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Research Letter

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ydfD encodes a novel lytic protein in *Escherichia coli*

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One sentence summary: Newly characterized putative transmembrane protein YdfD from *Escherichia coli* can induce cell lysis in a cell division-dependent manner.

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ABSTRACT

Bacteria carry a number of genes that cause cell growth arrest or cell lysis upon expression. Notably, defective prophages retain many lysis proteins. Here, we identified a novel lytic gene, *ydfD*, on the Qin prophage segment of the *Escherichia coli* genome. YdfD lyses 99.9% of cells within 2 h of its induction. The co-expression of the upstream gene, *dicB*, encoding a cell division inhibitor, as well as *sulA*, encoding another cell division inhibitor, abolished YdfD-induced cell lysis. These results imply that YdfD-induced lysis is a cell division-dependent event. We further found that by deleting the hydrophobic 22-residue N-terminal domain, the resulting 42-residue C-terminal domain was still toxic to cause cell lysis. We propose that YdfD, associated with the cytoplasmic membrane, inhibits an essential cellular process(s).

Keywords: YdfD; prophage; lysis; *Escherichia coli*

INTRODUCTION

Bacterial chromosomes carry multiple prophage segments on their genomes (Casjens 2003). It is estimated that 10%–20% of *Escherichia coli* genomes are prophage-related sequences. Notably, many of the defective prophage segments retain genes responsible for cell lysis and genes encoding toxic proteins (Srividhya and Krishnaswamy 2007; Yamaguchi, Park and Inouye 2011). Their roles in the host's physiology and evolutionary dynamics remain largely unknown. However, an increasing number of evidences suggest that these toxic proteins encoded by the prophage genes exert a significant impact on the host's fitness (Wang, Kim and Wood 2009; Wang *et al.* 2010).

At the end of the lytic cycle, phages escape from their hosts by inducing host cell lysis. Bacterial cell lysis is required for the release of viral particles from the host cells. Bacteriophages and active prophages lyse host cells by two distinct mechanisms; the holin–endolysin system and the single protein system (Wang, Smith and Young 2000). The holin–endolysin system is constituted by a protein-forming membrane pores and a cell wall-degrading enzyme. A pore-forming protein allows secretion of cell wall-degrading proteins into the periplasmic space from the cytoplasm, where cell walls will be degraded. In contrast, the single protein system does not require pore-forming proteins (Henrich, Lubitz and Plapp 1982; Coleman, Inouye and Atkins 1983). Examples of the single protein system include lysis protein E from bacteriophage φX174, Lysis (L) from MS2 phage and Kil protein of bacteriophage λ (Walderich and Holtje 1989; Conter, Bouche and Dassain 1996; Bernhardt, Roof and Young 2000). Single lysis proteins are small, and typically comprised of fewer than 100 amino acid residues. They are often associated with the membrane through a membrane-spanning domain (Walderich *et al.* 1988). Lytic proteins themselves do not

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possess any enzymatic activity (Coleman, Inouye and Atkins 1983).

Lysis proteins lyse the host cells by inducing the host's autolysis system (Lubitz, Halfmann and Plapp 1984; Halfmann and Lubitz 1986; Walderich *et al.* 1988). The L protein of MS2 expression was shown to induce structural changes of the murein, which triggers autolysis (Walderich *et al.* 1988). The expression of the L protein was also shown to increase the number of adhesion sites between inner and outer membranes (Walderich and Holtje 1989). However, the mechanisms for the induction of autolysis and the structural changes of cell wall caused by the L protein remain unknown. The lysis protein E from bacteriophage φX174 is also a small transmembrane protein (Altman *et al.* 1985). It inhibits translocase I (MraY), an essential enzyme that catalyzes the formation of the first lipid-linked intermediate in the cell wall biosynthetic pathway (Bernhardt, Roof and Young 2000). Lastly, the Kil protein of λ phage interferes with cell division (Haeusser *et al.* 2014). The Kil protein blocks the proper assembly of the cell division apparatus through interaction with FtsZ and ZipA. Thus, expression of Kil results in cell filamentation and eventual cell death.

