific to *S. flexneri*.

Selection of genes for functional characterization. The *icgR* gene was selected for further characterization as it had not been previously implicated in *Shigella* virulence and represented an unidentified regulatory pathway that could be exploited for potential therapeutic or vaccine development. The gene was inactivated in *S. flexneri* 2457T using the lambda red system (31) and replaced with a chloramphenicol resistance cassette (see Fig. S1A in the supplemental material). Growth curves were examined to ensure that the mutations of this regulatory pathway did not generally impact the growth of *S. flexneri*. No *in vitro* growth defect in the Δ*icgR* mutant was detected compared to growth of the wild-type 2457T (see Fig. S1B).

Growth and invasion of the wild-type and Δ*icgR* bacteria were also investigated in HCT-8 cells at 30 min and 3 h postinvasion. At 3 h, there were significantly more Δ*icgR* bacteria recovered than wild type (Fig. 2A), indicating increased intracellular growth of the Δ*icgR* mutant compared to the wild type. Complementation of the Δ*icgR* mutant from a plasmid carrying a version of *icgR* restored the wild-type phenotype (Fig. 2A). A plaque assay revealed similarly sized plaques caused by wild-type and Δ*icgR* bacteria, indicating that the increased recovery of the *icgR* mutant was not due to increased cell-to-cell spread (Fig. 2B and C).

Transcriptional impact of the deletion of the *icgR* gene *in vitro*. To examine the transcriptional impact of deleting the *icgR* gene, three *in vitro* growth stages were interrogated by microarray (early log phase, OD_{600} of 0.2; mid-log phase, OD_{600} of 0.5; and late log phase, OD_{600} of 1.0). As *icgR* is annotated as a putative DeoR family transcriptional regulator and as these regulators are typically repressors (40), it was expected that deletion of *icgR* would result in increased expression of the genes it normally represses. Compared to the wild-type transcriptome at the same three growth phases, the greatest number of array features, 65, was increased in the Δ*icgR* strain at the mid-log phase of growth (see

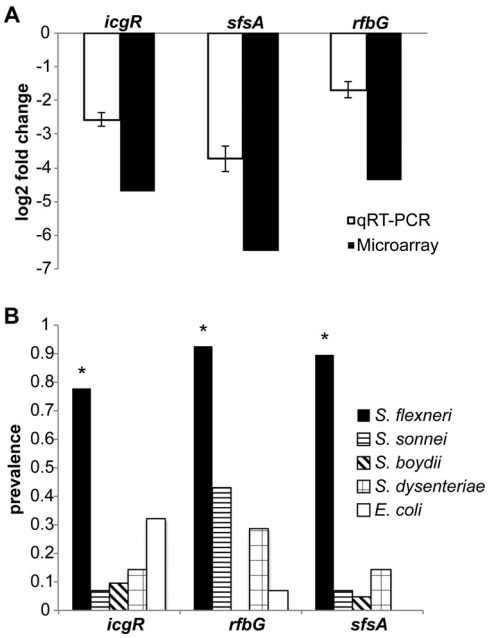

FIG 1 Expression levels of three *S. flexneri* genes are downregulated *in vivo* versus *in vitro*. (A) Expression of three *S. flexneri*-specific genes, all of which exhibited decreased expression, as determined by microarray, during intracellular growth compared to growth in broth, was verified by quantitative real-time PCR (qRT-PCR). These genes were the following: *S. flexneri* 2457T S4473, an uncharacterized member of the DeoR family of transcriptional regulators, which is now named *icgR*, for intracellular growth regulator; *S. flexneri* 2457T S4810, a hypothetical protein now named *sfsA*, for *Shigella flexneri*-specific A; and *rfbG*, which encodes a rhamnosyl transferase. (B) The prevalence of these three genes in *Shigella* species and diarrheagenic *E. coli* was determined by PCR screening. A total of 66 *S. flexneri*, 14 *S. sonnei*, 21 *S. boydii*, 7 *S. dysenteriae*, and 77 *E. coli* isolates were screened. All three genes were significantly more prevalent in *S. flexneri* than in any other species (*, $P < 0.0005$).

