Characterization of Acr2, an H-NS-like protein encoded on A/C2-type plasmids

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Abstract

Conjugation plays an important role in the horizontal movement of DNA between bacterial species and even genera. Large conjugative plasmids in Gram-negative bacteria are associated with multi-drug resistance and have been implicated in the spread of these phenotypes to pathogenic organisms. A/C plasmids often carry genes that confer resistance to multiple classes of antibiotics. Recently, transcription factors were characterized that regulate A/C conjugation. In this work, we expanded the regulon of the negative regulator Acr2. We developed an A/C variant, pARK01, by precise removal of resistance genes carried by the plasmid in order to make it more genetically tractable. Using pARK01, we conducted RNA-Seq and ChAP-Seq experiments to characterize the regulon of Acr2, an H-NS-like protein. We found that Acr2 binds several loci on the plasmid. We showed, *in vitro*, that Acr2 can bind specific promoter regions directly and identify key amino acids which are important for this binding. This study further characterizes Acr2 and suggests its role in modulating gene expression of multiple plasmid and chromosomal loci.

1. INTRODUCTION

The IncA/C plasmid type has spread globally and is now a major contributor to multidrug

resistance in enteric pathogens (1–4). This plasmid type is modular in nature, containing several variable regions encoding antimicrobial resistance mechanisms and a conserved backbone of core genes for plasmid replication and maintenance (2, 4–6). Recent work characterized several transcriptional regulators encoded by the core backbone (7). These regulators were found to regulate conjugative transfer of IncA/C plasmids. Interestingly, one of the negative regulators, Acr2, is similar to H-NS, a chromosomally encoded, global transcriptional regulator (2).

There are several nucleoid-associated proteins encoded by *E. coli*. H-NS is among the most well studied (8). H-NS binds preferentially to DNA with a low G + C content (9). These sequences serve as sites of initial binding. After these initial interactions, other copies of H-NS can bind to each other via protein-protein interactions, as well as the DNA, forming large complexes (9, 10). This is achieved through two distinct domains. The N-terminus of H-NS contains an oligomerization domain and the C-terminus contains a DNA binding domain (11). Primarily, H-NS binding results in silencing of adjacent gene transcription (12, 13).

Chromosomal copies of H-NS play an important role in regulation of horizontally acquired DNA, such as pathogenicity islands (14–16). In some cases, mobile elements encode their own copies of H-NS homologs (17–19). These horizontally encoded homologs of H-NS have been shown to antagonize the binding of the chromosomal copies of H-NS to horizontally acquired DNA (13, 20). These antagonistic H-NS homologs have been found only on genomic islands of pathogenic *E. coli*, which is

intuitive, given their specific function. There have been a few studies focusing on plasmid encoded H-NS homologs (17, 18, 21, 22). H-NS homologs from plasmid pSfR27 and pCAR (Sfh and Pmr, respectively) seem to play roles in regulating a diverse set of genes, some of which are regulated by the chromosomally encoded H-NS copies (17, 18, 22). It has been proposed that uncontrolled expression of these genes, caused by plasmid acquisition, could lead to a reduction in fitness and subsequent loss of the plasmid from the population. The H-NS homolog encoded on the R27 plasmid of *E. coli*, H-NS_{R27}, has been shown to directly interact with the plasmid's origin of replication, *oriT* and other transfer associated genes to regulate conjugation (21). H-NS_{R27} was shown to be involved in an intricate interplay of chromosomally encoded H-NS homologs to thermally regulate the expression of the conjugative transfer apparatus of R27. These recent studies exemplify the diverse roles these plasmid encoded H-NS homologs serve. Given how widely distributed these homologs are amongst plasmid types, the true diversity of roles for H-NS homologs is unknown (23).

In this study, we characterize Acr2, an H-NS-like protein that was found to negatively regulate conjugative transfer. RNA-Seq and ChAP-Seq (Chromatin Affinity Precipitation-Seq) were used to characterize Acr2 binding sites and regulatory network. We show that Acr2 binds multiple loci on the plasmid, specifically in regions of transfer genes and transposons carried by the plasmid. Additionally, we found that Acr2 binds several loci on the host bacterial chromosome and may directly alter host gene expression. Our sequence analysis indicates that Acr2 shares a DNA binding motif with that of other H-NS homologs and using site-directed mutagenesis we demonstrate that

these amino acids are critical for its function as a repressor of conjugation.

2. MATERIALS AND METHODS

2.1 Bacterial strains, plasmids and growth conditions.

The bacterial strains and plasmids are listed in Table 1. All strains were routinely grown in Difco™ Luria-Bertani (LB) broth or LB agar at 37° C unless otherwise noted. Broth cultures were grown in a shaking incubator at 37° C with shaking (200 RPM) unless otherwise noted. Supplementation of ampicillin (Amp, 100 µg/mL), chloramphenicol (Cm, 20 µg/mL), nalidixic acid (Nal, 30 µg/mL), kanamycin (Kan, 100 µg/mL), rifampicin (Rif, 100 µg/mL) and tetracycline (12.5 µg/mL) were used as needed. Counter-selections were done using M9 minimal agar was supplemented with 0.2% rhamnose. Arabinose induction of pBAD22 vector constructs was achieved with a final concentration of 0.02 %. X-gal was added to agar plates at a concentration of 40 µg/mL. Diaminopimelic acid (DAP) was add to a final concentration of 300 µM to facilitate growth of WM3064.

2.2 Strain construction via recombineering.

All deletions and mutations were done via λ-Red mediated recombination (recombineering) with some variations. In order to delete the resistance genes from pAR060302, strain DY331 was used because it expresses recombineering genes from the chromosome and not from a plasmid which must be selected for. To ease transfer of pAR060302, it was moved into a nalixidic acid resistant variant of strain WM3064, which is a DAP auxotroph. To obtain a nalidixic acid resistant WM3064, it was grown at

37° C with shaking (200 RPM) in LB broth supplemented with DAP overnight. This culture was used to inoculate a new 10 mL LB+Dap broth culture, which was incubated for 4 hours at 37° C with shaking (200 RPM). The cells were pelleted by centrifugation at 8000 RPM for 10 minutes. The pellet was resuspended with 100 µL of LB broth and used to inoculate an LB agar plate supplement with Nal and Dap to select a spontaneous nalidixic acid resistant mutant (WM3064nalR). This plate was incubated at 37° C overnight. Isolated colonies were streak purified on a new LB+Nal+Dap agar plate.

