A Prophage-Encoded Small RNA Controls Metabolism and Cell Division in *Escherichia coli*

Divya Balasubramanian,a,b Preethi T. Ragunathan,a Jingyi Fei,c,d Carin K. Vanderpoola

Department of Microbiology, University of Illinois at Urbana–Champaign, Urbana, Illinois, USAa; Department of Microbiology, New York University School of Medicine, New York, New York, USAb; Department of Physics, Center for the Physics of Living Cells, University of Illinois at Urbana—Champaign, Urbana, Illinois, USAc; Department of Biochemistry and Molecular Biology, the University of Chicago, Chicago, Illinois, USAd

**ABSTRACT**

Hundreds of small RNAs (sRNAs) have been identified in diverse bacterial species, and while the functions of most remain unknown, some regulate key processes, particularly stress responses. The sRNA DicF was identified over 25 years ago as an inhibitor of cell division but since then has remained uncharacterized. DicF consists of 53 nucleotides and is encoded by a gene carried on a prophage (Qin) in the genomes of many *Escherichia coli* strains. We demonstrated that DicF inhibits cell division via direct base pairing with *ftsZ* mRNA to repress translation and prevent new synthesis of the bacterial tubulin homolog FtsZ. Systems analysis using computational and experimental methods identified additional mRNA targets of DicF: *xylR* and *pykA* mRNAs, encoding the xylose uptake and catabolism regulator and pyruvate kinase, respectively. Genetic analyses showed that DicF directly base pairs with and represses translation of these targets. Phenotypes of cells expressing DicF variants demonstrated that DicF-associated growth inhibition is not solely due to repression of *ftsZ*, indicating that the physiological consequences of DicF-mediated regulation extend beyond effects on cell division caused by reduced FtsZ synthesis.

**IMPORTANCE**

sRNAs are ubiquitous and versatile regulators of bacterial gene expression. A number of well-characterized examples in *E. coli* are highly conserved and present in the *E. coli* core genome. In contrast, the sRNA DicF (identified over 20 years ago but remaining poorly characterized) is encoded by a gene carried on a defective prophage element in many *E. coli* genomes. Here, we characterize DicF in order to better understand how horizontally acquired sRNA regulators impact bacterial gene expression and physiology. Our data confirm the long-hypothesized DicF-mediated regulation of *ftsZ*, encoding the bacterial tubulin homolog required for cell division. We further uncover DicF-mediated posttranscriptional control of metabolic gene expression. Ectopic production of DicF is highly toxic to *E. coli* cells, but the toxicity is not attributable to DicF regulation of *ftsZ*. Further work is needed to reveal the biological roles of and benefits for the host conferred by DicF and other products encoded by defective prophages.

**KEYWORDS:** DicB, DicB, FtsZ, FtsZ, Hfq, Hfq, RNase E, RNase E, cryptic prophage

Many bacterial small RNAs (sRNAs) were fortuitously discovered starting in the 1980s, but it is only in the past decade that their roles as posttranscriptional regulators have been characterized in some detail. sRNA regulators that base pair with mRNA targets can exert positive or negative effects on mRNA translation or stability and typically require an RNA chaperone (Hfq) (1–3) to carry out their functions. Many bacterial sRNAs are produced in response to a specific stress (4–8), and sRNA-mediated
regulation promotes adaptation to stress. Other sRNAs regulate housekeeping functions that help bacteria maintain metabolic homeostasis (9). The sRNA DicF was discovered in the 1980s, when it was observed that dicF in multicopy configuration inhibited cell division and caused filamentation (10, 11). The dicF gene is present in an operon encoding six gene products: DicF and five small proteins (12, 13). The promoter-proximal genes, ydfA, ydfB, and ydfC, encode small proteins of unknown function; these genes are followed by a long untranslated region containing dicF and then by the genes dicB and ydfD (13, 14). DicB, a 64-amino-acid protein, is the only other characterized gene product encoded by this operon (referred to historically and in this study as the dicBF operon). DicB is also a cell division inhibitor and carries out this function by (indirectly) preventing polymerization of FtsZ, the protein that forms the contractile ring for bacterial cell division (13, 15).

The 5’ end of DicF is produced by RNase E-mediated processing of the polycistronic transcript (13). Two different 3’ ends have been reported. Rho-independent transcription termination downstream of ydfC generates the 3’ end of a 53-nucleotide (nt) DicF sRNA. RNase III-mediated processing of the full-length dicBF mRNA yields a 3’ end that results in a 72-nt DicF molecule (13). DicF binds Hfq (16, 17) and was previously shown to inhibit ftsZ translation, an effect that was postulated to occur via an antisense base pairing mechanism (14). Consistent with this putative regulation, overproduction of DicF inhibited FtsZ protein synthesis and caused cell filamentation. Moreover, DicF overproduction led to aberrant nucleoid separation and to strong growth inhibition of Escherichia coli (14). Together, these observations indicate that overproduction of DicF is toxic to E. coli. However, the mechanistic basis of this toxicity has not been defined, and aside from ftsZ, no other putative targets of DicF have been identified.

DicF is encoded by a gene carried on Qin, a defective lambdoid prophage in the E. coli chromosome. Qin is one of nine E. coli K-12 defective prophages (18, 19) that have lost genes (by mutation or deletion) required for induction of the lytic life cycle, excision from the host chromosome, and/or production of progeny virions (20, 21). There are a number of well-characterized cases where genes carried by functional prophages are beneficial to the bacterial host (22–24). In contrast, the impacts of defective prophage genes on bacterial physiology have remained enigmatic. A recent study implicated E. coli defective prophages in modulation of many facets of cell growth and physiology, particularly in response to stresses (25). In that study, gene products encoded by Qin (particularly DicB) promoted resistance to the antibiotics azlocillin and nalidixic acid (25).