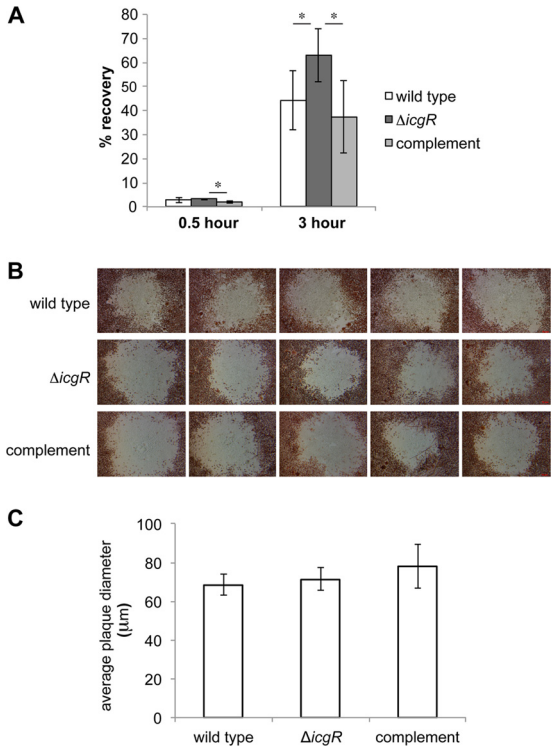

FIG 2 (A) The *S. flexneri* Δ*icgR* strain replicates to higher levels than the wild type in HCT-8 cells. Wild-type and Δ*icgR S. flexneri* strains were allowed to invade HCT-8 cells, and extracellular bacteria were killed with gentamicin (100 μg/ml for 30 min). HCT-8 cells were then lysed at 30 min or 3 h and plated for CFU counts. The number of CFU recovered was divided by the inoculum to calculate the percent recovery. Wild-type and Δ*icgR S. flexneri* bacteria invaded at similar rates at 30 min, but significantly (*, $P < 0.05$) more Δ*icgR* bacteria were recovered after 3 h of intracellular growth. Error bars represent standard deviations. (B) A plaque assay (to measure intracellular spread) was performed with the wild-type, Δ*icgR*, and *icgR* complemented bacteria. Plaques shown represent 2 days postinfection of L2 fibroblasts at ×10 magnification. (C) Average plaque diameter was calculated based on four measurements per plaque and five plaques per strain. Error bars represent standard deviations.

Fig. S2 and Table S6 in the supplemental material). For this reason, the mid-log-growth phase was chosen for further transcriptional analysis by RNA-seq. Wild-type and Δ*icgR* bacteria were grown in TSB to an OD_{600} of 0.5, and RNA was isolated for cDNA synthesis and sequencing. Over 60 million Illumina reads were obtained for each strain. Approximately 70% of the reads were mapped to the chromosome, and 18% were mapped to the virulence plasmid. Genome-wide coverage plots of each strain and fold change in gene expression are depicted for the chromosome and virulence plasmid in Fig. 3A. Consistent with the predicted function of *icgR* as a transcriptional regulator, its deletion resulted in altered expression of numerous genes throughout the genome. Following Bowtie (26) alignment and DEseq (27) analysis, 176 genes were found to be significantly upregulated, and 65 genes were found to be downregulated in the Δ*icgR* mutant compared to the wild type (see Tables S7 and S8 in the supplemental material). Twenty-six of these genes were also found to be upregulated by microarray. The altered expression of several genes was also confirmed by qRT-PCR (see Fig. S3A). The differentially expressed genes were divided into functional Clusters of Orthologous Group (COG) categories (41), as shown in Fig. 4A. Three COG categories of interest, which contained a large number of upregulated genes, were inorganic ion transport and metabolism, amino acid transport and metabolism, and energy production and conversion. The inorganic ion transport and metabolism COG includes the *bfr-bfd* operon, which was increased 4- to 8-fold. The *bfr-bfd* operon encodes bacterioferritin and bacterioferritin-associated ferredoxin, which are responsible for iron storage. The amino acid transport and metabolism category includes the *livKHMGF* operon, which was increased 2- to 4-fold. The *livKHMGF* operon encodes leucine, isoleucine, and valine transporters. The energy production and conversion group includes the *narKGHJI* and *nirDBC* operons, which were increased 2.2- to 6.7-fold and 2.6- to 3.8-fold, respectively. The *narKGHJI* operon encodes genes for nitrate reductase, and the *nirDBC* operon encodes genes for nitrite reductase.