The pAR060302 parental *E. coli* strain, AR060302, was mated with WM3064nalR. Briefly, WM3064nalR was struck on an LB+Dap plate and the WT strain AR060302 was struck over the top. The plate was incubated overnight. The resulting growth was struck onto an LB+Nal+CM+Dap plate to select for WM3064nalR(pAR060302) transconjugants.

WM3064nalR(pAR060302) was used to transfer pAR060302 into strain DY331 in a similar manner except selection for transconjugants was achieved by growth on LB+Cm plates (No DAP) to select for DY331(pAR060302). PCR was used to generate the *tet* knockout amplicon by amplifying the *neo-ccdB* cassette from pKD45 using the primers pARdeltaTet-fw and rv (Table 2) (12.5 µL Phusion 2x master mix (Life Technologies™), 500 nM of each primer, 1 uL of template (boiled cells), PCR conditions: 95° C 5 min, 25 cycles of 95° C 30 sec, 55° C 30 sec, 72° C° for 2 min; then a final incubation at 72° C for 10 min). The resulting amplicon was purified with a Qiagen™ PCR cleanup kit. The *neo-ccdB* cassette from pKD45 allows for selection with kanamycin and then removal of

the cassette (scar-less or otherwise) using counterselection as expression of *ccdB* (via growth on minimal rhamnose plates) is lethal.

Strain DY331(pAR060302) was grown in LB+Cm broth at 32° C with shaking (200 RPM) for overnight. The overnight culture was used to inoculate a new 50 mL LB+Cm broth culture (1:100 dilution). This was grown until an OD600 ~0.4 at which time it was moved to a 43° C shaking water bath and incubated for 20 min to prime cells for recombineering. After the incubation the flask was cooled on ice for 10 min. The cells were pelleted by centrifugation at 8000 RPM for 5 min at 4° C. The cell pellet was resuspended with 25 mL ice cold H₂O. The cells were collected again by centrifugation and washed an additional time. The final cell pellet was resuspended in 200 µL of ice cold H₂O. A 40 µL aliquot of washed cells was electroporated with 0.5 – 1 µg of purified amplicon. Cells were recovered with LB broth and incubated at 32° C for 4 hours. After recovery cells were plated on LB+Kan plates. Isolated colonies were checked for growth on LB+Tet plates to confirm disruption of the *tet(A)* gene. Successful mutants were mated on LB plates with *E. coli* strain K-12 MG1655 carrying pSIM5-Tet at 32° for overnight. Successful transconjugants were selected on LB+Cm+Tet plates to select for pAR060302(Δ *tet::neo-ccdB*) and pSIM5-tet. This strain was used to remove the *neo-ccdB* cassette by recombineering using a ssDNA substrate (24). Cells were primed for recombineering as above and the 70 nt oligonucleotide DeltaTetRepairOligo (Table 2) was electroporated. Cells were recovered and plated on M9+Rhamnose+Cm plates. This resulted in pAR060302(Δ *tet*), where the *tet* locus was deleted and the *neo-ccdB* cassette was removed. Isolated colonies were checked for mutations via PCR. The entire

insertion-removal process was then done a second time to target and remove the *bla*_{CMY-2} gene using the appropriate primers. This resulted in pARK01, a tetracycline and ampicillin susceptible variant of pAR060302.

Deletions of predicted transcriptional regulators on pARK01 were done recombineering in strain K12(pARK01, pSIM5-tet). The plasmid pKD4 (25) carrying the FRT-*neo*-FRT cassette was used as a template for PCR (12.5 µL Phusion 2x master mix (Life Technologies™), 500 nM of each primer, 1 uL of template (boiled cells), PCR conditions: 95° C 5 min, 25 cycles of 95° C 30 sec, 55° C 30 sec, 72° C° for 2 min; then a final incubation at 72° C for 10 min). PCR amplicons were subjected to a DpnI digestion to eliminate template plasmid and then electroporated into cells primed for recombineering. Resulting colonies were checked by PCR for mutations. Successful mutants were then grown and made electrocompetent and the plasmid pCP20 was electroporated. These transformants were grown at 32° C for 48 hours to “flip” out the *neo* cassette, leaving a copy of FRT. The plasmid pCP20 was lost via incubation at 37° C for 48 hours. Colonies were screened via PCR and amplicons were verified by sequencing.

Alterations in the AT-Hook motif of Acr2 were achieved by first inserting the *neo-ccdB* cassette (amplified using delATHOOKAcr2-kancdB-fw and -rv primers) into *acr2*, and then through another round of recombineering using the appropriate ssDNA oligo and counterselection on minimal rhamnose plates to remove the cassette and alter the coding sequence as desired.

2.3 Molecular cloning methods.

For constructs using pLUXtet, containing a promoterless *lux* operon, cloning was done via double digestion with *Spe*I and *Bam*HI and ligation with DNA ligase (NEB).

Oligonucleotides used to generate PCR amplicons of promoter regions with flanking ends containing restriction sites are listed in Table 2.

To clone genes from pAR060302 into pBAD22 (26), *in vivo* cloning via recombineering was used (27). In order to generate a template for PCR, pBAD22 was digested overnight using *Bam*HI. The resulting digestion was used as template for PCR (12.5 μ L Phusion 2x master mix (Life Technologies™), 500 nM of each primer, 1 μ L of template, PCR conditions: 95° C 5 min, 25 cycles of 95° C 30 sec, 55° C 30 sec, 72° C° for 6 min; then a final incubation at 72° C for 10 min). Primers used for *in vivo* cloning were designed to amplify the vector pBAD22, and add 40 bp regions of homology starting with 2nd codon of the gene of interest to the reverse primer and 40 bp of homology ending with the codon next to the stop codon for the forward primer. PCR amplicons were purified with a Qiagen™ PCR Cleanup Kit and electroporated into K-12(pARK01, pSIM5-tet) cells that were primed for recombineering. After 4 hours of recovery in LB broth at 32° C, cells were plated on LB+Amp+Cm plates to select for strains still carrying pARK01 as well as the newly recombined pBAD22 with appropriate insertion. Subsequent colonies were checked via PCR for insertions in pBAD22. Successfully cloned plasmids were confirmed via DNA sequencing. The pSIM5-tet plasmid was lost by incubation at 37° C for 24 hours.

