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ZipA Is Required for FtsZ-Dependent Preseptal Peptidoglycan Synthesis prior to Invagination during Cell Division

Lakshmi-Prasad Potluri,* Suresh Kannan, and Kevin D. Young

Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA

Rod-shaped bacteria grow by a repetitive cycle of elongation followed by division, and the mechanisms responsible for these two processes have been studied for decades. However, little is known about what happens during the transition between the two activities. At least one event occurs after elongation ends and before division commences, that being the insertion of new cell wall peptidoglycan into a narrowly circumscribed ribbon around midcell where septation is destined to take place. This insertion does not depend on the presence of the septation-specific protein PBP3 and is therefore known as *P*BP3-*i*ndependent *p*eptidoglycan *s*ynthesis (PIPS). Here we report that only FtsZ and ZipA are required to generate PIPS in wild-type *Escherichia coli*. PIPS does not require the participation of other members of the divisome, the MreB-directed cell wall elongation complex, alternate peptidoglycan synthases, the major peptidoglycan amidases, or any of the low-molecular-weight penicillin binding proteins. ZipA-directed PIPS may represent an intermediate stage that connects cell wall elongation to septal invagination and may be the reason ZipA is essential in the gammaproteobacteria.

As *Escherichia coli* elongates, new cell wall peptidoglycan is inserted diffusely into the cylindrical side walls (11, 19, 22, 72), but when division begins, most peptidoglycan synthesis is redirected to the invaginating septum (72, 73). This two-step cycling between elongation and division was described first by Schwarz et al. (57) and later developed into the idea that the elongation and septation machineries might compete so strongly with one another that only one could be active at a time (12, 55). Although this proposal may overstate the case, there is general agreement that the two stages of cell wall synthesis are distinct. For *E. coli* and other rod-shaped bacteria, the prevailing view is that the elongation complex is composed of peptidoglycan-synthesizing enzymes whose localization and spatial orientation are controlled by the cytoskeletal protein MreB in conjunction with MreCD, PBP2, RodA, RodZ, and others (13, 14, 16, 19, 26, 29, 43). Conversely, during the invagination stage, the peptidoglycan-synthesizing enzymes are believed to be redirected to work with the cytoskeletal protein FtsZ and its associated proteins, which constitute the divisome (19). Of particular importance is that penicillin binding protein 3 (PBP3, also known as FtsI) is absolutely required for synthesis of septal peptidoglycan during the latter period.

Although a great deal is known about these two stages of cell wall synthesis, a major open question is how the transition from one to the other is regulated and what happens during this transition. Somehow the relevant peptidoglycan-synthesizing enzymes must transfer from the MreB-directed elongation apparatus to the FtsZ-dominated division machinery. Wientjes and Nanninga provided the first hints about this intermediate stage by observing that cell division is composed of two separate and sequential peptidoglycan-synthesizing reactions (72). The first is a PBP3-independent reaction that occurs prior to any visible constriction at the division site, and the second is the well-known PBP3-dependent reaction that accompanies septal invagination (72). These authors concluded that "more than one switch in peptidoglycan synthesis takes place during constriction: one to initiate the constriction process and a second to activate PBP3 to continue it" (72). Thus, there appear to be at least two transitional events interposed between cell wall elongation and division. The first initiation event is now referred to as PIPS (*P*BP3-*i*ndependent *p*eptidoglycan *s*ynthesis) (48), and it may represent the earliest detectable signature of the transition from elongation to division. Of particular note is that this poorly understood stage of septal maturation may be the time at which bacteria are most susceptible to antibiotics that interfere with cell wall synthesis (21).

PIPS was described for *E. coli* in 1989 to 1991 (48, 49, 72). More recently, Aaron et al. found an analogous insertion of peptidoglycan at the midcell prior to invagination of *Caulobacter crescentus* (1), implying that PIPS may be a common transitional phase in different bacterial genera. However, since it was first described, there has been little movement toward understanding the nature of this reaction, which is surprising because an essential and substantial reorganization of the cell wall synthetic apparatus must occur within this interval. What is known is that immediately prior to any visible invagination, new peptidoglycan is incorporated into a ring (the "PIPS band") around the cell's center, that formation of this band requires FtsZ, and that this preseptal peptidoglycan insertion proceeds even when PBP3 (FtsI) is inactive (22, 48, 49, 72). To date, the only other proteins tested and shown not to be required are FtsA and FtsQ (22).

Here, to further dissect this important transitional event, we determined which of the known divisome or cell wall elongation components were required for FtsZ-directed PIPS. We found that only ZipA, one of the first two members of the divisome, was required for PIPS in wild-type *E. coli*, which is consistent with the idea that PIPS represents an early stage in the transition between

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Address correspondence to Kevin D. Young, kdyoung@uams.edu.

