YtfB, an OapA Domain-Containing Protein, Is a New Cell Division Protein in *Escherichia coli*

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ABSTRACT While screening the Pfam database for novel peptidoglycan (PG) binding modules, we identified the OapA domain, which is annotated as a LysM-like domain. LysM domains bind PG and mediate localization to the septal ring. In the Gram-negative bacterium *Escherichia coli*, an OapA domain is present in YtfB, an inner membrane protein of unknown function but whose overproduction causes cells to filament. Together, these observations suggested that YtfB directly affects cell division, most likely through its OapA domain. Here, we show that YtfB accumulates at the septal ring and that its action requires the division-initiating protein FtsZ and, to a lesser extent, ZipA, an early recruit to the septosome. While the loss of YtfB had no discernible impact, a mutant lacking both YtfB and DedD (a known cell division protein) grew as filamentous cells. The YtfB OapA domain by itself also localized to sites of division, and this localization was enhanced by the presence of denuded PGs. Finally, the OapA domain bound PG, though binding did not depend on the formation of denuded glycans. Collectively, our findings demonstrate that YtfB is a cell division protein whose function is related to cell wall hydrolases.

IMPORTANCE All living cells must divide in order to thrive. In bacteria, this involves the coordinated activities of a large number of proteins that work in concert to constrict the cell. Knowing which proteins contribute to this process and how they function is fundamental. Here, we identify a new member of the cell division apparatus in the Gram-negative bacterium *Escherichia coli* whose function is related to the generation of a transient cell wall structure. These findings deepen our understanding of bacterial cell division.

KEYWORDS cell division, septal ring, peptidoglycan, bacterial morphology, OapA domain

Bacterial cell division in the Gram-negative bacterium *Escherichia coli* involves the splitting of a mother cell to generate two equivalent daughter cells. This process is mediated by the septal ring, a multiprotein complex composed of more than 30 proteins that constrict three layers of the cell envelope, the cytoplasmic (inner) membrane, the peptidoglycan (PG) cell wall, and the outer membrane (1–3). Constriction requires the synthesis and splitting of septal PG, new cell wall material laid down between developing daughter cells. Separation of the septum is achieved by the combined action of several classes of cell wall-degrading enzymes, including amidases (4, 5), lytic transglycosylases (6), and endopeptidases (6–8). To maintain synchronous division, the progress of cell division must be communicated among the three layers of the cell envelope. Thus, many septum-localizing proteins are equipped with PG binding domains (e.g., amidase N-terminal [AMIN], LysM, and sporulation-related repeat [SPOR] domains) (9).

Disruption of the operation of the septal network stalls constriction and, depending on the stage affected, causes cells to grow with altered morphology (e.g., as chains of

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unseparated cells, long filaments, etc.). If left unresolved, these delays often result in cell death, a phenotype that has been repeatedly leveraged to identify new cell division proteins. Classically, this screening involved exposing cells to chemical or physical agents (10), and such methods were instrumental in identifying most of the essential cell division proteins (11, 12). One drawback to this approach is that the secondary screen measures growth (e.g., colony formation), but growth is often unaffected in many mutants lacking nonessential cell division proteins. Thus, new methods (e.g., high-throughput microscopy, flow cytometry, etc.) have been used to measure morphology when screening or selecting for mutants (13–18), resulting in the discovery of new factors connected to cell division and morphogenesis. However, many of the morphological phenotypes discovered in these reports have no mechanistic explanation, and some lack functional annotation altogether.

Here, we identify and characterize YtfB from *E. coli* as a new cell division-related protein. YtfB is a bitopic inner membrane protein of unknown function whose notable feature is a C-terminal OapA domain that is annotated as being a LysM-like domain (19). We identified YtfB via its OapA domain while screening the Pfam database for novel PG binding domains (19). YtfB was previously identified in a misexpression screen that identified novel factors affecting cell division (13), though how YtfB does this is unknown. We demonstrate that YtfB localizes to the septum and that a mutant produces a synthetic shape defect with DedD, a cell division protein. In addition, the OapA domain derived from YtfB localizes to sites of septal PG synthesis and binds PG *in vitro*. Interestingly, the OapA domain is not required to localize YtfB to the septum. We conclude that YtfB is a cell division protein whose function is linked to the late stages of cytokinesis.

RESULTS

Search for new PG binding domains identifies the OapA domain. To identify potentially new or overlooked PG binding determinants, we conducted a keyword search in the Pfam database (version 31.0) for domains having similarities to known PG binding modules (19). As our query, we chose the LysM domain, which binds *N*-acetylglucosamine residues found in PG (20) and mediates septal localization (17, 21–24). The search yielded 121 unique domain families across four sections of the Pfam database. As expected, the majority of domains listed were those that cooccur with the LysM domain, many of which directly interact with PG (e.g., cysteine- and histidine-dependent amidohydrolase/peptidase [CHAP], SPOR, soluble lytic transglycosylase [SLT], etc.). However, our interest was piqued by the opacity-associated protein A domain (OapA, PF04225), a domain of unknown function that is described as being a LysM-like domain. The OapA domain gets its name from the *Haemophilus influenzae* protein OapA, which is required for the expression of colony opacity, thus opacity-associated protein A (25). According to the Pfam database, the OapA domain is present in 529 sequences spread across 383 species found almost exclusively in the class *Gammaproteobacteria*. An examination of the domain architectures that include the OapA domain revealed that >70% of the sequences with an OapA domain also contain domains involved in breaking PG cross-links (i.e., M23 and NlpC). Altogether, these observations suggested that the OapA domain may interact directly with PG.