2.4 Conjugation experiments.

E. coli strain DH10 was used as a recipient in all conjugation assays. Donor and recipient cells were grown overnight at 37° C in LB broth with the appropriate selection. The overnight cultures were used to inoculate new 5 mL cultures in LB broth with no selection. The new cultures were incubated for 4 hours; if arabinose induction was needed it was done at 2 hours of incubation. After 4 hours of incubation, 0.5 mL of donor and recipient cells were added to 1.5 mL centrifuge tube, mixed by pipetting and incubated at 37° C without shaking for 1 hour. The mating reactions were then vortexed and placed immediately on ice. They were subsequently diluted in 1x phosphate buffered saline (PBS) and plated to select for transconjugants and donors.

2.5 Lux reporter assays.

Overnight cultures were grown with the appropriate selections in LB broth at 37° C with shaking (200 RPM). These cultures were diluted 1:100 in new broth with selection. These cultures were grown under the same conditions for 2 hours. At that time, the cultures were split in half. One half was treated with arabinose (0.02%) and the other half received no treatment. The cultures were allowed to grow for 2 hours. All cultures were then aliquoted into a 96-well plate, 200 µl per well. Plates were then read on a Bio-Tek plate reader. Cell density and arbitrary light units were measured. Bioluminescence was standardized for cell density by dividing light units by the OD600 absorbance. Each value represents the mean of 3 experiments.

2.6 RNA isolation and sequencing for RNA-Seq.

Strains DH10B(pAR) and DH10B(pAR Δ acr2) were grown until an OD₆₀₀ of 0.5 was achieved. Cells were pelleted and RNA was purified using a commercially available RNA extraction kit (Qiagen). Treatments were included to remove DNA contamination (Qiagen) and ribosomal RNA (MicrobExpress, Ambion). Two biological replicates for each strain were pooled for paired-end library sequencing (either 50 or 100 bp reads) via Illumina Genome Analyzer II at the Biomedical Genomics Center at the University of Minnesota. All of the sequencing data are publically available under the NCBI BioProject ID PRJNA273283.

2.7 RNA-Seq Analysis.

All Perl scripts and other computational biology resources used in this study can be found at <https://github.com/kevinslang>. cDNA reads were first trimmed so that the quality at each base position was above 30 and then mapped to the appropriate genome or plasmid sequence (for pAR060302, Genbank accession no. NC_012692, for DH10, the *E. coli* K-12 MG1655 published sequence was used Genbank accession no. NC_000913) Read mapping was done using BOWTIE (28). For each host, transcriptome maps of pAR060302 were constructed using Circos (29). To achieve this, a table was generated containing the average number of reads mapped per 250 bp of plasmid sequence. Each average was then normalized per 1 million total reads in the cognate sequence library. These averages were then log transformed and plotted as a line plot. For statistical testing of differentially expressed genes, the total number of reads mapped to each coding sequence (CDS) was calculated using Perl. These values were then analyzed using the R

package EdgeR (30, 31). We conservatively estimated the dispersion at 0.001. A fold-change cutoff of > 2 or < -2 and an adjusted p-value of < 0.05 were used to define significantly differentially expressed genes.

2.8 ChAP-Seq experiments.

K12(pARK01, pBacr2^{6xHis}) was grown overnight in LB+Cm+Amp at 37° C with shaking (200 RPM). The overnight culture was used to inoculate a new 10 mL LB+Cm+Amp culture (1:100 dilution). This was grown for 2 hours at 37° C with shaking (200 RPM). Arabinose was added to a final concentration of 0.02% and the culture was incubated for 2 additional hours. Cells were fixed with the addition of formalin to a final concentration of 1%. Fixed cells were incubated at RT for 20 min. The formalin was quenched by addition of glycine to a final concentration of 0.5 M and incubation for 5 min at RT. Cells were collected by centrifugation at 8000 RPM for 5 min. The cell pellet was washed 1x with 10 mL of cold PBS and collected again. The resulting pellet was resuspended in 1X lysis solution (MagnaHIS Kit™, Promega). The cells were sonicated on ice at 5 watts for 30 second intervals and 1 min rest times. This was done 10 times. The cell lysates were spun at 12,000 g for 5 min and the supernatant was moved to a 1.5 mL centrifuge tube containing 50 µL of MagneHIS particles. This suspension was taken through the MagneHIS kit protocol. After elution, cross-linked DNA was released via incubation at 65° C for 18 hours. The resulting DNA was purified with a Qiagen™ PCR Clean up Kit and sent for paired-end sequencing using the Illumina MiSeq platform. The library generation and sequencing was done at the UMGC at the University of Minnesota. In total, 2 biological replicates were sequenced separately. As a negative control, two

genomic DNA preparations of the same cells were done using a Qiagen™ DNeasy™ kit and ~400 ng of each was sent for sequencing.

The reads that resulted from the MiSeq run were all trimmed to 50 bp from the 3' end using the tool Trimmomatic (32). The trimmed reads were then mapped to either the pAR060302 sequence or the K-12 genome sequence using BWA using the default parameters (33). Reads that were mapped correctly were filtered using SamTools (34). The replicate libraries were then merged using SamTools. The resulting read alignments files were then analyzed using MACS (35). Peak summit coordinates were used to extract 200 bp of sequence surrounding them using BedTools (36). These sequences were combined into a multifasta file and submitted to MEME for motif analysis (37). ChAP-Seq read alignments were also subjected to read counting on 150 bp windows of the pAR060302 sequence for visualization in Circos (29).