Qin is one of the three lamboid prophages on the *E. coli* K12 genome. Unlike Rac prophage, another lamboid prophage that remains excision-proficient, Qin is considered to be defective due to a lack of active integrase (Blattner *et al.* 1997; Liu *et al.* 2015). The excision of Qin so far has not been reported.

Here, we have identified a novel lytic gene in *E. coli*, *ydfD*. The *ydfD* gene is a part of the Qin defective prophage (Zhou and Rudd 2013). We have shown that its expression causes 99.9% of cells to lyse within 2 h of induction. Interestingly, YdfD-induced cell lysis was abolished by co-expression of its upstream gene, *dicB*, or another gene for cell division inhibitor, SulA. We, thus, propose that YdfD-induced cell lysis requires functional cell division. We further demonstrated that the cellular target of YdfD is unique, different from targets of other known single-protein lysis systems.

MATERIALS AND METHODS

Strains and growth conditions

Escherichia coli BW25113 (ΔaraBD) (Datsenko and Wanner 2000) and BL21 (DE3) were grown at 37°C in M9 medium supplemented with 0.2% casamino acids, 1 mM thiamine and 0.5% glycerol. For examining the growth pattern, a designated amount of arabinose and/or IPTG was added to the culture when the OD_{600} reached mid-log phase. Cell morphology was observed using an Olympus BX40 microscope.

Molecular cloning

The full length YdfD was cloned into pBAD24 plasmid and designated as *ydfD_pBAD24*. The truncated mutant of YdfD, lacking the 22-residue N-terminal domain, was constructed by cloning the segment from residue 23 to residue 66 into pBAD24 (*ydfD23–63_pBAD24*) or pET21c plasmid (*ydfD23–63_pET21c*). A fragment containing the coding sequence of both *ydfD* and *dicB* was also cloned into pBAD24 (*dicBydfD_pBAD24*). IPTG inducible expression of *dicB* or *sulA* alone using pCN24N was achieved by using the clones obtained from Aska library (Kitagawa *et al.* 2005).

Transmembrane domain prediction

The transmembrane domain of YdfD was predicted using TMHMM (Sonnhammer, von Heijne and Krogh 1998).