We next conducted a literature search to identify additional phenotypes associated with proteins harboring the OapA domain. Interestingly, a screen to identify factors that affect cell division in *E. coli* discovered that overproducing a fragment of YtfB, including its OapA domain, caused cells to grow as long filaments (13). However, aside from its inclusion in a handful of large-scale studies, little is known about YtfB. Structurally, YtfB is a predicted bitopic inner membrane protein which, in addition to a C-terminal OapA domain, contains an N-terminal opacity-associated protein A N-terminal motif (OapA_N, PF08525) (Fig. 1A) that overlaps the transmembrane sequence. YtfB is nonessential (26), and a deletion mutant exhibits only mild phenotypes when challenged with various chemicals (27). Ribosomal profiling (28) indicates that the abundance of YtfB in the cell is relatively low (~275 to 500 molecules per cell generation), most likely because too

FIG 1 YtfB overproduction disrupts FtsZ ring assembly. (A) Predicted domain architecture of YtfB from *E. coli*. TM, transmembrane domain (residues 34 to 50); OapA, OapA domain (residues 127 to 212). Note that YtfB harbors an opacity-associated A N-terminal like domain (residues 23 to 50) that overlaps the TM domain. (B) Phase-contrast and fluorescence micrographs showing that YtfB overproduction disrupts FtsZ ring assembly. Overnight cultures were diluted 1:100 in LB containing 25 μ M IPTG, grown at 37°C for 2.5 h, and imaged. The fluorescence micrograph was inverted to enhance visualization of the mVenus fusion protein. Arrows point to FtsZ rings. Triangles point to examples of fluorescent foci. The bar represents 5 μ m. The strains shown are MAJ784 (vector only) and MAJ785 ($P_{trc}::ytfB$).

much YtfB filaments the cell (13). Collectively, these findings pointed to some role for YtfB in cell division. Thus, we set out to study the OapA domain in the context of YtfB.

YtfB overproduction causes cells to filament, most likely by disrupting FtsZ ring assembly. To confirm that YtfB overproduction filaments cells, we cloned the full-length protein under the control of isopropyl- β -D-1-thiogalactopyranoside (IPTG). As expected, *ytfB* overexpression filamented cells in a dose-dependent manner (see Fig. S1 in the supplemental material; also, data not shown). These results suggested that too much YtfB disrupts the assembly of the septal ring, so we monitored FtsZ localization in cells overexpressing *ytfB* by using the functional sandwich fusion FtsZ-mVen^{SW} (29). As expected, FtsZ localized to the septum in control cells (Fig. 1B, vector only) but failed to form rings in cells overexpressing *ytfB* (Fig. 1B, $P_{trc}::ytfB$). Interestingly, in 35% of cell filaments, FtsZ-mVen^{SW} formed fluorescent foci ($n = 71$) (Fig. 1B, bottom images). What causes the formation of these foci is unknown. The absence of FtsZ rings in these filaments (Fig. 1B) suggested that YtfB overproduction might have affected FtsZ stability, but Western blotting revealed that YtfB overproduction had no effect on FtsZ levels (Fig. S2). Though we considered it unlikely, we tested whether YtfB induced cell filamentation by triggering the DNA damage response by producing the FtsZ antagonist SulA (30). However, YtfB overproduction readily filamented cells lacking SulA (not shown). Similar results were also obtained in cells lacking the FtsZ antagonist MinC (not shown) (31). Thus, YtfB probably filaments cells by disrupting FtsZ ring assembly

FIG 2 YtfB localizes to the septal ring in *E. coli*. (A) Phase-contrast (left) and fluorescence (right) micrographs of live $\Delta ytfB$ mutant cells producing GFP-YtfB from plasmid pMAJ77. Overnight cultures were diluted 1:50 in LB containing 5 μ M IPTG, grown to an OD_{600} of ~ 0.4 , and imaged. The bar represents 3 μ m. (B) Demographic representation of GFP-YtfB fluorescence intensity from cells ($n = 202$) in panel A. On the y axis, 0 represents midcell and -1 and 1 represent the cell poles. AU, arbitrary units. The strain shown is MAJ678 ($\Delta ytfB$ /pMAJ77).

independent of the SOS response and the Min system (although these studies cannot rule out other indirect effects).

YtfB localizes to the septal ring. To determine if YtfB directly affects cell division by localizing to the septal ring, we constructed an N-terminal green fluorescent protein (GFP) fusion to YtfB (GFP-YtfB) in a vector that allows leaky expression from an IPTG-inducible promoter, P_{204} (32). After purifying the clones, however, we observed the formation of large and small colonies, which were composed of normal and filamentous cells, respectively. While the large-colony phenotype was stable upon repurification, the small-colony phenotype was unstable, giving rise to large colonies. Sequencing revealed that the plasmids derived from the small colonies were error free. Conversely, plasmids isolated from the large colonies harbored mutations in *ytfB*, which, in all cases, encoded protein variants lacking the C-terminal OapA domain. Interestingly, fluorescence microscopy revealed sporadic septal localization of GFP-YtfB in cells grown from the small colonies (data not shown).

To circumvent the selection of inactive YtfB variants, we cloned *gfp-ytfB* into a similar expression vector, except that the production of GFP-YtfB was under strict control of IPTG. Using this approach, we readily obtained clones that were free of mutations. To localize GFP-YtfB, we produced the fusion in cells lacking YtfB ($\Delta ytfB$ mutant, described below) to prevent competition with the native protein. When inducing gene expression, IPTG was added at 5 μ M, because higher concentrations induced filamentation. Strikingly, fluorescence microscopy revealed that GFP-YtfB localized to the septum in 83% of the cells ($n = 202$) (Fig. 2A), suggesting that it was an early recruit to the septosome. Indeed, demographic representation of fluorescence intensities showed septal localization in cells that were nondividing (unconstricted) and dividing (constricted) (Fig. 2). Collectively, the data demonstrate that YtfB localizes to the septal ring prior to the onset of constriction and remains at the midcell throughout cytokinesis.