2.9 Protein purification and EMSA.

The pBacr2^{6xHis} construct was introduced into strain were transformed into the BL21 (DE3) strain. The resulting strains were cultured in LB+AMP at 37° C for 1 hour with shaking (250 RPM). Arabinose was added to a final concentration of 0.2% prior to growing the cultures for 5 h at 28°C. Cells were spun at 4500×g for 30 min, resuspended in 5 mL cell lysis buffer (20 mM Tris pH 8, 500 mM NaCl, 5 mM imidazole, 5 mM β-mercaptoethanol) and sonicated. The cellular debris was removed by centrifugation at 12,000 g for 15 min. 200 µL of MagnePURE beads were added to the supernatant and these were incubated for 1 h on a rocking platform, washed twice with high salt washing

buffer (20 mM Tris pH 8, 1 M NaCl, 5 mM β -mercaptoethanol) and twice with low salt washing buffer (20 mM Tris pH 8, 0.5 M NaCl, 30 mM imidazole, 5 mM β -mercaptoethanol), and then eluted with 100 μ L elution buffer (20 mM Tris pH 8, 500 mM NaCl, 500 mM imidazole). PefablocTM (Roche) and glycerol were added to final concentrations of 1 mg/mL and 5%, respectively. Purified proteins were analyzed by SDS-PAGE gel electrophoresis and stored at -80° C.

Two DNA fragments were used for EMSA analysis. The region upstream of *acr1* (~480 bp) and the *floR* promoter (~150 bp). These fragments were amplified by PCR using the primers Orf184-EMSA-F and Orf184-EMSA-R (*acr1*) and Flo-F and Flo-R (*floR*). The amplicons were purified using a PCR cleanup kit (Qiagen). Various concentrations of purified Acr2 were incubated with 10 nM DNA in binding buffer (15 mM HEPES pH 7.9, 40 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol) for 30 minutes. The reactions were separated by gel electrophoresis for 2.5 h at 70 V on a 7.5% native polyacrylamide gel at 4°C (buffered with Tris glycine pH 8.0). Gels were stained with SYBR Green for 20 minutes at room temperature, washed twice with ddH₂O, and DNA complexes were visualized with ultraviolet light.

2.10 Development of *acr1-lacZ* fusion construct and detection of LacZ activity.

Recombineering was used to replace the *E. coli* chromosomal genes *lacY* and *lacA* with an FRT-*neo*-FRT cassette (Figure 21a). The *lacZ* gene, starting at the 4th codon was then amplified, along with the FRT-*neo*-FRT cassette with primers that contained 40 bp of homology with *acr1* and the region upstream of *acrC*. This amplicon was used to replace

acrI-acrC starting with the 4th codon of *acrI* in strain MC4100(pARK01, pSIM5-Tet) via recombineering and the *neo*-FRT cassette was removed by introduction of pCP20 expressing the FLP recombinase. In the same strain, *acr2* was disrupted via recombineering. These reporter cells were then transformed with constructs over expressing Acr2 and the relevant Acr2 variants. They were struck onto LB+Amp+Xgal plates with and without arabinose.

3. RESULTS

3.1 Acr2 is an H-NS-like protein that represses conjugative transfer of IncA/C plasmids.

To further investigate IncA/C plasmid conjugation, we developed a plasmid variant by systematically deleting the tetracycline and beta-lactam resistance genes (see Materials and Methods) to generate pARK01. We demonstrate that this variant is no different in terms of conjugation frequency than the wild-type plasmid (Figure S1A). Recently, several transcriptional regulators on the IncA/C plasmid pVCR94ΔX were characterized in terms of their ability to regulate conjugative transfer (7). However, pVCR94ΔX was isolated from a *Vibrio* isolate and a deletion of unknown content was made in its development for genetic studies (6). To be certain that the functions were conserved in our IncA/C plasmid, pAR060302 (isolated from an *E. coli*), we repeated several experiments using mutants of pAR060302. Our results were mostly consistent with what was previously found (Figure S1C and S1D), which indicated that pAR060302 was regulated in a similar matter to pVCR94ΔX. The putative protein encoded by *orf183* shares homology only with hypothetical proteins. Because of its proximity to the other

predicted transcriptional regulators, we wondered if it may be involved in regulating conjugative transfer (Figure S1B). Deletion of this gene does result in a slight increase in transconjugants, however we could not rule out that this was due to a polar effect of the deletion (Figure S1C). Given the small magnitude of the effect, the putative protein produced by *orf183* is unlikely to be a major contributor to repression of conjugation. In our experiments, deletion of *acr1* did not alter the frequency of conjugative transfer of pARK01. However, given the strong evidence previously reported on *acr1* (7), we acknowledge that this could be due to differences in our experimental set up.

To further investigate Acr2, we generated an Acr2^{6xHis} construct and confirmed its ability to complement an *acr2* deletion mutant by conducting transfer experiments. Plasmids lacking *acr2* exhibit a 10-fold increase in conjugation frequency compared to wild-type (Figure 1). The observation, from our data and that of other's (7), that Acr2 negatively regulates conjugation corroborated our RNA-Seq results comparing WT and Δ *acr2* plasmids. Nearly all of the genes that are predicted to be involved in conjugative transfer were significantly up-regulated (at least 2-fold and p-value < 0.05) in the Δ *acr2* strain (Figure 2, 4th ring). In addition to genes involved with transfer being up-regulated, the region encoding many putative hypothetical proteins and putative phage-like proteins (bp positions ~82k-100k) were also up-regulated in our experiment. Again these results are congruent with what has previously been reported, demonstrating that pARK01 is regulated in the same manner as other IncA/C plasmids and that our Acr2^{6xHis} construct is functionally analogous to the wild-type protein.

3.2 Characterization of Acr2 binding sites.

We performed a ChAP-Seq experiment *in vivo* to better understand where Acr2 binds on both the plasmid and host bacterial chromosome. Using nickel affinity chromatography to pull down Acr2^{6xHis}, cross-linked DNA was extracted and subsequently sequenced.

Analysis of the ChAP-Seq data revealed that Acr2^{6xHis} binds to several loci on the plasmid (Figure 2, rings 1 and 2). Some of the ChAP peaks overlapped with genes of unknown function, such as near base pair coordinates 19k, 45k, 140k, and 145k. The regions that showed greatest binding were the entire *ISEcp1* region, the regions within the *traFHG* and the region upstream of *acr1*. Binding upstream of *acr1* would suggest a direct repression of the operon encoding the positive regulators *acrDC*.

Our ChAP data suggests that Acr2^{6xHis} binds to chromosomal DNA sequences. However, like other H-NS-like proteins, the interactions are not easily interpretable. For example, we only found 3 instances of binding of genes that were subsequently found to be significantly differentially expressed in our RNA-Seq data (Figure 3). Interestingly, all three of these genes have functions associated with metabolism, most notably the *glcB* gene which was found previously as being up regulated in *E. coli* carrying pAR060302, compared to cells lacking the plasmid (38).