Overall, the impact of the deletion of *icgR* during *in vitro* growth appeared to be largely metabolic in nature.

Transcriptional impact of the deletion of the *icgR* gene *in vivo*. To determine if any of the *in vitro* differentially expressed genes were also regulated during intracellular growth, where increased replication is observed in the *icgR* mutant, RNA-seq was performed on *S. flexneri* wild-type and *icgR* mutant strains grown in HCT-8 cells. At 3 h postinfection, RNA from the Δ*icgR* and wild-type bacteria was isolated. Eukaryotic messenger and rRNA were depleted prior to cDNA synthesis and sequencing. An average of 78 million Illumina reads was obtained for each sample. Approximately 13% of the reads were mapped to the chromosome, and 1.4% were mapped to the virulence plasmid. The lower percentage of reads mapping to the bacterial reference genome is likely due to the presence of eukaryotic RNA that remained even after depletion. Genome-wide coverage plots of each strain and fold change in gene expression are depicted for the chromosome and virulence plasmid in Fig. 3B. It was determined that under *in vivo* growth conditions 500 genes were upregulated and that 378 genes were downregulated in the Δ*icgR* mutant compared to the wild-type strain (see Tables S9 and S10 in the supplemental material). Increased expression of several genes was confirmed by qRT-PCR (see Fig. S3B). When classified by functional category, there were a large number of upregulated genes found in COGs associated with metabolism and energy production, including inorganic ion transport and metabolism, amino acid transport and metabolism, carbohydrate transport and metabolism, and energy production and conversion (Fig. 4B). The increased expression of genes associated with metabolism and energy production is consistent with the increased intracellular replication observed in the *icgR* mutant. There were 34 genes that were upregulated and 9 genes that were downregulated under both *in vitro* and *in vivo* conditions in the *icgR* mutant. These upregulated genes included the *bfr-bfd* operon (increased 3- to 7-fold) and *livH*, *livM*, *livG*, and *livF* of the *livKHMGF* operon (increased 3.7- to 4.5-fold).

These analyses identify different but overlapping regulons controlled by *icgR* under different environmental conditions.

DISCUSSION

The goal of this study was to use a reverse genetics approach to identify previously uncharacterized genes involved in the pathogenesis of *S. flexneri*. Nine genes were identified that are transcriptionally altered during growth within epithelial cells and well conserved in *S. flexneri*. Genes with altered expression during intracellular growth are