Recruitment of YtfB to the septal ring requires FtsZ. Maturation of the septal ring occurs in several stages and requires the ordered recruitment of 10 essential proteins and more than 20 nonessential proteins (2). The timing of arrival can provide important functional cues. To determine the placement of YtfB in septal ring assembly, we localized GFP-YtfB in strains in which division proteins were inactivated or depleted. Inactivation of the first division protein FtsZ, but not later protein recruits, prevented the localization of GFP-YtfB (Fig. 3 and Table 1). However, we did observe an $\sim 60\%$ reduction in GFP-YtfB ring formation in cells inactivated for ZipA (an early recruit) when cells were grown under permissive or nonpermissive conditions (Table 1). Inactivation of FtsA or FtsQ had little effect on the formation of GFP-YtfB rings, as did depletion of FtsN (Table 1). Thus, YtfB is an early recruit to the septal ring.

YtfB has a synthetic phenotype with the $\Delta dedD$ mutant. We next sought to determine the morphological effect of deleting *ytfB*. However, visual inspection re-

FIG 3 YtfB requires FtsZ but not downstream division proteins for septal localization. Fluorescence micrographs of cells with the indicated genotypes producing GFP-YtfB from plasmid pMAJ77 grown at 30°C in LB (permissive) or 42°C in LB0N (nonpermissive) medium containing 5 μ M IPTG. Cells depleted for FtsN (*ftsN(dep)*) were grown at 30°C in LB with arabinose (permissive) or glucose (nonpermissive) and 5 μ M IPTG. The percentage of cells producing GFP-YtfB rings is noted in each micrograph and in Table 1. The bar represents 5 μ m. WT, wild type; ts, temperature sensitive. The strains shown are (top to bottom) MAJ646, MAJ696, MAJ712, MAJ697, MAJ699, and MAJ700.

vealed that a *ytfB* null mutation ($\Delta ytfB$) had no discernible impact on morphology (Fig. 4A) or cell size (Fig. 4B) in LB, in LB lacking NaCl, or in minimal medium (not shown). Additionally, no shape or growth defects were observed when cells were cultured at lower (30°C) or higher (42°C) temperatures (not shown). In the absence of any obvious phenotypes, we looked for synthetic phenotypes with known division mutants. While the loss of YtfB had little to no effect on a variety of *fts* mutants (Fig. S3), cells lacking YtfB and DedD (but not other SPOR domain-containing proteins [data not shown]) looked filamented (Fig. 4A). When examined by flow cytometry, the distribution of the side-scatter area of the $\Delta ytfB \Delta dedD$ mutant was shifted to the right compared to that of the $\Delta dedD$ mutant (Fig. 4B), a further indication that the cells were filamented (17). A closer inspection revealed that the $\Delta ytfB \Delta dedD$ double mutant was 65% longer than the *dedD* null mutant, and the standard deviation grew by 154% due to the formation of exceedingly long filaments (Table 2). Since DedD and DamX localize to the septum

TABLE 1 Characteristics of GFP-YtfB ring formation in *fts* mutants

Strain background ^a	Strain	Temp (°C)	NaCl	Sugar ^b	Length (avg ± SD) (μm) ^c	No. of cells evaluated	% of cells with rings	Total no. of rings	Ring spacing ^d
Wild type	MAJ646	30	+	—	5.9 ± 1.4	223	91	203	6.4
		42	—	—	5.4 ± 2.0	216	80	173	6.8
<i>ftsZ</i> (Ts)	MAJ696	30	+	—	6.6 ± 2.3	223	87	195	7.5
		42	—	—	38.9 ± 10.1	101	0	0	>3,933
<i>zipA</i> (Ts)	MAJ712	30	+	—	8.0 ± 3.7	231	26	59	29.2
		42	—	—	39.6 ± 11.7	136	32	43	41.1
<i>ftsA</i> (Ts)	MAJ697	30	+	—	7.3 ± 2.7	200	93	185	7.4
		42	—	—	56.0 ± 15.8	117	78	91	13.1
<i>ftsQ</i> (Ts)	MAJ699	30	+	—	7.0 ± 3.3	255	93	237	7.5
		42	—	—	41.6 ± 18.7	112	86	96	9.5
<i>ftsN</i> (dep)	MAJ700	30	+	Ara	10.1 ± 3.9	202	99	266	7.6
		30	+	Glu	30.3 ± 17.5	157	85	419	11.4

^adep, depletion.^bLB medium was supplemented with 0.2% L-arabinose (Ara) or 0.2% D-glucose (Glu).^cNote the low level of expression of *gfp-ytfB* slightly filamented cells.^dTotal lengths of cells divided by the total number of GFP-YtfB rings.

(33, 34) by binding septal PG (35), we wondered whether the simultaneous absence of YtfB and DedD might have created more sites for DamX binding (assuming that not all DamX is at midcell), which could filament cells (36). However, filamentation was not related to DamX because a loss of DamX did not reverse the distribution of the side-scatter area of a $\Delta ytfB \Delta dedD$ double mutant (Fig. S4, compare $\Delta 3$ to $\Delta ytfB \Delta dedD$). Similarly, these results indicated that the filamentation of a $\Delta damX \Delta dedD$ mutant (33, 34) is not primarily driven by YtfB, since the distribution of the side-scatter area was similar between the triple mutant and the $\Delta damX \Delta dedD$ double mutant (Fig. S4, compare $\Delta 3$ to $\Delta damX \Delta dedD$). However, these studies do not rule out the possibility that other septum-localizing proteins accumulate at the septum and interfere with cell division. Finally, production of the GFP-YtfB fusion protein reversed the cell shape defect of the $\Delta ytfB \Delta dedD$ double mutant, an indication that the fusion was functional (Fig. S5).