In this study, we probed the function of DicF by using global approaches to identify mRNA targets and by combining these with phenotypic comparison of cells expressing wild-type (wt) and mutant DicF RNAs. We found that, in addition to ftsZ, DicF also directly represses xylR and pykA, encoding the xylose regulator and pyruvate kinase, respectively. We demonstrated that the toxic effects of DicF are not solely attributable to regulation of ftsZ, suggesting that DicF regulation of other targets has important physiological consequences. Comparing the relative roles of DicF and DicB in growth inhibition of E. coli cells when the dicBF operon is ectopically expressed, we found that while both contribute, DicB is a more potent growth inhibitor than DicF.

RESULTS
To identify putative targets of DicF, we used global computational and experimental approaches. Four computational programs, TargetRNA (26), IntaRNA (27), CopraRNA (28), and sTarPicker (29), which utilize different methods to predict base pairing interactions between sRNAs and potential target mRNAs (30), were used to generate a list of putative DicF targets. Targets predicted by at least two algorithms (with a P value of <0.03) are shown in Table S1 in the supplemental material. We also conducted transcriptome sequencing (RNA-Seq) analyses of an E. coli ΔdicF mutant harboring vector or Plac-dicF plasmids. RNA-Seq data were analyzed from 3 independent biological replicates and differentially expressed genes identified as described previously (31) (see Table S2). Candidates prioritized for further study had ≥100 normalized read
counts (reads per kilobase per million [RPKM] values) under at least one set of experimental conditions and were 3-fold upregulated or downregulated in their levels compared to a vector control (*q* value of <0.05) (see Table S2).

**Validation of posttranscriptional regulation by DicF.** Chromosomal translational lacZ fusions were constructed for a subset of candidates (Table 1). Fragments encompassing the 5′ untranslated region (UTR) and 10 to 20 amino acids of coding sequence (CDS) of each target were placed under the control of an inducible promoter (P_{BAD} [32]) or a constitutive promoter (Cp19 [33]) (Fig. 1A) to eliminate indirect effects of DicF on target gene transcription. Wild-type *E. coli* reporter strains carrying either the vector control or a dicF plasmid were induced with IPTG (isopropyl-β-D-
thiogalactopyranoside), and β-galactosidase activity was monitored. The activity of several fusions was slightly altered by ectopic production of DicF (Fig. 1B), but only \textit{xylR} and \textit{pykA} fusions exhibited a \(\approx 2\)-fold difference in activity in response to DicF. RNA-Seq showed that levels of \textit{ftsZ} mRNA were reduced by \(\approx 1.7\)-fold in DicF-expressing cells (see Table S2 in the supplemental material), and since previous studies suggested \textit{ftsZ} mRNA as a DicF target, we included it in downstream analyses.

Posttranscriptional regulation of targets by DicF does not require other \textit{Qin} functions. Several genes carried on \textit{Qin} were differentially regulated upon DicF production (see Table S2 in the supplemental material). To determine whether \textit{xylR}, \textit{pykA}, and \textit{ftsZ} regulation by DicF required other \textit{Qin}-encoded functions, we examined DicF regulation of these targets in a host strain where the entire \textit{qin} prophage (~20 kb) was deleted (constructed as described in reference 25). As shown in Fig. 2, \textit{xylR}, \textit{pykA}, and \textit{ftsZ} were regulated by DicF in the presence and absence of \textit{qin}, indicating that regulation of these targets by DicF is not mediated indirectly by other \textit{Qin}-encoded functions.

Characterization of DicF regulation of \textit{ftsZ} mRNA. The 3’ region of DicF is predicted to base pair with the \textit{ftsZ} mRNA ribosome binding site (RBS) (14) (Fig. 3A). To test this prediction, we examined regulation of an \textit{ftsZ}’-\textit{lacZ} translational fusion by wt DicF, DicF9, and DicF3 (Fig. 3A). The DicF9 mutation disrupts the predicted DicF-\textit{ftsZ} mRNA interaction, while the DicF3 mutation does not. Wild-type DicF and DicF3 strongly repressed \textit{ftsZ} translation, while DicF9 failed to regulate \textit{ftsZ} (Fig. 3B). To further confirm direct interactions between DicF and the \textit{ftsZ} mRNA, we constructed DicF23 and \textit{ftsZ\textsubscript{comp23}}’-\textit{lacZ} (\textit{ftsZ\textsubscript{comp23}} contains compensatory mutations to restore pairing with DicF23) mutations to disrupt and restore the base pairing interaction upstream of the \textit{ftsZ} RBS (Fig. 3A). DicF23 did not repress \textit{ftsZ} translation as efficiently as wt DicF; likewise, wt DicF did not efficiently repress \textit{ftsZ\textsubscript{comp23}} (Fig. 3C). Importantly, DicF23 regulated \textit{ftsZ\textsubscript{comp23}} at nearly wt levels (Fig. 3C). These data are consistent with the
long-held hypothesis (14) that DicF directly represses the essential E. coli gene *ftsZ* via direct base pairing interactions.

Many sRNAs require Hfq (34–36) for their stability and for interactions with targets (e.g., SgrS [37, 38], RydC [39], and OxyS/RprA [40]), and the RNase E degradosome is

![Fig 2](image_url)

**FIG 2** Regulation of *ftsZ*, *xylR*, and *pykA* by DicF does not require other factors carried on Qin prophage. β-Galactosidase activity of *ftsZ*−*lacZ*, *xylR*−*lacZ*, and *pykA*−*lacZ* after DicF expression was assayed in the indicated strain backgrounds. The specific activities in Miller units (indicated at the bottom) were normalized to the corresponding vector control strains to yield relative-activity data for the experimental strain.