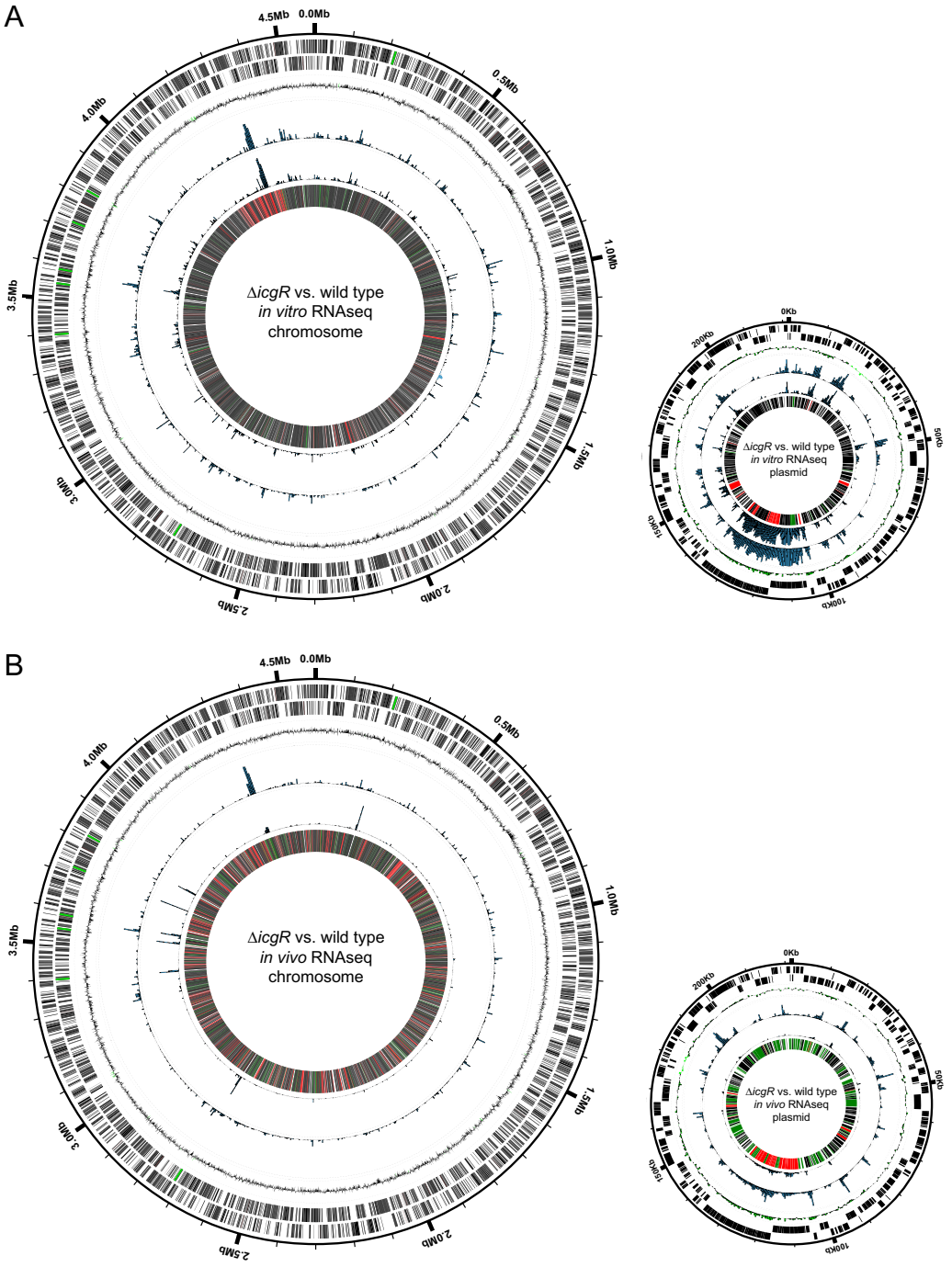

FIG 3 Global RNA-seq analysis of the Δ*icgR* strain versus the wild type *in vitro* (A) and *in vivo* (B). Circular visualization was created using Circleator (J. Crabtree et al., unpublished data). The outermost ring represents forward and reverse genes. The green ring represents GC skew. The blue histograms depict the number of mapped reads using 5-kb nonoverlapping windows for the chromosome and 500-bp windows for the plasmid. The outer histogram is wild-type reads, and the inner histogram is Δ*icgR* mutant reads. The scale for all histograms is from 0 to 8,000. The innermost ring shows the fold change in gene expression in the Δ*icgR* mutant versus the wild type. Genes with increased expression are red, and genes with decreased expression are green.

likely to have a role in pathogenesis, as replication in epithelial cells is a surrogate marker of virulence. Of note, there was a greater proportion of genes identified with a decreased transcriptional response when *in vivo* and *in vitro* transcriptomes were compared. To validate our findings, multiple genes were examined by qRT-PCR and demonstrated the same trend in expression, suggesting that the microarray data are robust. It is also noteworthy that many previously identified genes associated with *Shigella* virulence are downregulated during intracellular growth, including genes from the entry region of the virulence plasmid (*ipa*, *mxi*, and *spa* genes) as well as several *osp* genes, *virA*, and *icsA* (*virG*). While this result was initially unexpected, it is consistent with a previously reported microarray study in HeLa