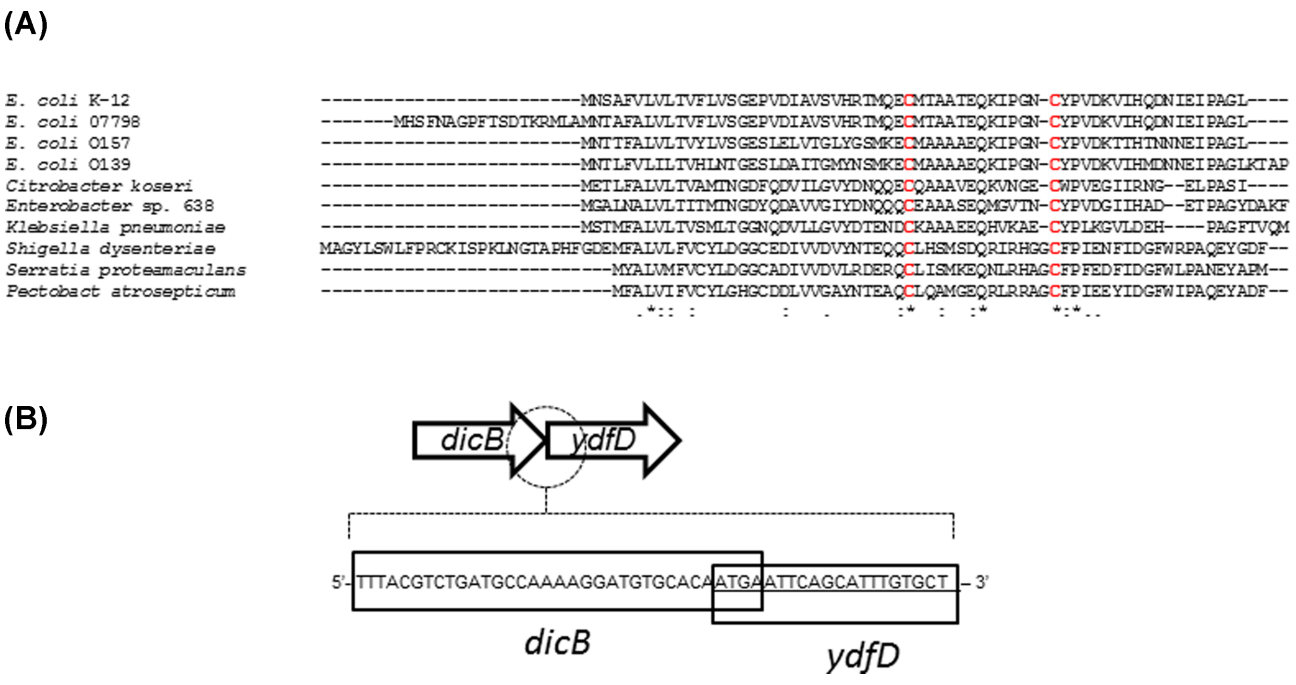

Figure 1. Protein sequence of YdfD and *dicB ydfD* operon structure. (**A**) Alignment of YdfD sequences from enterobacteral strains. Sequences from *E. coli* strains K-12, 07798, O157, O139, *Citrobacter koseri* ATCC BAA-895, *Enterobacter* sp. 638, *Klebsiella pneumoniae* subsp. pneumoniae WGLW2, *Shigella dysenteriae* Sd197, *Serratia proteamaculans* 568 and *Pectobacterium atrosepticum* are shown. (**B**) Overlapping coding sequence of *dicB* and *ydfD*. Sequences corresponding to *dicB and ydfD* are designated by solid lines.