We used MACS (Model-based Analysis of ChAP-Seq) (35) to determine significantly enriched peaks in DNA pulled down with Acr2^{6xHis}. The sequence surrounding the summits of each of the significant ChAP peaks found by MACS was submitted to MEME (Multiple Em for Motif Elicitation) to determine if there were common motifs within the

significant peaks. The motif discovered in a majority of the peaks is A+T rich, similar to that of other H-NS-like proteins (Figure 4B). Electromobility shift assays (EMSA) were used to confirm that Acr2^{6xHis} binds to the DNA fragment upstream of *acrI* containing the motif, but not a different A+T rich promoter sequence located upstream of the *floR* gene on plasmid pAR060302 (Figure 4A). Analysis using the tool Bprom (39) to computationally predict bacterial promoters shows a predicted transcriptional start site 287 bp upstream of the start codon of *acrI* (Figure 4C). These results demonstrate that Acr2 directly represses transcription of the operon containing *acrDC* by binding to the sequence upstream of *acrI* and that there is sequence specificity to Acr2 binding regardless of A+T content.

3.3 The C-terminal domain of Acr2 is crucial for its activity.

The C-terminal domain of Acr2 is similar to that of other H-NS-like proteins (Figure 5A). It contains an AT-hook motif, with conserved amino acids Q/RGR, that has been shown in structure studies to be critical to contact with DNA (40). We examined the possibility that this motif was important for Acr2 activity by making mutations in this region using recombination with a ssDNA substrate (see Materials and Methods). An in-frame deletion of the codons for QGRRPD (Δ AT-hook) resulted in abolishment of the ability of Acr2 to repress conjugative transfer (Figure 5B). Although substitution of alanine for glutamine at position 116 resulted in increased transfer frequency, it was not as dramatic as the increase observed for alanine substitutions for the arginine residues at positions 118 and 119 (Figure 5B). Given that these substitutions could have led to proteins targeted for degradation, which could also explain the *acr2*⁻ phenotypes

observed, we cloned each of these Acr2 variants in an arabinose inducible vector. We used these constructs to test the ability to repress the LacZ activity of an *acr1-lacZ* fusion construct (Figure 5C). Only the WT Acr2 and the Q116A variant were able to repress LacZ activity. The Q116A result contrasts with that of the conjugation experiment. In the conjugation experiment the Acr2 variants were under native promotion. This is probably much lower than that achieved by arabinose induction of the pBAD vector, however, we cannot rule out the possibility of degradation. Taken together, these results suggest that the QGRR motif is critical for the ability of Acr2 to repress the promoter upstream of *acr1*.

4. DISCUSSION

4.1 The expanded Acr2 regulon.

Figure 6 displays our proposed model for the regulation of transfer built from the results of our work, as well as others (6, 7). We showed that this is, at least in part, due to a direct interaction between Acr2 and the sequence upstream of *acr1*. Our ChAP-Seq data suggest that Acr2 binds upstream of other genes that are predicted to be important for transfer, namely *traF* and *traG*. There are a few hypothetical proteins that are annotated on IncA/C plasmids that are located near or within the transfer-associated loci. For example, *orf97* and *orf98* are located just downstream of *traN*, both of which have no known function nor are similar to any protein sequence with known functions in GenBank. We found a ChAP-Seq peak on the 3' end of *traN* near *orf97*. It is difficult to understand the function of such an interaction due to the ambiguity of hypothetical proteins such as *orf97*.

There are other mechanisms that contribute to the repression of conjugation. In this work we found that *orf183* represses conjugation by a mechanism that is unclear. The predicted protein sequence encoded by *orf183* shares no similarity to proteins with known functions in protein databases. Certainly, more work needs to be done to understand the product of *orf183* to understand if its role is truly to act as a repressor of conjugation. In our experiments, an *acr1* deletion did not exhibit an effect on conjugative transfer. This contrasts elegant experiments recently published showing that Acr1 represses conjugation and binds the sequence upstream of *acr1* (7), and we wonder if subtle differences in our experimental design are responsible for the discrepancy in phenotype. We predict that there might be another factor involved in repressing conjugation of IncA/C plasmids. The *setR* gene of the integrative conjugative element SXT of *Vibrio* species is a strong repressor of conjugation, so much so that its deletion is lethal (41). None of the known repressors of IncA/C transfer, described in this work or elsewhere, exhibit this phenotype. Intuitively, if the cost of conjugation is so high, one would conclude that it must be turned on only when needed.

4.2 Acr2 is an H-NS-like protein with a conserved DNA binding motif.

Our results demonstrate that, like other H-NS homologs, Acr2 binds A+T rich DNA. Our *in vitro* results suggest that Acr2 binds in a sequence specific manner. It has been proposed that the amino acid composition in the AT-hook motif is the mechanism by which different H-NS homologs distinguish between chromosomal and horizontally acquired DNA (40). It is thought that the number or arrangement of positively charged

amino acids, such as arginine, determine the DNA recognition by H-NS-like proteins. Acr2 shares a DNA binding motif with that of other H-NS homologs and our results show that this set of amino acids is critical for its function as a repressor. When we removed or altered this DNA binding motif from Acr2, we found that, in some cases, the conjugation frequency increased higher than that of an $\Delta acr2$ mutation. This result could be explained in two ways: 1) Acr2 variants could be imparting a dominant negative effect on other negative regulators, such as Acr1. Or 2) Acr2 variants could bind the promoter upstream of *acr1* in such a way as to promote transcription. The AT-hook motif in Acr2 contains an additional arginine residue (R119) that is not shared with other H-NS homologs. We have shown that this arginine is important for the function of Acr2. This difference might result in Acr2 having a distinct propensity to bind specific regions of IncA/C plasmids to accomplish specific tasks, such as repressing conjugation.