The OapA domain preferentially localizes to regions of septal PG synthesis. The Pfam database annotates the OapA domain to be LysM-like. The Protein Homology/Analogy Recognition Engine (PHYRE2) (37) confirmed that the YtfB OapA domain was similar to a variety of LysM domains (not shown). Since LysM domains localize to septal regions (17, 21–24), we reasoned that the same might be true for the OapA domain. To determine if this was the case, we constructed an N-terminal GFP fusion to the OapA domain from YtfB so that it was exported to the periplasm by the twin-arginine transport system (^{TT}GFP-YtfB^{OapA}) via the signal peptide derived from TorA (38). As with the full-length fusion protein, we produced ^{TT}GFP-YtfB^{OapA} in cells lacking YtfB to prevent competition with the native copy of YtfB. As expected, the OapA domain sharply localized to sites of constriction (Fig. 5A, lower images, and B, solid line), whereas Tat-targeted GFP did not accumulate at these positions (Fig. 5A, upper images, and B, dashed line). Interestingly, the OapA domain only localized to septal sites in 43% of the cells ($n = 373$) and, of those, nearly all were highly constricted (Fig. 5A, lower images). Similar localization percentages were observed in wild-type cells (not shown). These results were surprising, since we previously observed that a fluorescent fusion to the full-length protein localized to septation zones in more than 80% of cells (Fig. 2A). To determine if the OapA domain was necessary for YtfB localization to the septal ring, we constructed a GFP fusion to a YtfB variant lacking the OapA domain (residues 1 to 125). Strikingly, we observed that in the absence of the OapA domain, YtfB localized to septal sites in 83% of the cells ($n = 196$) (Fig. S6). Thus, while the OapA domain is sufficient for septal localization, it is not necessary for YtfB localization. This suggests that other features of YtfB contribute to its septal localization.

At this point, two pieces of evidence suggested that the OapA domain localizes to the septum by binding denuded glycans, a transient form of PG that lacks stem

FIG 4 A $\Delta ytfB \Delta dedD$ double mutant forms filaments. (A) Cells with the indicated genotypes were diluted 1:2,000 in LB and grown at 37°C for approximately 10 doublings. The cells were then fixed and photographed by phase-contrast microscopy. The bar represents 3 μ m. (B) Live cells from panel A were examined by flow cytometry. Histograms of the forward-scatter area are from 100,000 events (cells). The dashed line represents the mean cell size of the wild type (WT) expressed in arbitrary units (AU). The strains shown are MAJ1 (WT), MAJ579 ($\Delta ytfB$ mutant), MAJ682 ($\Delta dedD$ mutant), and MAJ684 ($\Delta ytfB \Delta dedD$ mutant). The data are representative of two independent experiments.

peptides due to the enzymatic activity of cell wall amidases (35). First, septal localization of the OapA domain occurred primarily in cells undergoing constriction (Fig. 5A), a process driven by the coordinated activity of cell wall amidases (4, 38). Second, the OapA domain is similar to the LysM domain, which binds the PG backbone (20). Thus,

TABLE 2 Morphological phenotypes of *E. coli* *ytfB* and *dedD* null mutants^a

Genotype	Strain	No. of cells evaluated	Length (avg $\pm$ SD) (μ m)	Width (avg $\pm$ SD) (μ m)	% cells with indicated no. of constrictions		
					0	1	>1
WT	MAJ1	203	3.7 $\pm$ 1.0	1.1 $\pm$ 0.1	73	27	0
$\Delta ytfB$	MAJ579	227	3.8 $\pm$ 1.0	1.1 $\pm$ 0.1	73	27	0
$\Delta dedD$	MAJ682	204	5.4 $\pm$ 1.8	1.0 $\pm$ 0.1	68	30	2
$\Delta ytfB \Delta dedD$	MAJ684	206	10.6 $\pm$ 13.7	1.1 $\pm$ 0.1	53	39	7

to determine if denuded glycans played a role in OapA septal localization, we monitored OapA localization while altering the intracellular levels of denuded glycans. To do this, we first produced $\text{TTGFP-YtfB}^{\text{OapA}}$ in a mutant lacking multiple lytic transglycosylases (Δ LTs), which are required to degrade denuded glycans (i.e., this mutant has increased levels of denuded glycans) (35). Compared to otherwise-normal cells (Fig. 5A, lower images), it was readily apparent that a greater fraction of the OapA domain localized to septal regions in the Δ LTs mutant (Fig. 5C, lower images), which was confirmed by the fluorescence intensity profiles (Fig. 5B and D, compare solid-line profiles). Given these results, we reasoned that if increasing the levels of denuded glycans promotes OapA septal localization, decreasing their levels would have the opposite effect. As expected, there was virtually no OapA septal localization in a triple amidase mutant (Δ amiABC), which contains no denuded glycans (Fig. 5E, lower images, and F). Since AmiB and AmiC accumulate at the septum (38, 39), this left open the possibility that the OapA domain localizes by an interaction with one (or more) amidase. However, $\text{TTGFP-YtfB}^{\text{OapA}}$ localization in mutants lacking AmiAC, AmiAB, or AmiBC was similar to that of normal cells (see Fig. S7; compare to the results shown in Fig. 5A and B), demonstrating that no one amidase was required to localize the OapA domain to the septum. We also measured the levels of $\text{TTGFP-YtfB}^{\text{OapA}}$ across the suite of PG hydrolase mutants and found that the fusion protein was produced in comparable amounts in cells lacking YtfB (Fig. S8), indicating that changes in $\text{TTGFP-YtfB}^{\text{OapA}}$ localization were not due to protein instability. *In toto*, these results demonstrate that the generation of denuded glycans by the enzymatic activity of amidases is necessary for localizing the OapA domain to the septum, but the domain is not necessary for YtfB localization.