Many sRNAs require Hfq (34–36) for their stability and for interactions with targets (e.g., SgrS [37, 38], RydC [39], and OxyS/RprA [40]), and the RNase E degradosome is

![Fig 3](image_url)

**FIG 3** Genetic and molecular characterization of DicF-*ftsZ* mRNA interactions. (A) Base pairing predictions for *ftsZ* mRNA and DicF. The numbers for *ftsZ* are relative to the start codon (with A as +1). The numbers for DicF are relative to the 5' end of *dicF*. The numbers for *ftsZ* are shown in bold, and the start codon is underlined. The predicted base pairing interactions between *ftsZ* and DicF are marked by vertical lines, and mutations in *dicF* and *ftsZ* are indicated by boxes. All mutations were substitutions, and boxed residues were changed to the complementary nucleotides. Nonpaired nucleotides or gaps are indicated by sequences above or below paired nucleotides. (B) The -galactosidase activities of the *ftsZ*−*lacZ* fusion strains ectopically producing DicF, DicF3, or DicF9 were assayed. (C) The -galactosidase activities of the *ftsZ*−*lacZ* fusion and the compensatory *ftsZcomp23*−*lacZ* fusion were assayed upon overexpression of DicF and DicF23. (D) The levels of β-galactosidase activity of the *ftsZ*−*lacZ* fusion in the wild-type (WT) strain and in the *ne131* and Δ*hfq* mutants were assayed after overexpression of DicF. The specific activities of the fusions were normalized as described for Fig. 2.
important for degradation of sRNA-mRNA complexes in the context of negative regulation (41–43). To assess whether Hfq and the degradosome are necessary for regulation of \( ftsZ \) by DicF, we tested the activity of the \( ftsZ^{\text{wt}} \rightarrow \text{lacZ} \) fusion in \( \text{wt}, \text{hfq}, \) and \( \text{rne131} \) (degradosome mutant) backgrounds. Hfq was important for DicF regulation of \( ftsZ \), as repression in the \( \text{hfq} \) mutant was less stringent than that seen with the \( \text{wt} \) strain (Fig. 3D). However, \( ftsZ \) translation was still efficiently repressed by DicF in the \( \text{rne131} \) background, suggesting that DicF-mediated translational silencing of \( ftsZ \) does not absolutely require RNase E-mediated degradation. Nonetheless, in the context of the full-length \( ftsZ \) mRNA, translational repression and mRNA degradation may indeed be coupled.

**Characterization of DicF regulation of \( xylR \) mRNA.** \( XylR \) is a transcription factor that activates \( \delta \)-xylose import (\( xylFGH \)) and catabolism (\( xylAB \)) genes. A \( xylR \) translational fusion was strongly repressed by DicF (Fig. 1). While sequences near the 3’ end of DicF are involved in interactions with \( ftsZ \) mRNA, the 5’ end of DicF was predicted to interact with \( xylR \) mRNA (Fig. 4A). DicF3 and DicF9 were tested alongside \( \text{wt} \) DicF for regulation of \( xylR^{\text{wt}} \rightarrow \text{lacZ} \) (Fig. 4B). In contrast with the result for \( ftsZ \) (Fig. 3B), DicF9 still strongly regulated \( xylR \) translation, while DicF3 failed to regulate \( xylR \) (Fig. 4B). These results suggest that DicF interacts with \( xylR \) and \( ftsZ \) mRNAs using distinct residues in the 5’ and 3’ ends, respectively.

To further characterize DicF regulation of \( xylR \) and verify the predicted interaction (Fig. 4A), a translational fusion to the \( xylR \) start codon (lacking the predicted DicF
binding site) was constructed (xylR1 = - lacZ; Fig. 4A). As predicted, wt DicF failed to regulate the truncated fusion (Fig. 4C). Point mutations in dicF (DicF11) and xylR (xylRcomp11) that disrupted and then restored complementarity confirmed the regulation (Fig. 4C). The mutation in DicF11 relieved regulation of wt xylR, and the xylRcomp11 mutation likewise prevented regulation by wt DicF. Regulation was restored for the DicF11-xylRcomp11 compensatory pair (Fig. 4C). DicF-mediated translational regulation of the xylR reporter fusion did not require the RNase E degradosome but did require Hfq (Fig. 4D). There was little change in xylR mRNA levels in response to DicF (see Fig. S1A in the supplemental material), suggesting that the primary mechanism of xylR regulation is translational repression and not mRNA decay.

Since XylR is a transcription factor that activates expression of xylose uptake and catabolism genes (44), repression of xylR by DicF should limit growth of E. coli in minimal medium with xylose as the sole carbon source. Cells expressing wt DicF were severely growth inhibited (as observed previously [14, 45]) on both LB and xylose minimal medium (Fig. 5; +IPTG, dicF). To differentiate the ability of DicF to restrict growth of E. coli specifically on xylose medium due to repression of xylR from general restriction of colony-forming ability due to repression of ftsZ, we utilized the dicF3 and dicF9 mutants (Fig. 3A and 4A), which differentially regulate ftsZ and xylR, respectively (Fig. 5). Cells producing DicF3 were growth inhibited on both LB and xylose media, as expected because DicF3 represses ftsZ (Fig. 3B). DicF9, which does not repress ftsZ (Fig. 3B) but which still represses xylR (Fig. 4B), allowed growth on LB (Fig. 5) as well as on minimal glucose and fructose plates (see Fig. S2 in the supplemental material) but inhibited growth on minimal xylose plates (Fig. 5). The control xylR mutant strain grew well on LB but was unable to grow on xylose minimal medium (Fig. 5). These growth phenotypes are entirely consistent with genetic data indicating that DicF uses different residues to base pair with different targets, ftsZ and xylR. Further, the data indicate that independent regulation of these two different targets results in specific phenotypes consistent with known physiological roles of the mRNA targets.