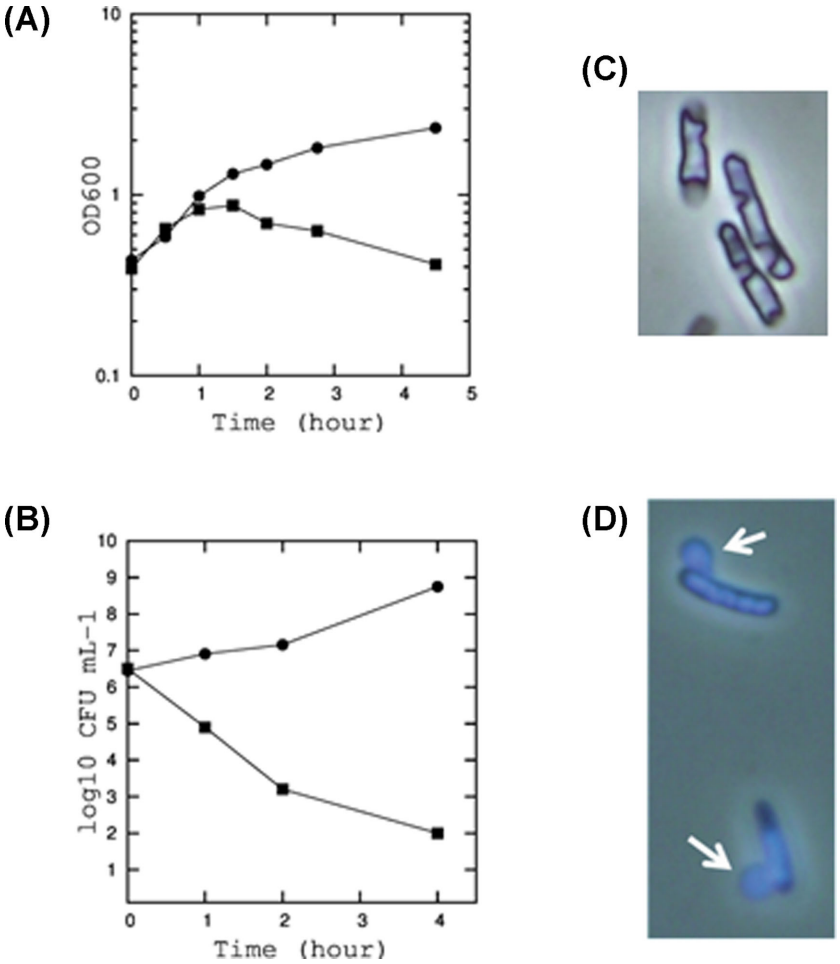

Figure 2. The growth inhibition and cell lysis by YdfD. (**A**) Growth curves of *E. coli* BW25113 expressing YdfD from pBAD24. The cells were incubated in M9 medium. When the optical density at 600 nm reached 0.4, 0.2% arabinose was added (square). Arabinose was also added to the cells containing empty pBAD24 vector as a control (circle). (**B**) The colony-forming unit (CFU) of cells at 0, 1, 2 and 4 h after the induction of YdfD expression (square) and in control (circle). (**C**) Cell morphology observed by a phase contrast microscope. (**D**) Cells stained with DAPI.

RESULTS AND DISCUSSION

YdfD gene structure and evolutionary conservation

The *ydfD* gene encodes for a short peptide, consisting of 63 amino acid residues, with two conserved cysteine residues (Fig. 1A). It is commonly found in sequenced genomes of *E. coli* and related enterobacterial strains and is a part of *dicB-ydfDF-insD-intQ* operon within the defective Qin prophage segment on the *E. coli* K-12 genome (Keseler *et al.* 2013). No homologues were identified in other prophage sequences on the K-12 genome.

The *ydfD* gene is located immediately downstream of a cell division inhibitor, *dicB*. The coding sequences of *dicB* and *ydfD* overlap by 4 bp (Fig. 1B), indicating that the expression of *ydfD* and *dicB* are transcriptionally coupled and under the control of transcriptional repressor, DicA (Béjar and Bouché 1985). The function of YdfD has not been investigated to date.

YdfD induces host lysis

In order to investigate the function of YdfD, we first examined the effects of its expression on *E. coli* physiology by the ectopic expression of YdfD from the arabinose inducible promoter in pBAD24. As seen in Fig. 2A, the induction of YdfD expression causes a rapid decrease in OD_{600} within 1 h. The colony-forming unit (CFU) also dropped significantly, killing 99.9% of cells within 2 h of induction (Fig. 2B). CFU continued to decrease down by four orders of magnitude within 4 h of induction. When examined under a phase-contrast microscope, cells became ruptured (Fig. 2C). We observed one or two incisions in the middle or at the posterior of the cells. Leakages of DNA from these incisions were observed using DAPI (4′,6-diamidino-2-phenylindole) staining (Fig. 2D). These results indicate that YdfD expression causes local cell rupture creating one or two large openings in the cell wall through which cytosolic materials were released. YdfD was also toxic to cells when its expression was induced during the growth on solid media (data not shown).

The C-terminal domain of YdfD is essential for lysis

YdfD is composed of a putative 22-residue N-terminal hydrophobic domain and a 41-residue C-terminal seemingly cytolasmic domain (Fig. 3A). Although the N-terminal domain consists of hydrophobic amino acids, it does not have a feature known for the signal peptide required for protein secretion across the membrane. It seems to be required for the localization of a protein in the inner leaflet of the cytoplasmic membrane. To examine the functional roles of these two domains, we first attempted to clone the C-terminal domain from residue