4.3 Other roles for Acr2.

We have generated evidence that Acr2 might have other roles in the biology of IncA/C plasmids. Our ChAP-Seq data suggest that Acr2 binds nearly the entire *ISEcp1* element that carries the *bla_{CMY-2}* gene. Comparative genomics studies have demonstrated that the acquisition of this transposon is a recent event in the evolution of IncA/C plasmids (2–4). Could Acr2 bind newly acquired mobile elements within IncA/C plasmid backbones? Given the propensity for chromosomally encoded H-NS to bind genomic islands, it seems plausible (12). Furthermore, Acr2 binds to the 3' end of the *rhs* gene located downstream of the class 1 integron (bp coordinate ~146,000). The *rhs* genes have repeat sequence elements that implicates them in genome shuffling (42). Recent work from Harmer *et al.*

suggests that the *rhs* homolog carried on IncA/C plasmids plays an important role in the diversity of newly integrated mobile elements (4). Binding by Acr2 may inhibit the ability for recombination to happen at the *rhs* locus. H-NS has recently been shown to play an important role in the evolution of *Salmonella*, providing stability for its genomic islands (15). Given broad spatial distribution of highly similar variants of IncA/C plasmids, Acr2 binding of *rhs* could be a mechanism driving IncA/C plasmid evolution.

Our ChAP-Seq experiment also yielded evidence that Acr2 binds chromosomal DNA. It is unclear what the true role of this binding is as nearly all genes bound showed no differential expression in RNA-Seq experiments. We cannot rule out that these peaks might be an artifact of overexpressing Acr2. The lack of differentially expressed genes could also be explained by the presence of chromosomally encoded nucleoid associated proteins, such as H-NS, IHF and others. It will take more sophisticated experiments to tease out the meaning behind these results. There were three bound genes, however, that were differentially expressed in our RNA-Seq data. All three have to do with different metabolic pathways. Interestingly, one of the genes was *glcB*, which is involved in the glyoxylate bypass pathway. We previously found this pathway to be modulated in several different host bacteria upon acquisition of IncA/C plasmids (38). This lends some credence to the possibility that IncA/C plasmids encode the ability to specifically alter host metabolic pathways to improve fitness of plasmid carrying cells. This presents an almost phage-like scenario where the plasmid co-ops the host bacterium to become adept at carrying and disseminating the plasmid. It is an interesting area of study that future work must explore.

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Figure Legends.

Figure 1. Conjugation frequency of *acr2* mutants. (+) and (-) represent presence or absence of arabinose in the media. Results represent the mean and standard deviation of 3 experiments. Means were compared using a 2-tailed Student's T-test. * indicates p-value > 0.05; *** indicates p-value < 0.05.

Figure 2. Acr2 binding sites on pAR060302. Outer ring (Ring 1) Base pair coordinates of pAR060302. (Ring2) Results of ChAP-Seq experiment. Scale (toward the outer circle) is

from 0 reads to 1500 on a linear scale. (Ring 3) Enriched peaks (purple bars) as found by MACS (35). (Ring 4) Differentially expressed genes in pAR060302 Δ *acr2* compared to pAR060302. Color indicates fold-change and FDR < 0.05. Grey indicates unchanged. (Ring 5) pAR060302 gene map. Genes on the map are positioned by what strand they are coded on and colored by function: gray, hypothetical protein; red, antibiotic resistance; yellow, mobile genetic elements; green, transcriptional regulation; light purple, recombination; purple, DNA binding; blue, conjugal transfer; light blue, transglycosylase; orange, replication. (Ring 6) G+C content along 150 bp windows of pAR060302.

Figure 3. Expression of *E. coli* chromosomal genes carrying either pAR060302 or pAR060302 Δ *acr2*. Normalized expression values were calculated using Perl and are displayed as Reads Per Kilobase of gene length per Millions of total reads (RPKM) on a log10 scale. Acr2 enriched peaks were found with MACS and annotated with BedTools.

Figure 4. Acr2 binds promoter region of *acrI*. a) Electromobility Shift Assay using either a fragment of DNA upstream of *acrI* (left) or *floR* (right) combined with either no protein, or increasing protein concentrations of 150 nM, 200 nM, 300 nM, 400 nM, 500 nM, 600 nM, 700 nM, or 850 nM. DNA-protein complexes were separated on a 7.5 % native acrylamide gel. The black triangle indicates a bound DNA complex. b) Consensus DNA binding sequence of Acr2 as found by MEME (37). 200 bp of sequence surrounding each peak summit found by MACS and compared by MEME. c) Promoter region of *acrI* 323 bp upstream of *acrI* start codon. -35, -10 and TSS sites indicated were

predicted by bprom (43). The underlined sequence is the predicted binding site of Acr2.

Figure 5. Activity of Acr2 variants. A) Alignment of the C-terminal domain of Acr2 and other H-NS-like proteins. Box indicates region of conserved amino acid positions. Red letters indicate the AT hook motif in Acr2. B) pARK01 encoding variants of Acr2 were used in conjugation experiments. Results represent the mean and standard deviation of 3 experiments. C) Alleles encoding Acr2 variants were cloned into pBAD and assayed for their ability to repress the *acrI-lacZ* fusion construct. Blue color indicates LacZ activity. White color indicates absence of LacZ activity.

Figure 6. Proposed model for the regulation of IncA/C conjugation and Acr2 regulon. Red indicates homology to *flhDC*. Green indicates predicted DNA binding domains. Blue indicates predicted transglycosylase activity. Acr2 and Acr1 have been shown to repress P_{acr1} and ORF183 represses conjugation by some unknown mechanism. Expression of *acaDC* results in the up-regulation of transfer genes as well as mobilization of other genomic islands. This also may express *acr2*, which, in turn, shuts down conjugation. Acr2 acts on other loci on the plasmid including on genes of unknown function and newly acquired DNA.