**Characterization of DicF regulation of pykA mRNA.** In E. coli, pykA encodes one of two pyruvate kinase isozymes that catalyze the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP. DicF is predicted to interact with sequences encompassing the pykA mRNA RBS (Fig. 6A). The point mutation in DicF23 completely
abolished repression of wt pykA-lacZ (Fig. 6A and B). A compensatory mutation in pykA (pykAcomp23) impaired regulation by wt DicF but restored regulation by DicF23 (Fig. 6B). These results validated the base pairing prediction and demonstrated that DicF directly regulates pykA. As for the other two targets, DicF regulation of pykA reporter translation requires Hfq but is not dependent on the presence of a functional degradosome (Fig. 6C). However, DicF was responsible for reduced levels of the pykA native transcript, as observed by Northern blot analysis (see Fig. S1B in the supplemental material), consistent with RNA-Seq analyses showing that pykA mRNA levels were strongly diminished upon DicF expression (Table 1). Together, these results suggest that, while translational regulation of pykA by DicF does not require the degradosome, the degradation of pykA mRNA may nevertheless play a role in regulation in vivo.

**Effects of DicF on E. coli growth and cell division.** A large number of genes were differentially expressed in control cells versus DicF-expressing cells (Table 1; see also Table S2 in the supplemental material). Our results thus far demonstrate that DicF directly regulates, at a minimum, three different mRNA targets in *E. coli*. While characterizing DicF regulation of ftsZ, xylR, and pykA, we isolated mutants that differentially
regulate these targets (e.g., DicF3 and DicF9; Fig. 3, 4, and 5). Mutant DicF RNAs were produced at levels comparable to those seen with wt DicF, confirming that the mutations did not substantially alter DicF stability (see Fig. S3). We therefore utilized dicF mutant strains to further probe DicF-associated phenotypes. DicF3 represses ftsZ but fails to repress xylR and pykA, whereas DicF21 (see Fig. S4) does not regulate ftsZ or pykA but retains the ability to silence xylR translation (Fig. 7A). Growing on LB plates, E. coli strains expressing wt dicF or dicF3 were unable to form individual colonies (Fig. 7B; + IPTG). In contrast, cells carrying the vector and cells expressing dicF21 grew similarly on LB plates (Fig. 7B; + IPTG). Strains expressing the same alleles were cultured in liquid LB medium.
without inducer until mid-log phase, and then dicF expression was induced and growth (Fig. 7C) and viability (Fig. 7D) were monitored. Compared to control cells, DicF-expressing cells were severely growth inhibited (Fig. 7C) and viability was reduced by ~10-fold (Fig. 7D). In contrast, DicF3 cells continued to increase in optical density (OD), and CFU counts remained stable over the course of the experiment (Fig. 7C and D). (We note that the phenotype for DicF3-producing cells seems less severe than would be expected based on growth on LB plates [Fig. 7B]. In liquid media, cells were allowed to grow without induction for several generations and then dicF alleles were induced for ~3 h. Cells growing on plates with IPTG expressed dicF immediately and constitutively upon subculture to the plates, which might exacerbate the growth inhibition phenotypes.) Both wt DicF and DicF3 repressed ftsZ, as evidenced by filamentation of cells expressing wt DicF or DicF3 (Fig. 7E). In contrast, cells producing DicF21, which regulates xylR but not ftsZ or pykA, showed growth (Fig. 7C), viability (Fig. 7D), and morphology (Fig. 7E) similar to those seen with control cells. Since wt DicF and DicF3 both repress ftsZ and inhibit cell division, but only wt DicF strongly inhibits growth of E. coli in liquid medium, we infer that DicF-dependent regulation of targets other than ftsZ (disrupted by the mutation in DicF3) substantially contributes to growth inhibition of cells by wt DicF. In other words, while DicF repression of ftsZ certainly inhibits cell division, this regulatory interaction does not account for the toxicity of DicF in E. coli cells.

Microscopy revealed subtle differences in morphologies between cells expressing wt DicF and those expressing DicF3 (Fig. 7E). Cells producing wt DicF were highly filamentous and also had a "bloated" morphology (Fig. 7E), appearing greater in width (diameter) than control cells. Cells expressing DicF3 were also filamentous but appeared to be more uniform and similar in width to control cells (Fig. 7E). DicF3 does not regulate xylR or pykA, so, formally, loss of repression of these targets could account for differences in morphology between DicF- and DicF3-expressing cells. However, xylR and pykA mutants have no overt growth phenotypes in the rich LB medium used for this experiment (46); thus, it is unlikely that regulation of these targets accounts for the loss of viability and the more dramatic effects on morphology in DicF-producing compared to DicF3-producing cells. We hypothesize that the mutation in DicF3 also relieves regulation of other gene products that impact cell shape or cell wall structure and that this accounts for the observed differences.