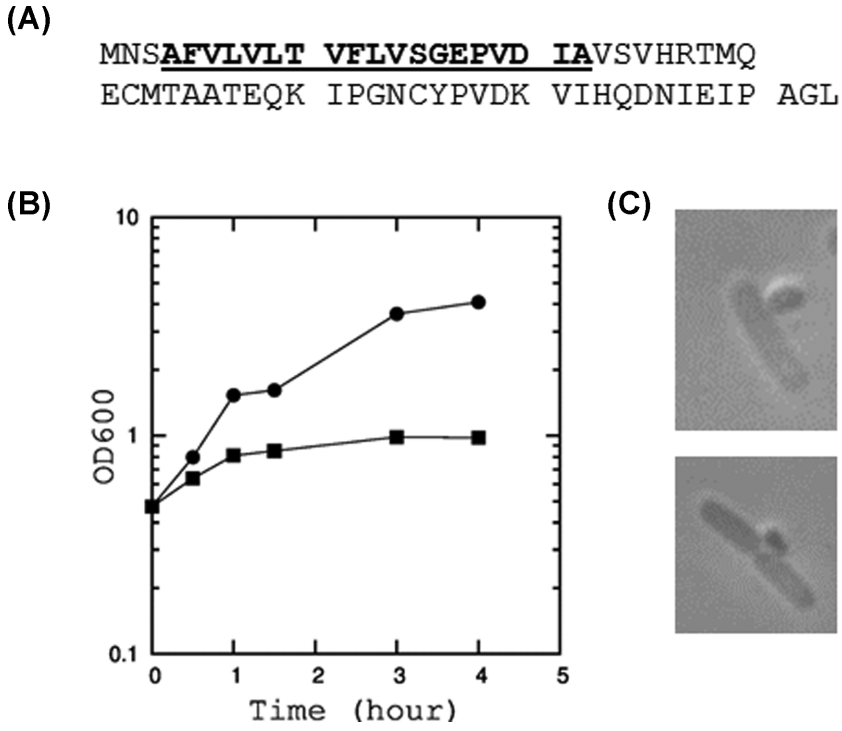

Figure 3. Amino acid sequence of YdfD and the effect of YdfD expression on cell growth and morphology. (**A**) Amino acid sequence of YdfD. The hydrophobic domain is shown in bold and underlined. (**B**) Growth curve of *E. coli* BL21 (DE3) expressing a truncated variant of YdfD, $YdfD_{23–63}$. 0.2% arabinose was added (square) to induce the expression from *ydfD23–63_pBAD24*. Arabinose was also added to the cells containing empty pBAD24 vector as a control (circle). (**C**) Morphology of cells expressing $YdfD_{23–63}$.

23 to 63 ($YdfD_{23–63}$) into expression vectors to express it in a soluble from in the cytoplasmic space. When $YdfD_{23–63}$ was cloned into pBAD24 and its expression was induced, cell growth was almost completely inhibited after 1 h (Fig. 3B), indicating that this 41-residue cytoplasmic fragment retains the toxicity of YdfD. When cells after 1 h induction of YdfD were examined under a microscope, leakage of the cytoplasm is observed, indicating that the cell wall is locally weakened (Fig. 3C). Truncated *ydfD* were also cloned into pET21c for a higher level of expression, and even more severe growth retardation was observed (data not shown). Notably, the secretion of the C-terminal domain in the periplasmic space using pCold-V did not show the toxic effect to the cells, indicating that the target(s) of the C-terminal domain of YdfD resides in the cytoplasm to inhibit a cytoplasmic process(s).

The lower toxicity of $YdfD_{23–63}$ compared to full length YdfD suggested that the membrane localization of YdfD is important for the full YdfD activity. It remains unknown whether the N-terminal domain merely serves as an anchor to the membrane or possesses additional functions.

YdfD-induced lysis is abolished by the co-expression of upstream gene, *dicB*

The *dicB* encodes DicB, an inhibitor of FtsZ polymerization (Cam *et al*. 1988). FtsZ is an essential protein, forming a ring-like structure at the septum and functioning as a scaffold for the assembly of a cell division complex (Bi and Lutkenhaus 1991). The cell division is inhibited when the polymerization of FtsZ is blocked. Hence, the expression of DicB causes extensive cell elongation (Johnson, Lackner and de Boer 2002), and ultimately cell growth is arrested. DicB overexpression is toxic to cells causing cell elongation, but does not cause immediate cell lysis (Fig. 4A).