* Present address: Lakshmi-Prasad Potluri, Department of Microbiology, University of Chicago, Chicago, Illinois, USA.

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TABLE 1 Bacterial strains

Strain	Genotype	Gene, P1 source × recipient	Source and/or reference
CS109	W1485 *glnV* (*supE*) *rpoS rph*		Lab collection (20)
MG1655	*ilvG rfb-50* (IS*5* insertion) *rph-1*		Lab collection (65)
W3110	*rph-1* IN(*rrnD rrnE*)1		J. Lutkenhaus (41, 50)
CS703-1	CS109 *mrcA*::*res dacB*::*res dacA*::*res dacC*::*res pbpG*::*res ampC*::*res ampH*::*res*		20
LP3-1	CS109 *envC*::*kan*	Δ*envC* from LP2[a] × CS109	This work
LP6-1K	W3110 *ftsA12 leu*::Tn*10 mrcB*::*kan*	Δ*mrcB* from BMCS20K-1[a] × PS236	This work
LP7-1	W3110 *ftsZ84 leu*::Tn*10*	*ftsZ*84 from WM1109[a] × W3110	This work
LP8-1K	W3110 *ftsA12 leu*::Tn*10 mrcA*::*kan*	Δ*mrcA* from BMCS04-1K[a] × PS236	This work
LP11-1	W3110 *ftsK44 aroA*::Tn*10*	*ftsK*44 from C600-4[a] × W3110	This work
LP19-1 pCX16	W3110 *mreB*::*kan*	Δ*mreB* from P2357[a] × W3110	This work
LP20-1 pCX16	W3110 *mreCD*::*kan*	Δ*mreCD* from FB14 pCH244[a] × W3110	This work
LP21-1 pCX16	W3110 *mreBCD*::*kan*	Δ*mreBCD* from P2394[a] × W3110	This work
LP35-1K pCX16	W3110 *rodA*::*kan*	Δ*rodA* from P3962[a] × W3110	This work
PS223	W3110 *zipA1*		J. Lutkenhaus (50)
PS234	W3110 *ftsA12 leu*::Tn*10 zipA1*		J. Lutkenhaus (50)
PS236	W3110 *ftsA12 leu*::Tn*10*		J. Lutkenhaus (50)
RP182	CS109 *amiC*::*kan*		54
SKW16-1	W3110 *ftsI23 leu*::Tn*10*	*ftsI23* from MCI23[a] × W3110	This work
SKW20-1	W3110 *ftsI23 leu*::Tn*10 mrcA*::*kan*	Δ*mrcA* from BMCS04-1K[a] × SKW16-1	This work
SKW27-1	W3110 *ftsA*(R286W) *leu*::Tn*10 zapB*::*frt zapC*::*aph zapA*::*cat*	Δ*zapA* from MC1000 *zapA*[a] × SKW21-1[a]	This work
SKW30-1 pCX16	W3110 *rodZ*::*aph*	Δ*rodZ* from P5439[a] × W3110	This work
SKW33-1	W3110 *ftsQ1 leu*::Tn*10*	*ftsQ1* from SKM1[a] × W3110	This work
SKW35-1	W3110 *pbp1C*::*res mtgA*::*res ynhG*::*frt ycbB*::*frt ftsA12 leu*::Tn*10*	*ftsA12* from PS236 × SKW32-1[a]	This work
SKW36-1	W3110 *ftsEX*::*cat ftsI23 leu*::Tn*10*	*ftsI23* from MCI23[a] × LP22-1[a]	This work
SKW41-1	W3110 *pbp1C*::*res mtgA*::*res ynhG*::*frt ycbB*::*frt ftsA12 leu*::Tn*10 mrcA*::*kan*	Δ*mrcA* from BMCS04-1K[a] × SKW35-1	This work
SKW42-1	W3110 *pbpC*::*res mtgA*::*res ynhG*::*frt ycbB*::*frt ftsA12 leu*::Tn*10 mrcB*::*kan*	Δ*mrcB* from BMCS20K-1[a] × SKW35-1	This work
SKW49-1	W3110 *zapB*::*frt zapC*::*frt zapA*::*cat ftsA12 leu*::Tn*10*	*ftsA12* from PS236 × SKW48-1[a]	This work
WM1657	MG1655 *ftsA*(R286W) *leu*$^+$ *zipA*::*aph*		W. Margolin (31)

[a] The strain is listed in Table S1 in the supplemental material.

elongation and division. PIPS did not require the participation of members of the MreB-directed cell wall elongation complex, nor was this preseptal synthesis affected by removing alternate peptidoglycan synthases, amidases, or penicillin binding proteins.