Physiological effects of DicF and DicB. DicF and DicB are produced from the same operon, and both have been reported to inhibit cell division (14). To further explore the physiological functions of these products, we examined growth phenotypes of cells expressing dicF and/or dicB in the context of the intact operon. Since no signal that induces expression of the dicBF operon has been identified (it is not induced by SOS-inducing compounds [25]) (P. T. Ragunathan and C. K. Vanderpool, unpublished data), we inserted an inducible, Plac promoter upstream of ydfA (Fig. 8A), thus replacing the promoter that is repressed by DicA and DicC (47, 48). We made constructs with a deletion of dicF or of dicB or of both genes (as previously described [49]), leaving only an 82-nt “FRT scar” sequence at each deletion site (Fig. 8A). We then assayed the growth of these strains on LB agar and in LB liquid medium upon induction of the operon. Induction of the wt operon (dicF positive [F+] and dicB+ [F+ B+] ; Fig. 8A) was extremely toxic, and no growth was observed on plates (Fig. 8B; + IPTG). Deletion of dicB (F-B-) in the context of this inducible operon largely relieved growth inhibition, whereas deletion of dicF (F-B-) allowed only very slight growth (Fig. 8B). Finally, deletion of both dicF and dicB (F-B-) relieved the growth inhibition, indicating that dicF and dicB are primarily responsible for the toxicity conferred by this operon. Using liquid medium, we observed that even in the absence of the inducer, the F+B+ strain was slightly growth inhibited (see Fig. 5SB in the supplemental material). When strains were grown in LB liquid medium and exposed to inducer in early log phase, the F+B+ strain was severely growth inhibited (Fig. 8C) and showed 100-fold-reduced viability after ~2.5 h of induction (Fig. 8D). The F+B+ strain also showed the extensively
filamented and bloated morphology (see Fig. S5C) observed in DicF-overproducing strains (Fig. 7E). The induced F^+H11002B^+H11001 strain was still growth inhibited but not as severely growth inhibited as the F^+H11001B^+H11001 strain (Fig. 8B). The F^+H11002B^+H11001 cells showed an ~10-fold reduction in viability (Fig. 8D) and displayed extensive filamentation (see Fig. S5C). Growth of the F^+H11001B^+H11002 cells was more inhibited than that of the control (wild-type [WT] and F^+H11002B^+H11002; Fig. 8C) strains, but the cells were less growth impaired than cells of either the F^+H11001 strain or the F^+H11002 strain (Fig. 8C). Moreover, F^+H11002 cells did not exhibit a decrease in CFU counts per milliliter at 2.5 h postinduction and in fact had grown to nearly wt levels by the end of the experiment (Fig. 8D; compare WT and F^+H11002 results). Consistent with this observation, the F^+H11002 cells were not filamentous (see Fig. S5C). In contrast with the DicF-associated filamentation observed when DicF was ectopically expressed on its own (Fig. 7), these results suggest that, expressed in the context of the intact operon, the presence of DicF by itself (i.e., without DicB) is not sufficient to strongly affect cell division. Instead, when the entire operon is induced, the cell division defect and most of the toxic effects appear to be due to the presence of DicB. Using Northern blots, we assessed levels of DicF in wt, F^+H11001B^+H11001, F^+H11002B^+H11001, F^+H11001B^+H11002, and F^+H11002B^+H11002 strains as well as in a strain expressing DicF from the Plac-dicF plasmid. While DicF was readily detected in cells carrying the Plac-dicF plasmid, it was not detected during IPTG-mediated induction of the chromosomal constructs shown in Fig. 8A (data not shown). This strongly suggests that the constructs illustrated in Fig. 8A do not produce DicF at levels sufficient to reproduce the DicF-associated phenotypes shown in Fig. 7. The F^+H11002 cells were not growth inhibited (Fig. 8C), and both CFU counts per milliliter (Fig. 8D) and morphology (Fig. S5C) resembled those seen with the wt strain.

**The dicF gene and the dicBF operon are present in many E. coli strains.** Since ectopic expression of DicF or the dicBF operon is toxic to E. coli cells, we wondered whether this sRNA or this operon would be conserved in other E. coli strains. The genetic region encompassing dicBF is similar to the bacteriophage P22 immC locus.
Sequencesthathybridizetoprobesfromthe
dicF
regionwerepreviouslyidentified
inseveral
E. coli
and
Shigella
species, but these sequences were not fully characterized
(12). More recently, conservation of sRNAs, including DicF, was examined for 27
E. coli
and
Shigella
strains (50). In this study, DicF was identified as a conserved sRNA in 17 of
the 27
E. coli
and
Shigella
genomes analyzed. We chose 10 different
E. coli
strains with
DicF-like sRNAs for further analysis. Sequences 5 kb upstream and downstream of the
predicted 5’=end of
dicF
were obtained from these 10 genomes and aligned using
Progressive Mauve (51), and genes neighboring
dicF
were examined (Fig. 9). First, we
noted that
dicF
and parts of the
dicBF
operon were present in multiple contexts within
different prophages (Fig. 9). Second,
E. coli
strains carrying prophages closely resem-
bling Qin (defined by NCBI and BioCyc annotations) from
E. coli
K-12 (UMNF18, CFT073,
IAI1, ATCC 8739, and APEC 078) possessed the same
dicF
flanking genes, namely,
ydfA,
ydfB,
ydfC
upstream and
dicB
and
ydfD
downstream (except the ATCC 8739 strain, which lacked
dicB).
Third, some pathogenic
E. coli
strains (the O157:H7 Sakai and
O157:H7 EDL933 strains) contained multiple copies of
dicF
and parts of the
dicBF
locus
in
different prophages (Fig. 9). Thus, it appears that despite their toxicity,
dicF
and the
dicBF
operon are widely retained in resident prophages in many
E. coli
strains and that
some strains in fact have multiple copies of this locus.

**DISCUSSION**

Despite being one of the earliest sRNAs identified in bacteria, DicF has remained poorly
characterized for more than two decades. In this study, we directly tested and validated
the hypothesis that DicF regulates
ftsZ
translation by an antisense base pairing mech-
anism. Moreover, we define two additional targets of DicF,
xyIR
and
pykA
mRNAs, and
show that different regions of DicF are responsible for regulating different targets. The
3’ end of DicF base pairs with
ftsZ
and
pykA
mRNAs, while the 5’ end is required for
pairing with
xyIR
mRNA. Analyses of viability and morphology of cells expressing DicF
revealed that wt DicF is toxic, causing both filamentation and loss of viability (Fig. 7).
Using mutant \textit{dicF} alleles, we demonstrated that repression of \textit{ftsZ} mRNA is responsible for filamentation but that the bloated cell morphology and loss of viability must be due to regulation of other unidentified targets (Fig. 7). When the entire operon containing \textit{dicF} was ectopically expressed, toxicity that was even more dramatic than that seen with expression of \textit{dicF} alone (Fig. 7) was observed (Fig. 8). Cell viability was reduced by more than 2 orders of magnitude when the entire \textit{dicBF} operon was expressed, and this effect was due in large part to \textit{dicB} (Fig. 8D). Interestingly, we identified DicF and DicB homologs in numerous \textit{E. coli} chromosomes, sometimes in multiple independent prophage-encoded loci (Fig. 9). In contrast, BLAST searches of bacteriophage genome databases (NCBI and EMBL, containing ~2,000 sequenced phage genomes) failed to identify hits with significant similarity to DicB or DicF (data not shown). These observations hint that whatever function the \textit{dicBF} operon served in the ancestral bacteriophage was less useful than the function it serves in extant \textit{E. coli} hosts, where it is conserved in the context of defective prophages.