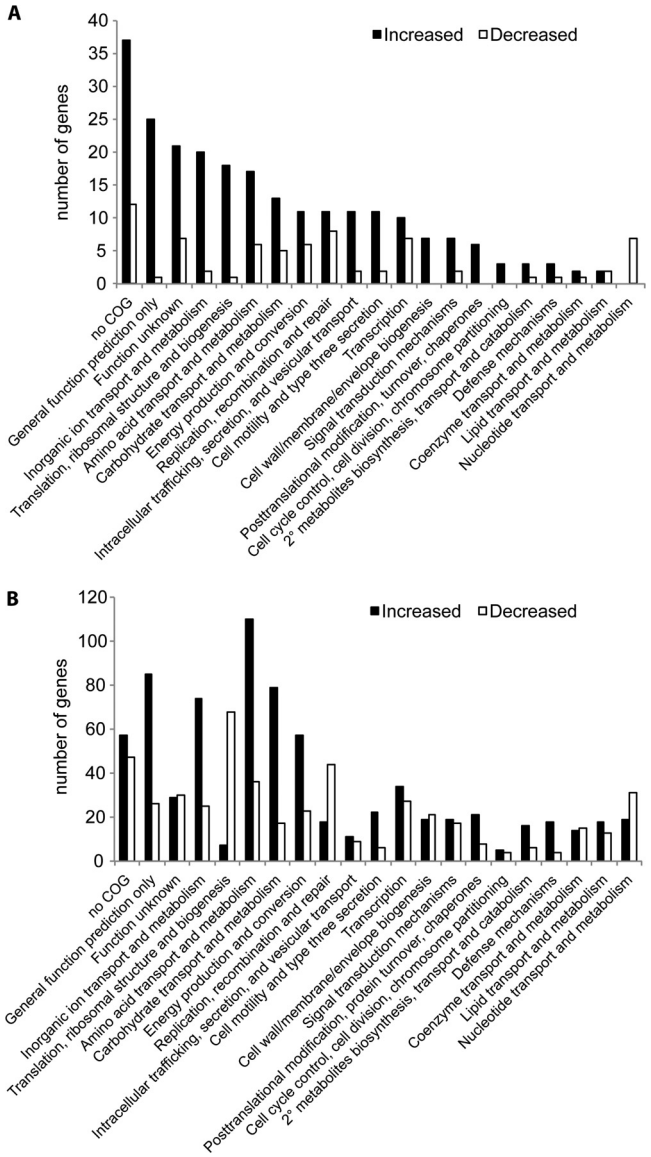

FIG 4 RNA-seq analysis of the Δ*icgR* strain versus wild-type bacteria grown *in vitro* and *in vivo*. (A) Δ*icgR* and wild-type cultures were grown in broth to mid-log phase (OD_{600} of 0.5). RNA-seq analysis identified genes that were increased or decreased more that 2-fold in the *icgR* mutant versus the wild type. These genes are classified according to Clusters of Orthologous Groups (COG), and the number of genes increased or decreased in each COG category is presented. (B) Δ*icgR* and wild-type bacteria were grown in HCT-8 cells for 3 h. RNA-seq analysis identified genes that were increased or decreased more that 2-fold in the *icgR* mutant versus the wild type, and the number genes increased or decreased in each COG is presented.

and U937 cells, which also found the *ipa-mxi-spa* locus, *ospB*, *ospF*, *virA*, and *icsA* (*virG*) to be decreased during infection (36). As Lucchini et al. hypothesized, it is likely that while these transcripts are decreased during intracellular growth, they are still present at levels sufficient for intracellular replication and spread (36).

With the ultimate goal of expanding the current understanding of *Shigella* pathogenesis, genes that were differentially expressed during invasion and present in a significant proportion of *S. flexneri* strains were examined. BLAST score ratio (BSR) analysis took advantage of the eight publicly available *Shigella* genomes to identify genes that were present in all of the *S. flexneri* genomes but absent in the genomes of other *Shigella* species. This analysis was extended by screening for three of the genes identified as *S. flexneri* specific by BSR (*icgR*, *rfbG*, and *sfsA*) in 108 isolates of *Shigella* and 78 isolates of diarrheagenic *E. coli* using focused PCR assays. This screening confirmed that all three genes had increased prevalence in *S. flexneri* and were not significantly present in other species of *Shigella* or pathogenic *E. coli*. Furthermore, the *Shigella* collection contained isolates of 11 different *S. flexneri* serotypes, and *icgR* was present in at least one isolate of each serotype (see Fig. S4 in the supplemental material).