Since the expression of *ydfD* and its upstream gene *dicB* are coupled, we wondered if there is a synergistic or antagonistic relationship between DicB and YdfD. As shown in Fig. 4B and C, when YdfD and DicB are co-expressed, YdfD overexpression no longer caused cell lysis. However, the cells were still elongated, suggesting that DicB still arrested cell division. This result suggested that YdfD-induced lysis cannot occur when cell division is inhibited. The same result was obtained when two proteins were encoded from a single transcript or when they were cloned separately (data not shown).

FtsZ polymerization inhibitor can inhibit YdfD-induced lysis

Multiple inhibitors of FtsZ polymerization have been described (Huisman, D'Ari and Gottesman 1984; Tan, Awano and Inouye 2011; Masuda *et al*. 2012). We examined if other FtsZ polymerization inhibitors could also abolish YdfD-induced cell lysis. As predicted, SulA did indeed neutralize YdfD-induced cell lysis (Fig. 5), supporting our hypothesis that YdfD-induced lysis is linked to cell division.

The cellular target of YdfD is distinct from the known prophage single protein lytic systems

We further examined whether YdfD may inhibit the same cellular target as other known lytic proteins. First, YdfD was compared with the lysis protein E, a membrane-associated lytic protein from bacteriophage φX174 (Maratea, Young and Young 1985). Lysis protein E inhibits the MraY-catalyzed step of the peptidoglycan biosynthetic pathway in a KBP-type peptidyl-prolyl

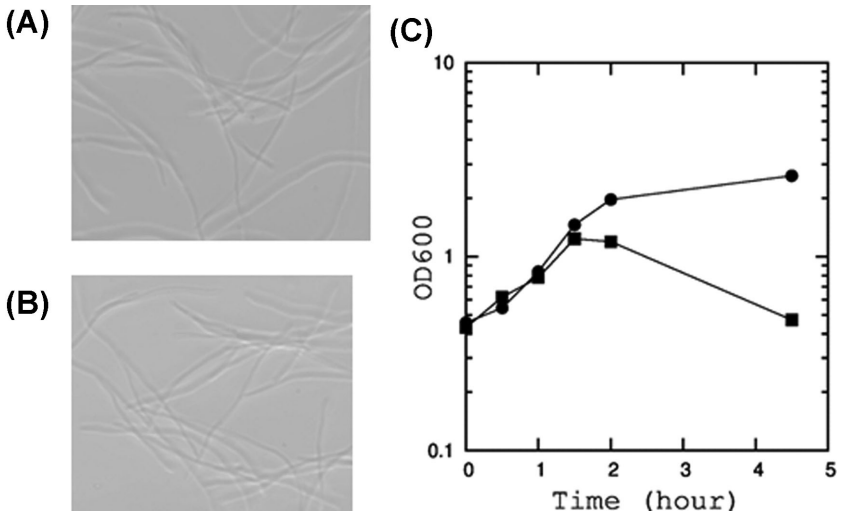

Figure 4. Neutralization of YdfD-induced toxicity by the expression of the upstream gene, *dicB*. (**A**) Cell morphology of *E. coli* BW25113 overexpressing DicB alone from pBAD24. (**B**) Cell morphology of *E. coli* BW25113 overexpressing DicB and YdfD from *dicBydfD*_pBAD24. (**C**) Growth curve of BW25113 expressing YdfD alone or YdfD and DicB. Cells were grown as described in Fig. 2. YdfD was expressed from *ydfD*_pBAD24 (circle). YdfD and DicB were co-expressed from *dicBydfD*_pBAD24 (square) by addition of arabinose at a final concentration of 0.2%.