It is interesting to contemplate how sRNAs like DicF, which were brought into bacterial chromosomes on mobile genetic elements, have evolved to regulate bacterial genes, including some in the “core genome” (the set of genes shared by all strains of a given species). The InvR sRNA is carried on \textit{Salmonella} pathogenicity island 1 (SPI1), and its expression is controlled by the major SPI1-encoded virulence regulator HilD (52). InvR, in turn, regulates the \textit{Salmonella ompD} gene, encoding the abundant outer membrane porin OmpD (52). Another example is IpeX sRNA, which is carried on the \textit{E. coli} cryptic prophage DLP12 (53). IpeX represses translation of \textit{E. coli ompC} and \textit{ompF} mRNAs, coding for the two major \textit{E. coli} porins (53). Like DicF, both InvR and IpeX require Hfq for the base pairing-dependent regulation of at least some of their target mRNAs (52, 53). These observations suggest that horizontally acquired regulatory RNAs have evolved to use host cofactors (e.g., Hfq and RNase E) in order to regulate genes on the host chromosome. Maintenance of these regulatory interactions through evolutionary conservation likely reflects that the regulation improves the fitness of the host under some conditions. Understanding those conditions and the fitness benefits conferred is an ongoing challenge.

The considerations mentioned above become even more complex when we consider that the \textit{dicBF} locus is present in multiple copies in several \textit{E. coli} strains (Fig. 9). In addition to being present in Qin-like prophages (as in \textit{E. coli} K-12), the \textit{dicBF} locus is found in prophage islands dubbed “CP-933,” for “cryptic prophage 933” (21, 54, 55). The CP-933O, CP-933M, and CP-933P islands that carry \textit{dicBF} are lambdoid prophages that are \textasciitilde{} 80 kb, 45 kb, and 57 kb in length, respectively (21, 55). Interestingly, the CP-933O prophage is thought to be a fusion of at least 2 prophages, one of which contains Qin-like genes (54, 55). Although these prophages are defective, their genomes are twice the size of Qin and are as large as or bigger than the genome of the ancestral lambda-like phage. A study analyzing the prophages of \textit{E. coli} O157:H7 revealed that some defective prophages actually retain the ability to be excised and can be transferred to other bacteria (56). Thus, although these prophages may contain some mutations or losses that prevent their full functionality as lambdoid phage, some clearly retain functions that could facilitate spread of their genes to other strains. It would be fascinating to study how newly horizontally acquired sRNA regulators integrate into the host’s existing regulatory circuitry.

We observed that, expressed ectopically, DicF and DicB are independently toxic to \textit{E. coli}. Expressed together by induction of the \textit{dicBF} operon from a heterologous promoter, their effects are compounded and reduce viability of cells by more than 100-fold. Our studies showed that wt DicF caused filamentation and a morphological defect suggestive of problems with the cell wall (Fig. 7E). Cells expressing a DicF mutant that still regulated \textit{ftsZ} were filamentous but otherwise had normal morphology and continued to increase in biomass (Fig. 7). Our interpretation of these results is that DicF targets other as-yet-unidentified genes involved in cell shape or chromosome segregation, since mutations in such genes have been shown to yield an elongated and bloated morphology (57) similar to that of cells expressing DicF. The only reported
function of DicB is inhibition of FtsZ ring assembly through interactions with MinC (58–61) and ZipA (62). We found that ectopic expression of DicB is very toxic (Fig. 8). Whether this toxicity is due simply to inhibition of cell division, which eventually causes lysis, or to other unknown functions of DicB remains to be discovered.

Toxin-antitoxin (TA) systems represent another notable example of toxic prophage-encoded functions that are evolutionarily conserved on bacterial chromosomes (63). The toxins of TA systems become active only under certain conditions, where they inhibit growth and can contribute to a dormant state that is associated with bacterial persistence (64). Like TA systems, the dicBF operon is not active under standard laboratory growth conditions, owing to repression by DicA (48). We speculate that, similarly to TA systems, the dicBF operon is expressed under very specific environmental conditions and that the activities of the sRNA DicF and small protein DicB are beneficial to the host (on either the single-cell level or population level) under those conditions. Wang et al. (25) published a study consistent with this hypothesis in which they showed that the cryptic prophages in E. coli enhanced resistance to a variety of environmental stresses. They reported that Qin prophage and, specifically, DicB increased the resistance of E. coli to certain β-lactam antibiotics (25). Though we could not reproduce this particular result (data not shown), perhaps due to our use of a different strain background, it remains our hypothesis that the dicBF locus is conserved in numerous E. coli strains because it confers a fitness advantage under some conditions.