The OapA domain binds PG. Finally, we determined if the OapA domain binds PG. For these studies, we cloned and purified a variant of the OapA domain (residues 117 to 212) harboring an N-terminal hexahistidine tag ($\text{His}_6\text{-YtfB}^{\text{OapA}}$). In a standard pull-down assay, $\text{His}_6\text{-YtfB}^{\text{OapA}}$ bound sacculi extracted from wild-type (WT) cells (Fig. 6), with approximately 34% of the $\text{His}_6\text{-YtfB}^{\text{OapA}}$ present in the pellet (PG) fraction. Since $\text{TTGFP-YtfB}^{\text{OapA}}$ localization was enhanced in cells harboring increased levels of denuded glycans (Δ LTs), we reasoned that the purified $\text{His}_6\text{-YtfB}^{\text{OapA}}$ protein might bind with greater affinity to sacculi derived from these cells. Indeed, $\text{His}_6\text{-YtfB}^{\text{OapA}}$ reproducibly bound with greater affinity to PG isolated from the Δ LTs mutant (Fig. 6), though the effect was quite modest (40% bound in the pellet, for a 20% increase). Finally, we tested $\text{His}_6\text{-YtfB}^{\text{OapA}}$ binding to sacculi purified from the Δ amiABC mutant. Surprisingly, $\text{His}_6\text{-YtfB}^{\text{OapA}}$ continued to bind to PG isolated from Δ amiABC mutant cells (31% in the pellet), despite the absence of denuded glycans. The results suggest that while denuded glycans may be a better binding substrate, they are not the only binding target. In sum, the OapA domain binds PG. Whether the OapA domain binds with greater affinity to denuded glycans is an open question.

DISCUSSION

Cells grow and divide through the coordinated actions of numerous proteins (1, 40). We now add YtfB, a gene of previously unknown function, to the growing list of proteins that function to support cell division (Fig. 7) (17, 41–43), though the exact

FIG 5 The OapA domain derived from YtfB preferentially localizes to septal PG. (A) Phase-contrast (left) and fluorescence (right) micrographs of live $\Delta ytfB$ mutant cells producing Tat-targeted GFP (top images) or Tat-targeted GFP-YtfB^{OapA} (bottom images). Overnight cultures were diluted 1:50 in LB medium, grown to an OD₆₀₀ of ~0.4, and imaged. (B) Average fluorescence profiles per cell plotted against normalized cell length for cells in panel A. The x axis represents 0.5 for midcell and 0.0 or 1.0 for the cell poles. The solid line is from $\Delta ytfB$ mutant cells producing Tat-targeted GFP-YtfB^{OapA}, and the dashed line is from $\Delta ytfB$ mutant cells producing Tat-targeted GFP. Fluorescence intensity was measured across 20 septa and is expressed in arbitrary units (AU). (C to F) Similar procedures were used to localize Tat-targeted GFP-YtfB^{OapA} in cells accumulating denuded glycans (ΔLTs) (C and D) and in cells lacking denuded glycan strands ($\Delta amiABC$) (E and F). Note that Tat-targeted GFP does not sharply accumulate at sites of constriction, indicating that Tat-targeted GFP-YtfB^{OapA} localization is not driven by an enlarged periplasmic space but rather the accumulation of septal PG. The strains shown are MAJ679 ($\Delta ytfB$ /T^TGFP), MAJ680 ($\Delta ytfB$ /T^TGFP-OapA), MAJ732 (ΔLTs /T^TGFP), MAJ733 (ΔLTs /T^TGFP-OapA), MAJ870 ($\Delta amiABC$ /T^TGFP), and MAJ871 ($\Delta amiABC$ /T^TGFP-OapA). The bar represents 3 μ m. Data are representative of two independent experiments.

details of how it does so remain to be elucidated. We also demonstrate that the OapA domain derived from YtfB independently localizes to the septum and that localization requires the production of denuded glycans by cell wall amidases. However, denuded glycans were not necessary for the OapA domain to bind PG *in vitro*, suggesting that these structures are a preferred but not a necessary binding substrate. Thus, YtfB is a cell division protein whose function is related to cell wall hydrolases.

Requirement for YtfB septal localization. Generally speaking, PG binding modules are necessary and sufficient for septal localization. For example, removing the LysM domain drastically reduces the septal localization of NlpD (17) and abolishes the septal localization of DipM (22–24). Similarly, AmiC variants lacking the AMIN domain fail to accumulate at sites of constriction (38). Finally, the SPOR domain is

FIG 6 His₆-YtfB^{OapA} binds PG. Purified His₆-YtfB^{OapA} was incubated with PG isolated from wild-type cells (WT, MAJ1), cells lacking multiple lytic transglycosylases (ΔLTs, MAJ718) or periplasmic amidases (ΔamiABC, RP77). A control reaction contained no PG (–). The reaction mixtures were incubated on ice for 1 h, followed by ultracentrifugation to pellet the sacculi. Pellets were washed once and repelleted. The fractions containing the supernatant, wash, and pellet were analyzed by SDS-PAGE, followed by staining with Coomassie brilliant blue. The predicted molecular mass of His₆-YtfB^{OapA} is 12 kDa. The results are representative of two independent experiments.

required for septal localization of FtsN (33). In light of these examples, YtfB is distinct in that it harbors a PG binding domain that is sufficient but not necessary for septal localization. Thus, other features of YtfB probably contribute to its septal localization (i.e., a small N-terminal domain, transmembrane helix, and/or the remainder of the periplasmic domain).

Relationship of YtfB to DamX. The characteristics of YtfB closely mirror that of another cell division protein, DamX. YtfB and DamX have a similar topology (but no sequence identity), their PG binding modules both require cell wall amidases for septal localization (33, 35), and both mutants produce synthetic phenotypes with the simultaneous absence of DedD (33, 34). In addition, overexpressing either protein induces the formation of long cell filaments (36). In the case of DamX, this (reversible) effect is required for colonization and infection by uropathogenic *E. coli* strains (44). Though a similar effect has not been observed for YtfB from *E. coli*, the YtfB homolog in *Haemophilus influenzae* (OapA) is required for efficient colonization in an infection model (25). Interestingly, the expression of *H. influenzae* *oapA* undergoes phase variation (25). Thus, one function of YtfB may be to induce morphological changes to evade the host immune system.

Septal PG as an important localization determinant for many PG binding domains. Septal PG is a general term used for the various types of PG present at the septum during the process of cell division. Though its exact chemical composition remains to be determined, the region is enriched for glycan strands that lack peptide side chains (aka denuded glycans) (35), and our *in vivo* data implicate these structures in septal localization of the OapA domain. Denuded glycans are expected to play a similar role for several other PG binding domains (9) and are the relevant binding substrate for the SPOR domain (35). The transient nature of these structures suggests that the function of YtfB might be related to the progress of the late stages of cell division.