MATERIALS AND METHODS

Strains, plasmids, media, and growth conditions. Bacterial strains are listed in Table 1 and were constructed using standard phage P1 transduction protocols. Bacteria were cultured overnight in Luria-Bertani (LB) broth supplemented with appropriate antibiotics, as required, to give the following final concentrations: kanamycin, 50 μg/ml; chloramphenicol, 20 μg/ml; spectinomycin, 50 μg/ml; or tetracycline, 10 μg/ml. Cultures were incubated at 37°C or at 30°C for temperature-sensitive mutants. Antibiotics and chemicals were purchased from Sigma, St. Louis, MO, unless otherwise noted. Plasmid pCX16 (*sdiA aadA*; spectinomycin resistant) was from P. de Boer (70); constitutive expression of *sdiA* from this plasmid increases the level of the FtsZ protein in *E. coli* (6). The *envC*, *mtgA*, and *ynhG* genes were deleted from *E. coli* by λ Red recombination, as described in Materials and Methods in the supplemental material.

PIPS assay: D-cysteine labeling. Cells were pulse-labeled with D-cysteine, sacculi were purified, and incorporated D-cysteine was detected by biotinylation and immunolabeling as described previously (22, 53, 64, 66) but with the following modifications. Overnight cultures were diluted (1:200) in fresh LB or LB-0.5% NaCl to give an initial starting optical density at 600 nm (OD_{600}) of ~0.015 to 0.02. The cultures were incubated for 10 min at 37°C or for 20 min at 30°C, at which time D-cysteine was added (200 μg/ml, final concentration). When the cultures reached an OD_{600} of 0.3 to 0.4 (after 3 to 4 generations), the cells were harvested by centrifugation at room temperature and resuspended in prewarmed LB without D-cysteine. When required, aztreonam (1 μg/ml) was added at the start of the chase period. Cells were harvested after one mass doubling, and sacculi were purified and immunolabeled. Temperature-sensitive strains were treated in the same way, except that the cells were grown in the presence of D-cysteine at 30°C before being resuspended in prewarmed 42°C LB (0.5% NaCl), incubated for one mass doubling during the chase period, and then chilled on ice and harvested by centrifugation at 4°C.

Sacculus purification, biotinylation, and immunolabeling. Cells were resuspended in 3 ml of LB containing aztreonam (as required) or in LB–0.5% NaCl (for temperature-sensitive strains) and added dropwise to 6 ml of boiling 6% sodium dodecyl sulfate (SDS). Samples were boiled for 4 to 5 h and incubated overnight at room temperature with gentle stirring. The next day, the samples were resuspended in 3 ml of 0.5% SDS, boiled for an additional 2 h, and then washed 3 times with distilled water to remove SDS. Washing was performed by centrifugation at 30°C at 65,000 rpm for 15 min in a Beckman Optima TLX ultracentrifuge with a TLA110 rotor. Sacculi were resuspended in 1× phosphate-buffered saline (PBS), α-chymotrypsin was added to 300 μg/ml, and the sacculi were incubated overnight at 37°C. The next day, SDS (0.5% final) was added, the samples were boiled for 2 h, pelleted, and resuspended in 50 mM sodium bicar-

bonate, and 1 mg sodium borohydride was added, followed by incubation at room temperature for 30 min to reduce the D-cysteine thiol groups. The sodium borohydride was inactivated by slowly adding 20% phosphoric acid to reduce the pH of the sample to 4.0, after which the sacculi were pelleted and resuspended in 0.6 ml of 25 mM sodium bicarbonate. To each of these samples was added 0.4 ml EZ-Link HPDP-biotin (2.2 mg/ml) (Pierce), which had been freshly prepared by dissolving in anhydrous dimethyl sulfoxide (DMSO), and the samples were incubated at room temperature for 1 h. The sacculi were pelleted, resuspended in 100 μl of distilled water, and stored at 4°C.

Biotinylated sacculi were immunolabeled on coverslips by adding antibiotin antibody (mouse IgG; Abcam, Cambridge, MA), diluted 1:100 in PBG (0.4% bovine serum albumin [BSA] and 0.2% gelatin in 1× PBS). Antibody was detected by secondary labeling with goat anti-mouse IgG conjugated to Alexa Fluor 568 (Invitrogen, California), diluted 1:250 in PBG. Total peptidoglycan was labeled with antimurein antibody (rabbit serum, a gift from Miguel de Pedro, Universidad Autónoma de Madrid, Madrid, Spain) diluted 1:200 in PBG and then detected by secondary labeling with goat anti-rabbit antibody conjugated to AF488 (Invitrogen) diluted 1:250 in PBG. The labeled sacculi were air dried, and the coverslips were inverted onto clean glass slides spotted with a drop of Prolong-Gold antifade reagent (Invitrogen), cured overnight in the dark at room temperature, and then sealed with clear nail polish.