As *icgR* was previously uncharacterized in terms of a role in pathogenesis and is predicted to have conserved protein domains, it was further characterized in this study. The *icgR* gene was originally annotated as a putative DeoR family transcriptional regulator (24). Members of this family of transcriptional regulators often act as repressors (40). They contain a helix-turn-helix DNA binding domain and a sugar phosphate isomerase domain. The canonical family member in *E. coli*, *deoR*, regulates genes encoding enzymes required for deoxyribonucleoside catabolism, and synthesis of these genes is stimulated by the presence of deoxyribose-5-phosphate (42). DeoR represses transcription by binding to DNA (as an octamer) at two distant regions to form a loop (42). DeoR family members have been characterized in a variety of both Gram-positive and Gram-negative bacteria, including *Corynebacterium glutamicum* (43), *Bacillus subtilis* (44), *Pseudomonas aeruginosa* (45), *Escherichia coli* (42), and *Salmonella enterica* serovar Typhi (46). Haghjoo and Galán demonstrated that a DeoR family member, IgeR, was involved in virulence of *Salmonella* Typhi (46). As with our observation of *icgR* in *Shigella*, the *Salmonella igeR* gene was also downregulated during intracellular growth, and overexpression of *igeR* led to a decrease in colonization in a mouse model (46). An *igeR* mutant had increased expression of several genes, including *pagC* (PhoP/PhoQ-activated gene required for virulence), *cdtB* (cytolethal distending toxin active subunit), and *yciG* (a gene of unknown function whose expression is altered by bile) (46). The similarities between *igeR* and *icgR* suggest that some DeoR family transcriptional regulators may play a role in virulence.

The *icgR* gene in *Shigella* was further characterized by first examining the transcriptional impact of its deletion. The loss of *icgR* resulted in increased expression of 176 genes and decreased expression of 65 genes during *in vitro* growth and increased expression of 500 genes and decreased expression of 378 genes during *in vivo* growth. The larger number of genes with increased expression in the *icgR* mutant suggests that IcgR is a repressor, like other DeoR family transcriptional regulators (40). Of particular interest was the increased expression of *livJ in vivo*, *livK in vitro*, and *livHMGF* under both growth conditions. LivJ is a leucine, isoleucine, and valine binding protein, which is part of the LIV-I transport system, and LivK is a leucine-specific binding protein, which is part of the LS transport system. LivHMGF are membrane components that are shared between both systems (47). Previous studies have demonstrated that *liv* mutants in *Campylobacter jejuni* are unable to colonize chicks (48) and that *liv* mutants in *Streptococcus pneumoniae* are attenuated in mice (49). These studies suggest a role for leucine, isoleucine, and valine transporters in *in vivo* growth and pathogenesis. The increased expression of *livJ* and *livHMGF* observed in the *icgR* mutant during intracellular growth

may be responsible for the increased intracellular replication of this mutant.

Also of note during *in vivo* growth were the 28 upregulated genes that were associated with the COG category inorganic ion transport and metabolism. Specifically, these genes were often involved with iron acquisition and storage. These included siderophores such as enterobactin and aerobactin, iron storage proteins like bacterioferritin, and the ferrous iron transporter encoded by *sitABCD*. Previous studies have demonstrated that single deletions of enterobactin, aerobactin, or Sit in *Shigella* did not produce defects during invasion or intracellular replication (34). While a deletion of bacterioferritin has not been examined in *Shigella* to date, deletion in *S. enterica* serovar Typhimurium did not affect virulence or bacterial burden in mice (50). Based on these studies, it is not expected that the increased expression of iron acquisition genes in the *icgR* mutant is contributing to increased replication but is perhaps a response to iron stress.

The transcriptional data suggest that IcgR, like other DeoR family regulators, acts as global repressor; however, the DNA sequence it recognizes and the precise mechanism of repression remain to be determined. For multiple, previously characterized DeoR family regulators, a phosphorylated sugar molecule acts as an inducer, and the oligomeric repressor binds two distant regions of DNA to form a loop (51). Further study of this mechanism and regulatory network may yield insight into potential therapeutic or vaccine targets and is ongoing.

This study demonstrates the feasibility of identifying previously uncharacterized genes affecting intracellular growth, a surrogate measure of virulence, in *S. flexneri* from transcriptomic and genomic data sets. While the function of one previously uncharacterized gene that was both differentially expressed and *S. flexneri* specific was investigated, there were several other potential target genes identified. Characterization of these genes may reveal additional roles in *Shigella* pathogenesis. This novel approach provides a framework for the further identification and characterization of new virulence factors and regulatory pathways based on genomic and transcriptomic data sets in other species.

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