YtfB, another bridge between early and late stages of cell division? We were surprised to find that YtfB is an early recruit to the septum because its PG binding domain appears to bind to a substrate generated only in the later stages of division. This is noteworthy because little is known about the coordination between early and late-division proteins. The best example linking these two sets of proteins is FtsN, the

FIG 7 Placement of YtfB in the assembly of the septal ring. The schematic depicts the order of recruitment for various septum-localizing proteins in *E. coli*. YtfB is an early recruit and is highlighted in purple. The OapA domain from YtfB localizes to the septal ring when cell wall amidases split septal PG, noted in gold.

last known essential division protein that localizes to the septal ring (45–47). FtsN interacts with ZapA, ZipA, and FtsA (48–50), early recruits that help stabilize FtsZ polymers and which anchor the FtsZ ring to the inner membrane (2). FtsN is also required for the septal localization of cell wall amidases (38), late recruits whose PG-degrading activities drive daughter-cell separation (Fig. 7). Thus, FtsN may be part of a mechanism that coordinates FtsZ ring constriction with the generation of septal PG (48, 51). Whether YtfB fulfills a similar role remains to be seen.

MATERIALS AND METHODS

Media. Bacteria were cultured in lysogeny broth (LB) containing 1% tryptone, 0.5% yeast extract, and 1% NaCl. Plates contained 1.5% agar. When necessary, antibiotics were used as follows: 50 or 100 $\mu\text{g} \cdot \text{ml}^{-1}$ ampicillin, 20 $\mu\text{g} \cdot \text{ml}^{-1}$ chloramphenicol, and 50 $\mu\text{g} \cdot \text{ml}^{-1}$ kanamycin.

Strain construction. Chromosomal deletions were generated using lambda Red recombination (52) or P1-mediated transduction (53). Eviction of kanamycin resistance markers was mediated by the FLP recombinase produced from pCP20 (54), leaving behind an *frt* scar. All gene deletions were verified by colony PCR. All strains used are listed in Table 3.

Plasmid construction. pMAJ56 ($P_{15}::\text{his6-ytfB}^{\text{OapA}}$) is a plasmid for the purification of the OapA domain derived from YtfB. pMAJ56 was constructed by amplifying the region corresponding to the OapA domain from *ytfB* (residues 117 to 212) with primers P354 and P355. The 318-bp product was cut with BamHI and HindIII and ligated to the same sites of pQE-80L. pMAJ76 ($P_{204}::\text{ytfB}$) is a plasmid for the overexpression of *ytfB*. pMAJ76 was constructed by amplifying full-length *ytfB* with primers P373 and P374. The 654-bp product was cut with EcoRI and PstI and ligated to the same sites of pDSW361. pMAJ77 ($P_{206}::\text{gfp-ytfB}$) is a plasmid for the localization of full-length YtfB. pMAJ77 was constructed by amplifying full-length *ytfB* with primers P374 and P445. The 666-bp product was cut with EcoRI and PstI and ligated to the same sites of pDSW209. pMAJ78 ($P_{204}::\text{TTgfp-ytfB}^{\text{OapA}}$) is a plasmid for the localization of the OapA domain from YtfB. pMAJ78 was constructed by amplifying the region spanning the OapA domain from *ytfB* (residues 123 to 212) with primers P446 and P447. The 300-bp product was cut with BamHI and HindIII and ligated to the same sites of pDSW962. pMAJ79 ($P_{206}::\text{TTgfp-ytfB}^{\text{OapA}}$) was similarly constructed in pDSW963. pMAJ97 ($P_{206}::\text{gfp-ytfB}[1-125]$) is a plasmid for the localization of YtfB lacking its OapA domain. pMAJ97 was constructed by amplifying *ytfB* (residues 1 to 125) with primers P445 and P619. The 405-bp product was cut with EcoRI and PstI and ligated to the same sites of pDSW209. All cloning was verified by sequencing at the University of Arkansas for Medical Sciences (UAMS) DNA Sequencing Core Facility using a 3500 genetic analyzer (Applied Biosystems). Reference sequences were obtained from EcoGene (version 3.0) (55) or EcoCyc (version 21.1) (56). The region corresponding to the OapA domain was determined by using the coordinates listed in the Pfam database (version 31.0) (19). All plasmids and primers are listed in Tables 4 and S1, respectively.

Protein localization. Cells producing GFP fused to full-length YtfB were grown overnight in LB containing ampicillin, diluted 1:50 into the same medium containing 5 μM isopropyl- β -D-1-thiogalactopyranoside (IPTG), and grown for 2 h. Live cells were then spotted onto 1% agarose pads and imaged by phase-contrast and fluorescence microscopy. Strains producing GFP fused to the OapA domain derived from YtfB were grown similarly, except that IPTG was omitted unless otherwise noted. Localization studies were carried out at 30°C. Demographic representations and line profiles of GFP fluorescence intensities from live cells were produced by using MicroBJ (57), an ImageJ plug-in (58).

Localization dependency. Temperature-sensitive mutants producing GFP-YtfB were grown overnight in LB medium containing ampicillin, diluted 1:50 in the same medium containing 5 μM IPTG, and grown at 30°C for 2 h. Cells were then diluted 1:10 into the same medium or into LB lacking NaCl (LB0N) and grown for 1 h at 30°C (permissive) or 42°C (nonpermissive). Cells were fixed with a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde (to prevent renaturation and localization of the temperature-sensitive proteins), washed with phosphate-buffered saline (PBS), spotted onto 1% agarose pads, and imaged by phase-contrast and fluorescence microscopy. For FtsN depletion experiments, cells producing GFP-YtfB were grown overnight in LB medium containing ampicillin and 0.2% arabinose, diluted 1:50 into the same medium, and grown at 30°C for 2.5 h. Cells were then washed to remove arabinose and resuspended in LB medium containing ampicillin, 10 μM IPTG, and 0.2% arabinose (permissive) or 0.2% glucose (nonpermissive) for an additional 2.5 h. Live cells were imaged as described above.