Finally, it is worth noting that limitations of widely used experimental and computational techniques inhibit faster progress in defining functions for bacterial sRNAs. In this study, we took a combined approach, using both RNA-Seq and biocomputational algorithms to identify DicF targets. Of 17 target candidates that we selected for further validation (based on experimental or computational predictions), we found only 2 new mRNAs that are directly regulated by base pairing with DicF (using a criterion of ≥2-fold regulation). Genetic and phenotypic analyses suggest that there are additional DicF targets that play important roles in the physiological effect of DicF. Transcriptomic approaches, including use of microarrays (65) or RNA-Seq (2, 66, 67), are certainly powerful approaches for defining sRNA target candidates, but they miss targets expressed at low basal levels or whose mRNA stability is not substantially changed by interactions with the sRNA. Moreover, there are often abundant indirect effects on gene expression from even short-term ectopic expression of sRNAs. Computational algorithms can in some cases accurately predict mRNA targets for sRNAs. Indeed, xylR was accurately predicted as a DicF target by computational prediction but did not appear in the RNA-Seq data because it was not highly expressed under our experimental conditions. However, this example represents an exception, since other computationally predicted targets were not posttranscriptionally regulated by DicF (Table 1 and Fig. 1). These results highlight the high rates of false negatives and false positives produced even using a combination of global approaches for sRNA target identification, and this has been typical of our experience in characterization of several sRNAs in E. coli (reference 68 and unpublished results). Development of new tools for the more rapid and accurate characterization of sRNA target regulons would greatly facilitate efforts to define functions for hundreds of fascinating bacterial sRNAs.

**MATERIALS AND METHODS**

**Strain and plasmid construction.** All strains and plasmids used in this study are summarized in Table S3 in the supplemental material, and the oligonucleotides (obtained from IDT) used in this study are listed in Table S4. The strains used in this study are derivatives of the Δlac DJ480 strain (D. Jin, National Cancer Institute), which was derived from MG1655. All lambda red recombination methods were performed as described in reference 69.

The predicted targets of DicF were recombined into PM1205 as described in reference 32. Briefly, sequences of targets spanning the +1 site to 10 or 20 amino acids of coding sequence were amplified with oligonucleotides with homology to the PBAD and lacZ sequences. Lambda red recombineering was used to generate the fusions described for Fig. 1. Sequences of the carb (O-DB429/430), rlmN (O-DB433/439), pgaA (O-DB427/428), ppp (O-DB425/426), psiE (O-DB391/392), clcA (O-DB393/394), glmS (O-DB441/442), cyoA (O-DB445/446), pykA (O-DB475/476), rbsD (PR108/109), rbsA (O-DB414-a/415), ptsP (O-DB447/...
recombineering of a transduction into DB189, DB228, and DB229 to yield DB206, DB238, and PR127, respectively.

The qin:kan deletion in Fig. 2 was created by lambda red recombination using primers O-DB413/O-DB414. This mutation was then moved into DB189 (xylR::lacZ), DB228 (pykA::lacZ), and DB229 (ftsZ::lacZ) via P1 transduction to create DB120, DB237, and PR124, respectively. The ftsZ::lacZ fusion containing only 4 amino acids (DB229) and ftsZcomp23::lacZ (PR130) were created in PM1205 as described above using oligonucleotides O-DB503/O-DB504 and O-PR151/O-PR152, respectively. The xylRcomp11::lacZ (DB202) fusion contained the +1 site to the first amino acid of xylR and was created with oligonucleotides O-DB385 and O-DB436. The xylRcomp17::lacZ fusion (DB227) was constructed in PM1205 with primers O-DB385 and O-DB458 (containing the xylR11 mutation). The rne131 mutant obtained from the Masse laboratory (EM1377 [33]) was linked to a kanamycin resistance cassette (inserted in the intergenic region between rne131 and the downstream fill gene) by Maksym Bobrovskyy in our laboratory to yield strain MB10 (68). This mutation was then moved into DB189, DB228, and DB229 to yield DB207, DB239, and PR125, respectively. Similarly, the Δhfr:cat mutation (37) was moved by P1 transduction into DB189, DB228, and DB229 to yield DB206, DB238, and PR127, respectively.

The ΔxylR strain was obtained from the Keio collection (46). The DB176 strain was created by recombineering of a dicF::kan PCR product amplified using O-DB358/O-DB359. The pykAcomp23::lacZ fusion was made as described above using oligonucleotides O-DB475/O-DB458. A Pmar promoter sequence linked to a chloramphenicol resistance cassette (70) was amplified using oligonucleotides O-DB479 and O-DB480 and recombined into a lac+ strain to make DB240. PCR products generated using oligonucleotides O-DB508/O-DB509 (to make a ΔdicF::kan deletion), and O-DB521/O-DB522 (to make a ΔdicF::lac deletion), were recombined independently into DB240 to create DB241 and DB252. Further, the kanamycin cassettes in DB241 and DB252 were removed using pCP20 (49), generating strains DB243 (ΔdicB) and DB247 (ΔdicF), respectively. Lastly, the dicF::kan PCR product was once again recombined into DB243 and the kanamycin cassette removed to create the double ΔdicF ΔdicB mutant (DB248).

The Pmar-vector and the Pmar-dicF plasmids were obtained from the Gottesman laboratory (45). The Pmar-dicF mutant alleles used in this study were generated using a QuikChange mutagenesis II kit (Stratagene). The oligonucleotides used to create the individual mutants are described in Table S4.

The Cp19-xylR strain was made by amplifying the kanamycin-linked Cp19 promoter from JNB304 (33) using oligonucleotides O-DB463 and O-DB464 and by recombineering of the linear PCR product in place of the xylR promoter. Cp19-xylR was moved to a lac+ strain to create DB223. The Cp19-pykA lac+ strain (DB224) was created in a similar manner using oligonucleotides O-DB459 and O-DB460. Strains DB223 and DB224 were used in experiments represented in Fig. S1A and S1B in the supplemental material, respectively.