Figure S1. Summary of experiments regarding the regulators of conjugation. A) Conjugation experiment showing conjugation frequency of donor strains WT strain AR060302 (WT AR), K-12 strain MG1655 carrying pAR060302 (K12 pAR) and K-12 strains MG1655 carrying pARK01 (K12 pARK01). The results represent the means and

standard deviations of 3 experiments. B) Gene map of the cluster of transcriptional regulators that regulate conjugation. Red indicates homology to *flhDC*. Green indicates predicted DNA binding domains. Blue indicates predicted transglycosylase activity. C) Conjugation frequency of mutants. (+) and (-) indicate the presence or absence of arabinose in the media. Results represent mean and standard deviation of 3 experiments. Means were compared using a 2-tailed Student's T-test. * indicates p-value > 0.05. ** p-value < 0.05. *** indicates not detected. D) Activity of promoter fusions to *lux* operon. Results represent mean and standard deviation of 3 experiments.

a)

Acr2^{6xHis}

P_{acr1}

P_{floR}

b)

c)

162,286

162,387

taaa**atcact**ttatacctaaacgct**atttaatat**cacaaa**accgca**actccccgtgcgct
 -35 -10 TSS

a)

```

90 -LNEMFTSRSEPDNAKYSKDGVSWSGQGRRRPDAFKGL---GAVELERYRIPQKK--- > Acr2
104 -AQRPAKYSYVDENG-----ETKTWTGQGRTPAVIKKAMDEQGKSLDDFLIKQ----- > H-NS
71 -MSTVPKYR---DPA---TGKTWSGRGRQPAWL-----GNDPAAFLIQPDLPAL > bv3
81 AAIREWARR-----NGHNVSTRGRIPADV-----IDAYHAAT----- > Lsr2

```

Table 1. Strains and plasmids used in this study.

Strain or Plasmid	Characteristics	Source
<i>E. coli</i> DH10B	Rif ^R strain used as a conjugation recipient	(25)
<i>E. coli</i> MC4100	lac ⁻ strain used for observing LacZ fusion activity	ATCC# 35695 Jeff
<i>E. coli</i> K12 MG1655	Model <i>E. coli</i> strain	Gralnick, University of Minnesota
<i>E. coli</i> DY331	<i>E. coli</i> with stable prophage that expresses <i>beta-gam-exo</i>	Don Court, NIH Jeff
<i>E. coli</i> WM3064	DAP auxotroph	Gralnick, University of Minnesota
<i>E. coli</i> WM3064nalR	nal ^R mutant of WM3064	This work
<i>E. coli</i> AR060302	Wild type host of pAR060302	(2)
pAR060302	IncA/C plasmid, FloR, CmR,	(2)
pARK01	pAR060302 derivative carrying deletion of <i>tetA</i> and <i>bla</i> _{CMY-2}	This work
pARK01-01	pARK01 carrying deletion of <i>orf183</i>	This work
pARK01-02	pARK01 carrying deletion of <i>acr1</i>	This work
pARK01-03	pARK01 carrying deletion of <i>acaDC</i>	This work
pARK01-04	pARK01 carrying deletion of <i>acr2</i>	This work
pARK01-05	pARK01 carrying lacZ fusion of the first 4 codons of <i>acr1</i> and <i>lacZ</i> and a deletion of <i>acr2</i>	This work
pARK01-06	pARK01 carrying the Acr2ΔA _{Thook} mutation	This work
pARK01-07	pARK01 carrying the Acr2(Q116A) mutation	This work
pARK01-08	pARK01 carrying the Acr2(R118A) mutation	This work
pARK01-09	pARK01 carrying the Acr2(R119A) mutation	This work
pARK01-10	pARK01 carrying the Acr2(R118A and R119A) mutation	This work
pBBRLux	Plasmid carrying a promoterless <i>lux</i> operon; Cm ^R	Fitnat Yildiz,

		UC Santa Cruz
pLux	pBBRLux with <i>tetRA</i> in place of <i>cat</i>	This work
pLuxFloR	pLux with <i>floR</i> promoter cloned in between SpeI and BamHI sites	This work
pLuxTraI	pLux with <i>traI</i> promoter cloned in between SpeI and BamHI sites	This work
pLuxTraF	pLux with <i>traF</i> promoter cloned in between SpeI and BamHI sites	This work
pLux190	pLux with <i>orf190</i> promoter cloned in between SpeI and BamHI sites	This work
pBAD22	arabinose inducible vector, Amp ^R	(32)
pB183	pBAD22 with <i>orf183</i> cloned	This work
pBacr2	pBAD22 with <i>acr2</i> cloned	This work
pBacaDC	pBAD22 with <i>acaDC</i> cloned	This work
pBacr2ΔA _{Thook}	pBAD22 with <i>acr2ΔA_{Thook}</i> cloned	This work
pBacr2Q116A	pBAD22 with <i>acr2Q116A</i> cloned	This work
pBacr2Q116A	pBAD22 with <i>acr2Q116A</i> cloned	This work
pBacr2R119A	pBAD22 with <i>acr2R119A</i> cloned	This work
pBacr2R118A,R119A	pBAD22 with <i>acr2R118A,R119A</i> cloned	This work
pCP20	plasmid expressing FLP recombinase, Amp ^R , <i>ts rep</i>	(31)
pSIM5-tet	plasmid expressing <i>beta-gam-exo</i> used for recombineering, tet ^R	(33)
pKD4	plasmid carrying FRT- <i>neo</i> -FRT cassette, Kan ^R , Amp ^R	(31)
pKD45	plasmid carrying <i>neo-ccdB</i> cassette, R6K <i>ori</i>	(33)

Table 2. Oligonucleotides used in this study.