Morphology of *ytfB*, *damX*, and *dedD* mutants. Overnight cultures were diluted 1:2,000 in LB medium and grown at 37°C to an optical density at 600 nm (OD_{600}) of ~ 0.5 . Cells were then fixed with 4% paraformaldehyde, washed with PBS, spotted onto 1% agarose pads, and imaged under phase-contrast microscopy. Cellular dimensions were measured using the cellSens Dimensions software (Olympus). Live cells were prepared for flow cytometry analysis, as previously described (59). Cells were assayed by using the forward- and side-scatter detectors of a BD LSRFortessa flow cytometer housed in the UAMS Flow Cytometry Core Facility. The forward-scatter measurement is proportional to cell size, and the side-scatter measurement is relative to the shape complexity (60), though it has been used to differentiate cells of different lengths (17). All flow histograms were plotted using FlowJo (version 10.1) software.

Assay to determine rescue of *fts* mutants. Overnight cultures were diluted to an OD_{600} of 1.0 in LB0N, and 10-fold serial dilutions were spotted onto LB plates at 30°C (permissive) or LB0N plates at 42°C (nonpermissive). Plates were incubated for 18 h and imaged the next day by using a ChemiDoc MP imaging system (Bio-Rad).

TABLE 3 Strains used in this study

Strain	Relevant features	Source or reference
MAJ1	MG1655 <i>wbbL::IS5</i>	Lab collection
MAJ473	MAJ1/pDSW361	This study
MAJ574	BL21(DE3)/pMAJ56	This study
MAJ579	MAJ1 $\Delta ytfB::frt$	This study
MAJ643	MAJ1/pMAJ76	This study
MAJ646	MAJ1/pMAJ77	This study
MAJ675	RP161/pDSW962	This study
MAJ676	RP161/pMAJ78	This study
MAJ677	MAJ579/pDSW209	This study
MAJ678	MAJ579/pMAJ77	This study
MAJ679	MAJ579/pDSW962	This study
MAJ680	MAJ579/pMAJ78	This study
MAJ681	MAJ1 $\Delta damX::kan$	This study
MAJ682	MAJ1 $\Delta dedD::kan$	This study
MAJ683	MAJ579 $\Delta ytfB::frt \Delta damX::kan$	This study
MAJ684	MAJ579 $\Delta ytfB::frt \Delta dedD::kan$	This study
MAJ685	MG1655 $P_{BAD}::ftsN kan$	65
MAJ693	MAJ1 <i>ftsA12 leu::Tn10</i>	This study
MAJ696	WM1109/pMAJ77	This study
MAJ697	MAJ693/pMAJ77	This study
MAJ699	SKM1/pMAJ77	This study
MAJ700	MAJ685/pMAJ77	This study
MAJ703	MAJ681 $\Delta damX::frt \Delta dedD::kan$	This study
MAJ704	MAJ695 $\Delta ytfB::frt \Delta damX::frt \Delta dedD::kan$	This study
MAJ712	PS223/pMAJ77	This study
MAJ718	MG1655 $\Delta rlpA::frt \Delta mltA::frt \Delta slt::frt \Delta mltD::frt \Delta mltE::frt \Delta mltC::frt$	35
MAJ732	MAJ718/pDSW962	This study
MAJ733	MAJ718/pMAJ78	This study
MAJ742	WM1109 $\Delta ytfB::kan$	This study
MAJ743	MAJ693 $\Delta ytfB::kan$	This study
MAJ744	MCI23 $\Delta ytfB::kan$	This study
MAJ745	SKM1 $\Delta ytfB::kan$	This study
MAJ754	RP75/pDSW962	This study
MAJ755	RP75/pMAJ78	This study
MAJ756	RP273/pDSW962	This study
MAJ757	RP273/pMAJ78	This study
MAJ778	BW27783 <i>ftsZ</i> [55mVen56i]	29
MAJ784	MAJ778/pDSW361	This study
MAJ785	MAJ778/pMAJ76	This study
MAJ788	MAJ684/pDSW209	This study
MAJ789	MAJ684/pMAJ77	This study
MAJ812	PS223 $\Delta ytfB::kan$	This study
MAJ868	MAJ579/pMAJ97	This study
MAJ870	RP77/pDSW963	This study
MAJ871	RP77/pMAJ79	This study
MCI23	MG1655 <i>ftsI23 leu::Tn10</i>	66
PS223	W3110 <i>zipA1</i>	67
RP75	CS109 <i>amiA::cam amiB yje25::res</i>	68
RP77	CS109 <i>amiB yje25::res amiA::cam amiC::kan</i>	68
RP161	CS109 <i>amiA::cam amiC::kan</i>	68
RP273	CS109 <i>amiB yje25::res amiC::kan</i>	5
SKM1	MG1655 <i>ftsQ1 leu::Tn10</i>	69
WM1109	MG1655 <i>ftsZ84 leu::Tn10</i>	70

Protein purification. His₆-YtfB^{OapA} was produced and purified from the *E. coli* overproduction strain BL21(DE3). Overnight cultures grown in LB medium containing ampicillin were diluted into the same medium and grown at 30°C to an OD₆₀₀ of 0.5. Overproduction was induced by adding 1 mM IPTG, and growth was continued for 3 h, after which cells were harvested and lysed by sonication. The His₆-tagged protein was captured over Talon affinity chromatography resin charged with cobalt (Clontech). Purified protein was dialyzed into buffer containing 25 mM HEPES, 150 mM NaCl, and 5% glycerol. Protein concentration was determined by averaging the values from measuring UV absorbance at 280 nm and