Computational predictions of DicF targets. The four sRNA target prediction programs, TargetRNA (26), IntaRNA (27), StarPicker (29), and CopraRNA (28), were used to generate lists of potential DicF targets. The search window interrogated for potential interactions with DicF was set at 100 nucleotides (nt) upstream of the start codon to 20 amino acids into the coding sequence. Genes with interactions that were predicted by at least two programs with a P value of ≤0.05 were chosen for further analyses.

RNA-Seq experiments and data analyses. A ΔdicF lac+ strain harboring the vector control or the plasmid containing dicF was grown to an optical density at 600 nm (OD600) of ~0.1 in LB with ampicillin. Three biological replicates were performed. IPTG (0.5 mM) was added to the cultures to induce DicF production. RNA was harvested 20 min after induction, treated with DNase (Ambion), and checked for integrity on a 1% agarose gel. Library construction and sequencing were performed at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana—Champaign. Ribosomal RNA was removed from 1 μg of total RNA using a Ribozero rRNA Removal Meta-Bacteria kit (Epicentre Biotechnologies), and the mRNA-enriched fraction was converted to indexed RNA-Seq libraries with a ScriptSeq v2 RNA-Seq library preparation kit (EPICENTRE Biotechnologies). The libraries were pooled in equimolar concentrations and were quantitated by quantitative PCR (qPCR) using a library quantification kit (Illumina compatible; Kapa Biosystems) and sequenced for 101 cycles plus 7 cycles for the index read using a HiSeq 2000 sequencing system and TruSeq SBS version 3 reagents. The Fastq files were generated with Casava 1.8.2 (Illumina). The computational program Rockhopper (31) was used to analyze the RNA-Seq data. Details of normalization procedures can be found in the publication describing Rockhopper (31). The cutoffs of >=100 normalized sequence reads and >=2-fold differential expression were chosen based on our experience with other sRNAs. We have found that results from candidate targets showing very low normalized read counts or low fold changes between control and experimental samples in RNA-Seq experiments are more likely to be false positives. RNA-Seq data were submitted to the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI).

β-Galactosidase assays. Strains were grown overnight in Terrific broth (TB) medium (with 100 μg/ml ampicillin for plasmid selection) and were subcultured into fresh media with antibiotics and grown to mid-logarithmic phase. When cultures reached an OD600 of ~0.3, IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma Aldrich) was added at a final concentration of 0.1 mM. Samples were harvested after 1 h and assayed for β-galactosidase activity as described previously (71). All experiments were conducted in triplicate.

RNA extraction and Northern blot analysis. Strains carrying plasmids were grown overnight in LB with ampicillin. They were then subcultured into fresh media with antibiotics and grown to mid-log phase. When cultures reached an OD600 of ~0.3, 0.5 mM IPTG was added, and samples were harvested at different time points. RNA was extracted by the hot phenol method as described in reference 72.
Northern blot analysis was carried out as described in reference 73. Briefly, 7 μg total RNA (for DicF) or 10 μg total RNA (for xylR and pykA mRNAs) was run on acrylamide gels and 1% agarose gels using 1× Tris-acetate-EDTA (TAE) or 1× MOPS (morpholinepropanesulfonic acid) buffer, respectively. RNA in acrylamide gels was transferred to a 0.2-μm-pore-size membrane (Whatman) in 0.5× TAE buffer by electrophoresis. RNA in agarose gels was transferred by capillary transfer using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Following transfer, the membranes were probed overnight with biotinylated DNA oligonucleotides (IDT) complementary to the respective RNAs. Detection was carried out according to the instructions for the Brightstar Biodetect kit (Ambion).

**LB growth inhibition.** Strains carrying plasmids with wt dicF, dicF mutants, or chromosomal P_xyl dicF constructs were grown to an OD_600 of ~0.1. IPTG (0.5 mM) was used to induce expression of DicF or the dicF operon. Growth was monitored over time by measuring the OD_600 of cultures until they reached stationary phase.

**Xylose, glucose, and fructose growth inhibition.** ΔdicF ΔlacI strains harboring the vector or the dicF, dicF3, or dicF9 plasmid were streaked on M63 xylose, glucose, and fructose medium with and without 0.5 mM IPTG. Plates were imaged after 22 h of incubation.

**Phase-contrast microscopy.** Cultures were grown to the indicated time points. Cell cultures (500 μl to 1 ml) were collected by centrifugation. The cell pellet was resuspended with 1× phosphate-buffered saline (PBS), washed, and resuspended in 1× PBS. The resuspended cells (1 μl) were pipetted onto a 24- by 50-mm no. 1.5 coverslip (Fisher Scientific; catalog no. 12-544E). A 1.5% agarose gel pad (in 1× PBS) was laid on the cells for immobilization. Cells were then imaged using an inverted epifluorescence microscope (Nikon Instruments Eclipse E800-E) and an electron microscopy charge-coupled-device (EMCCD) camera (Photometrics; Cascade 512). A 1×1 numerical aperture (NA) 1.40 oil immersion phase-contrast objective (Nikon Instruments Plan Apo 100×/1.4 Oil) was used in conjunction with a ×2.5 lens in front of the camera. The microscope and camera were controlled using Metamorph software (Molecular Devices). Each sample was imaged at multiple locations.

**Microarray data accession number.** RNA-Seq data were submitted to the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/geo/); the GEO accession number is GSE76916.

### SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at [http://dx.doi.org/10.1128/mSystems.00021-15](http://dx.doi.org/10.1128/mSystems.00021-15).

- Figure S1, PDF file, 0.3 MB.
- Figure S2, PDF file, 0.6 MB.
- Figure S3, PDF file, 0.2 MB.
- Figure S4, PDF file, 0.02 MB.
- Figure S5, PDF file, 1.9 MB.
- Table S1, DOCX file, 0.02 MB.
- Table S2, XLSX file, 0.4 MB.
- Table S3, DOC file, 0.1 MB.
- Table S4, DOCX file, 0.02 MB.

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### REFERENCES