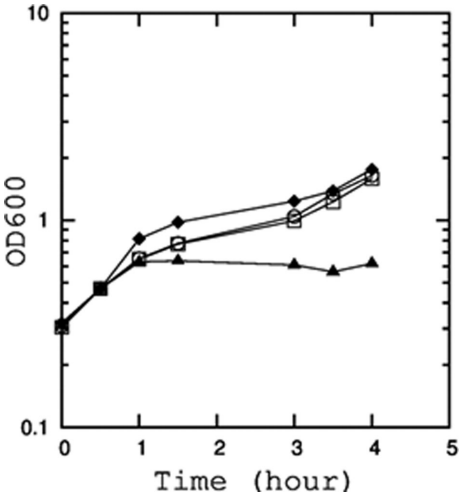

Figure 5. Inhibition of YdfD-induced cell lysis by the co-expression of cell division inhibitor, SulA. Growth curve of *E. coli BL21* (DE3) co-transformed with *ydfD_pBAD24* and *SulA-pCA24N*. The cultures were treated with no addition (open square), 1 mM IPTG (open circle), 0.2% arabinose (triangle), or 1 mM IPTG and 0.2% arabinose (diamond).

cis-trans isomerase (SlyD) dependent manner (Roof and Young 1995). SlyD is proposed to be essential for the stability of lysis protein E (Roof and Young 1995; Bernhardt, Roof and Young 2002), and lysis protein E does not induce lysis in a Δ*slyD* strain (Maratea, Young and Young 1985). Unlike lysis protein E, YdfD remained toxic in the Δ*slyD* strain.

Lysis protein L from RNA phage MS2 causes cell lysis at cell membrane adhesion sites (Walderich and Holtje 1989). When cells were grown in media at pH 5, lysis protein L did not induce cell lysis. In contrast, YdfD remained toxic even when cells were grown at pH 5 (data not shown), indicating that YdfD and lysis protein L utilized different mechanisms. Our results, however, do not rule out the possibility that YdfD may also be localized at the membrane-adhesion sites.

The Kil protein of λ phage inhibits cell division, causing cell elongation. Unlike Kil protein, YdfD does not induce cell elongation. Instead, the inhibition of cell division had a negative effect on the function of YdfD. A homologue of Kil protein is also found in another cryptic lambdoid prophage, Rac, on *E. coli* K-12 genome, located on the equivalent position of *dicB* in Qin. Although there is no sequence similarity between DicB and Kil, they both inhibit cell division by blocking septum formation. In Rac prophage, a small uncharacterized gene, *ydaE,* is found immediately downstream of Kil, which is the equivalent genetic location of *ydfD*. There is also no sequence homology between YdfD and YdaE, however, YdaE also carries conserved cysteine residues. The presence of conserved cysteine residues, small size and transcriptional coupling with cell division inhibitors (DicB and Kil, respectively) suggest that YdfD and YdaE may share a similar function. Further work is required to examine this possibility.

The strong conservation of *dicB- ydfD* operonic structure, despite diverged gene orders in neighboring regions suggests the strong linkage between the two proteins. The operon consisted of a toxic gene and a gene that neutralize the toxin's function is reminiscent of the toxin–antitoxin (TA) systems. It is possible that these two proteins function as a TA pair, ensuring the maintenance of the Qin prophage.

In summary, we have identified a novel lytic protein on the *E. coli* chromosome. Its toxicity can be neutralized by DicB as well as by another cell division inhibitor, SulA, indicating that YdfD-induced lysis is a cell division-dependent event. During cell division, many processes, including the degradation of preexisting cell walls and the synthesis of new cell walls, occur. Such processes have to be both spatially and temporally coordinated (Cabeen and Jacobs-Wagner 2005). Uncoupling any of these processes could lead to rapid osmotic lysis. YdfD appears to inhibit or uncouple such processes to induce cell lysis. We also discovered that the C-terminal cytoplasmic domain is important for YdfD-induced lysis. Our data support that YdfD inhibits a unique cellular target, distinguishable from other known phage lytic proteins. Further research is currently being conducted to

identify the exact cellular target of YdfD and its mechanisms of action.

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