Name	Sequence
pARdeltaTet-fw	CCGGCATTTCGCTGCGCTTATGGCAGAGCAGGGAAAGGAATTGCCGGGCTAGAGCCTGACATTTATATTCC
pARdeltaTet-rv	GGCTGCAACTTTTGTCATGCTTGACACTTTTATCACTGATAAACATAATATGGTCCCGCTCAGAAGAAGCTC
deltaTetRepairOligo	CTTATGGCAGAGCAGGGAAAGGAATTGCCGGGCTACATATTATGTTTATCAGTGATAAAGTGTCAGCAT
pARdeltaBlaCMY2-fw	CTGACGGGCCCCGGACACCTTTTTTGCTTTTAATTACGGAACTGATTTTCATGGAGCCTGACATTTATATTCC
pARdeltaBlaCMY2-rv	AAAAAAGAGAAAGAAAGGAGGCCCAATATCCTGGGCCTCATCGTCAGTTAGTCCCGCTCAGAAGAAGCTC
deltaCMYrprOligo	ACCTTTTTGCTTTTAATTACGGAACTGATTTTCATGTAAGTACGATGAGGCCAGGATATTGGGCCTCCT
183_KOchk-F	TCGACGGAAGCTGGAGACCGT
183_KOchk-R	TGATGCCGTGGTAAACTGGAGGT
183_koCHK-F	CCGCGCAACGCGGATAAAGC
183_koCHK-R	GCTGGCTCCCTTCGGCGTTT
pBAD22_orf183-rv	ATTCCCAGAAGCACCATTCCCAGCAGAAGAATATTGACCACATGTTTCATGGTGAATTCCTCCTGCTAG
pBAD22_orf183-fw	ATTCCTATGATTTTGACCAGCGCTTCCATATTGGAGCTGGAGACGAATAAGTACCCGGGGATCCTCTAG
pbadseq-2	GGCTGAAAATCTTCTCT
pbadseq-1	CTGTTTCTCCATACCCGTT
acr1_KO-F	TGTATGGCTAACAACGCAGTTTAAAGGGTGGCAAACAATGGTGTAGGCTGGAGCTGCTTC
acr1_KO-R	GCCGCAGACAGAACTAGCGCCTTGGCTTTGTTCTTCATCAATGGGAATTAGCCATGGTCC
acr1_KOchk-F	ACAACGCAGTTTAAAGGGTGGCA
acr1_KOchk-R	GCCGCAGACAGAACTAGCGCC
acaDC_KO-F	ATCTATCGCAACCTTCGTGATTTGTGAGGGGGGCGGAATGGTGTAGGCTGGAGCTGCTTC
acaDC_KO-R	GGGCGCTCATCTTCTGGTCCGAAATGTCATAGTCTACTCAATGGGAATTAGCCATGGTCC
acaDC_KOchk-F	GCCGCTATCACGCCTGGGAA
acaDC_KOchk-R	GGGCGCTCATCTTCTGGTCCG
pBAD22_acaDC-rv	TGTGAGCTGTAACCCAGCGACCCAATGTACCAGAGTTGCCAATGTTTCATGGTGAATTCCTCCTGCTAG
pBAD22_acaDC-fw	TATTGAAAAAACTGCAACAGTCTCTACAGGGACGTGGGAGGTGGAGATGAGGCTGTTTTGGCGGATGAGA
pBBR-F	CGCGCGTAATACGACTCACT
pBBR-R	TCGGGAAAGATTTCAACCTG

cat_frt-tet-frt-fw	GACCGTTCAGCTGGATATTACGGCCTTTTTAAAGACCGTAGAATTCGAGCTCGGTACCCG
cat_frt-tet-frt-rv	ATTGGCTGAGACGAAAAACATATTCTCAATAAACCCCTTTAGCTATGACCATGATTACGCC
BamHI-190p-rv	GGATCCATAACATGAAATCCTCCAAAGATT
SpeI-190p-fw	GATACTAGTTGCTGGAGGGCAAGCGGTTC
BamHI-floRp-rv	GGATCCATAACATGACGAATCAACCCTCG
SpeI-floRp-fw	GATACTAGTAAAGATAATCGGATAAAATG
BamHI-traFp-rv	GGATCCATAACACGTATTTTCCCATGCTCG
SpeI-traFp-fw	GATACTAGTGGACACGACCAAATAGTTTG
BamHI-traIp-rv	GGATCCATAACATGTAGGTTGACCATATTC
SpeI-traIp-fw	GATACTAGTTCGTTCCCTCATAAAAAAATTG
acr2_ko-F	AGAAGATGAGCGCCCAAACCTTTGCGGCGCTCATTTTTTTTAGTGTAGGCTGGAGCTGCTTC
acr2_ko-R	GATTCACTTAAAGGAAAACCTGATGGGCTCCGTATGACATGATGGGAATTAGCCATGGTCC
acr2_chk-F	AGGAAGGCATGGTGGTGGGGA
acr2_chk-R	ATTCGCCAGTGCAGACTGACGC
pBAD22_acr2_6xhis-fw	AGCTGTTCGAGCTGGAGCGCTACCGTATCCACAGAAGAAGCATCACCATCA CCATCACTAAGGCTGTTTTGGCGGATGAGA
pBAD22_acr2-rv	CAGCCGCTGATAGCGTAGAGAACTCCTCGTGGTCTTTCATGGTGAATTCCTCCTGCTAG
acr1-EMSA-F	CCCTAAAATCACTTATACC
acr1-EMSA-R	CAATTGGCATTTATCCATTG
floRp-rv	ACATGACGAATCAACCCTCG
floRp-fw	AAAGATAATCGGATAAAATG
delATHOOKAcr2-kancedB-fw	CTCCAGCTCGACAGCTCCCAACCCCTTGAAAGCATCCGGGGTCCCGCTCAGAAGAATC
delATHOOKAcr2-kancedB-rv	ATTCAAAAGACGGAGTTTCGTGGAGTGGGCAAGGACGTCGGAGCCTGACATTTATATTCC
delATHOOK-repair	CGCTCCAGCTCGACAGCTCCCAACCCCTTGAAAGCCCCACTCCACGAAATC CGTCTTTTGAATATTTAG
Acr2Q116A	CTAAATATTCAAAAGACGGAGTTTCGTGGAGTGGGGCGGGACGTC GCCCCGATGCTTTCAAGGGGTGGGAGC
Acr2R118A	AATATTCAAAAGACGGAGTTTCGTGGAGTGGGCAAGGAGCGCGCC CGGATGCTTTCAAGGGGTGGGAGCTGT

Acr2R118A-R119A	AATATTCAAAAGACGGAGTTTCGTGGAGTGGGCAAGGA GCGGCGCCGGATGCTTTCAAGGGGTGGGAGCTGTCGA
Acr2R119A	ATTCAAAAGACGGAGTTTCGTGGAGTGGGCAAGGACGTG CGCCGGATGCTTTCAAGGGGTGGGAGCTGTCGA
deltalacAY-fw	GCCTGATAAGCGCAGCGTATCAGGCAATTTTATAATTTAGTGTAGGCTGGAGCTGCTTC
deltalacAY-rv	CAGGCCATGTCTGCCCCGTATTCGCGTAAGGAAATCCATTATGGGAATTAGCCATGGTCC
acr1-lac-rv	TTTGGGCGCTCATCTTCTGGTCCGAAATGTCATAGTCTACCGTGTAGGCTGGAGCTGCTT
acr1-lac1-fw	AACAACGCAGTTTAAAGGGTGGCAAACAATGGATAAATGCCCCGTCGTTTTACAACGTCG