TABLE 4 Plasmids used in this study

Plasmid	Relevant genotype or characteristics	Origin	Source or reference
pCP20	$\lambda_{PR}::FLP$ λ cl857 <i>bla</i> <i>cat</i> Rep(Ts)	pSC101	54
pDSW204	IPTG regulation P_{204} <i>lacI^q</i> <i>bla</i>	pBR	32
pDSW209	$P_{206}::gfp$ <i>lacI^q</i> <i>bla</i>	pBR	32
pDSW361	pDSW361 is a Kan ^r derivative of pDSW204	pBR	32
pDSW962	$P_{204}::^{ss}torA$ - <i>gfp</i> <i>lacI^q</i> <i>bla</i>	pBR	65
pDSW963	$P_{206}::^{ss}torA$ - <i>gfp</i> <i>lacI^q</i> <i>bla</i>	pBR	David Weiss
pKD13	<i>bla</i> <i>FRT</i> - <i>aph</i> - <i>FRT</i>	R6K γ	52
pKD46	$P_{araB}::gam$ - <i>bet</i> - <i>exo</i> <i>bla</i> Rep(Ts)	pSC101	52
pMAJ56 ^a	$P_{T5}::his6$ - <i>ytfB</i> ^{OapA} (117–212)	ColE1	This study
pMAJ76 ^b	$P_{204}::ytfB$	pBR	This study
pMAJ77 ^c	$P_{206}::gfp$ - <i>ytfB</i>	pBR	This study
pMAJ78 ^d	$P_{204}::^{TT}gfp$ - <i>ytfB</i> ^{OapA} (123–212)	pBR	This study
pMAJ79 ^e	$P_{206}::^{TT}gfp$ - <i>ytfB</i> ^{OapA} (123–212)	pBR	This study
pMAJ97 ^c	$P_{206}::gfp$ - <i>ytfB</i> (1–125)	pBR	This study
pQE-80L	$P_{T5}::his6$ <i>lacI^q</i> <i>bla</i>	ColE1	Qiagen

^aDerivative of pQE-80L.^bDerivative of pDSW361.^cDerivative of pDSW209.^dDerivative of pDSW962.^eDerivative of pDSW963.

the bicinchoninic acid assay (Thermo Scientific), which were in good agreement. Visual purity was judged to be ~95% by staining with Coomassie brilliant blue following SDS-PAGE.

PG purification. PG sacculi were purified as previously described (35), with modifications. Overnight cultures were diluted 1:100 in 500 ml LB and grown at 30°C to an OD₆₀₀ of ~0.5. Cells were then chilled, harvested, and resuspended in 3 ml 1% NaCl. The cells were added dropwise to an 8% SDS solution in a 16 by 150 mm borosilicate culture tube (Fisher Scientific) containing a stir bar and were in turn housed in a boiling water bath. The suspension was boiled for 2 h and then incubated overnight with stirring at room temperature. The next day, the suspension was boiled for an additional 2 h, and the sacculi were recovered by spinning at 21,000 $\times g$ for 10 min in a tabletop centrifuge (Eppendorf model no. 5424). The translucent pellet containing the recovered sacculi was washed repeatedly with water until residual SDS was removed, as determined by the methylene blue assay (61). To remove Braun's lipoprotein, sacculi were resuspended in 0.1 M Tris-HCl containing 200 $\mu g \cdot ml^{-1}$ α -chymotrypsin and incubated overnight at 37°C. The next morning, SDS was added to 1%, and the samples were boiled for 1 h in a heating block. SDS was removed by repeatedly washing with water and pelleting, as described above.

PG quantification. PG sacculi were quantified as previously described (62), with slight modifications. Isolated sacculi were hydrolyzed with 6 N HCl in a borosilicate glass screw-thread sample vial (Wheaton) at 95°C for 10 h. The resulting PG monomers were dried via evaporation in a fume hood and then resuspended in water. These samples were mixed with >99% acetic acid and ninhydrin reagent (250 mg of ninhydrin [Sigma] in 4 ml of 0.6 M phosphoric acid and 6 ml of >99% acetic acid) in a ratio of 1:1:1 and placed at 100°C for 5 min. The reaction of the ninhydrin reagent with diaminopimelic acid (mDAP) results in a yellow color, whose absorbance at 434 nm was measured and compared to standards of 1 to 25 μg mDAP (Sigma) (63).

PG binding assay. A standard binding reaction contained binding buffer (10 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl [pH 8.0]), 100 μg PG, and 12 μg protein in 100 μl (PG was omitted in control reactions) (64). The reaction mixtures were incubated on ice for 1 h and then centrifuged at 4°C for 45 min in a Beckman Coulter TLA110 fixed-angle rotor at 50,000 rpm (~104,000 $\times g$). Pellets were washed in an equal volume of binding buffer and pelleted, as described above. The supernatant (S), wash (W), and pellet (P) were resuspended in Laemmli sample buffer containing 5% β -mercaptoethanol and boiled for 10 min, and 10 μl was loaded onto a 12% SDS-PAGE gel. His₆-YtfB^{OapA} was visualized by staining with GelCode Blue Safe protein stain (Thermo Scientific) and imaged by using a ChemiDoc MP imaging system (Bio-Rad). Protein bands were quantified using ImageJ (58).

Western blotting. Cells (1 ml) were harvested at an OD₆₀₀ of ~0.5, pelleted, and resuspended in 0.2 ml Laemmli sample buffer containing 5% β -mercaptoethanol. The samples were boiled for 10 min, and 10 μl were loaded onto a 12% SDS-PAGE gel. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane. Rabbit anti-FtsZ serum (a gift from David Weiss, University of Iowa) and anti-GFP (Thermo Fisher Scientific) were applied at dilutions of 1:10,000 and 1:2,000, respectively. Primary antibody was detected by using the goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluor 488 (1:5,000; Thermo Fisher Scientific). Blots were imaged by using a ChemiDoc MP imaging system (Bio-Rad).

Microscopy and figure construction. Cells were visualized by using an Olympus BX60 microscope coupled to an XM10 1.4 MP monochrome fluorescence charge-coupled-device (CCD) camera. Image brightness and contrast were adjusted linearly using ImageJ. Figures were assembled in Adobe Illustrator.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00046-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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