Microscopy. Immunolabeled sacculi were imaged by using a wide-field epifluorescence Zeiss Axio Imager.Z1 microscope fitted with a 100× differential interference contrast objective (1.45 numerical aperture [NA]). Images were acquired with a Zeiss Axiocam MRm cold charge-coupled device camera, using appropriate filter cubes for fluorescence image acquisition. High-resolution confocal images were obtained with a Zeiss LSM510 Meta microscope fitted with a 63× plan-Neofluar oil immersion objective (1.45 NA) and driven by the AIM software program. Images were visualized with the Zeiss LSM image browser and AxioVision software programs for confocal and bright-field images, respectively, and were processed with Adobe Photoshop software to adjust brightness and contrast.

RESULTS

ZipA is required for preseptal peptidoglycan synthesis in wild-type *E. coli*. When PBP3 (FtsI) is inhibited by inactivating a temperature-sensitive variant or by adding a PBP3-specific antibiotic, such as aztreonam, *E. coli* grows as filaments that synthesize regularly spaced hoops of preseptal peptidoglycan located at potential division sites (22). These PIPS bands can be detected by using the D-cysteine pulse-label-and-chase assay, in which segments of preseptal synthesis appear as discrete unlabeled zones in purified bacterial sacculi (22, 23). This is currently the simplest assay for PIPS. When we inhibited division by shifting a strain containing a temperature-sensitive FtsI protein (FtsI23) to 42°C for one mass doubling, more than 85% of the cells exhibited distinct PIPS bands (Fig. 1A and Table 2, row 1), which is consistent with previous reports (33, 23). Most of the time, three bands appeared—one representing the original potential division site at midcell, with two additional zones at the one- and three-quarter positions of the filament (Fig. 1A and D and Table 2, row 1).

We reconfirmed that PIPS requires FtsZ by observing a mutant carrying the temperature-sensitive allele *ftsZ84*. As expected, virtually no PIPS bands were observed in sacculi grown in the absence of active FtsZ84 (Fig. 1B and Table 2, row 2). However, when such cells are grown at 42°C, not only does the Z ring disappear but the FtsZ84 monomer may be inactivated (3). Thus, it was possible, though unlikely, that PIPS might not require a complete Z ring but only the presence of unpolymerized but otherwise wild-type FtsZ. To distinguish between these alternatives, we took advantage of the fact that Z rings form if either FtsA or ZipA is present but do not form if these last two proteins are inactive (50, 71). When FtsA12 and ZipA1 were heat inactivated simultaneously, fewer than 7% of cells exhibited PIPS bands (Fig. 1C and Table 2, row 3). This indicated that PIPS required the presence of a Z ring and not just the presence of cytoplasmic monomers of wild-type FtsZ.

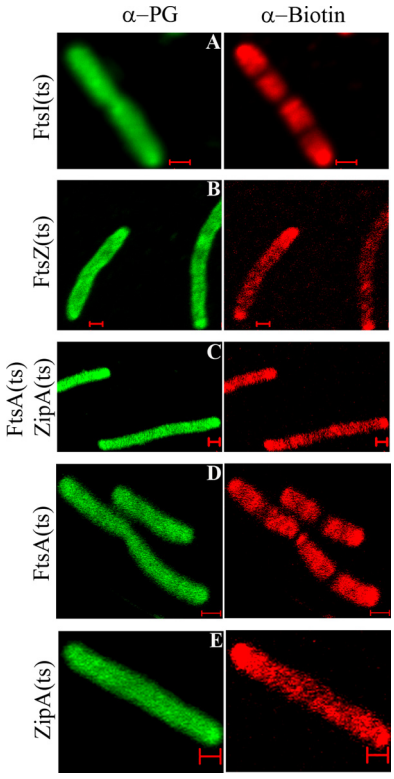

FIG 1 ZipA is essential for PIPS. Confocal images of *E. coli* sacculi from cells carrying one or more temperature-sensitive proteins, as noted to the left of each set of panels. Cells were shifted to 42°C to inhibit the respective protein, and sacculi were isolated and immunolabeled with antimurein to label total peptidoglycan (α-PG, in green) and with antibiotin antibodies to label older peptidoglycan made prior to the temperature shift (α-Biotin, in red). The uninterrupted labeling by α-PG indicates that the peptidoglycan was uniform and intact along the length of these sacculi. The dark and narrow unlabeled zones in the α-Biotin panels of A and D represent newly synthesized peptidoglycan that accumulated at potential septation sites (PIPS bands). Generalized peptidoglycan insertion occurred along the length of sacculi in panels B, C, and E, but no well-defined PIPS bands were present. The following *E. coli* strains were processed: SKW16-1 (A), LP7-1 (B), PS234 (C), PS236 (D), and PS223 (E). In each panel, bar = 1 μm.

Additional divisome proteins associate with FtsZ in a well-defined order, beginning with the formation of the nascent Z ring composed of FtsZ, FtsA, and ZipA (17, 34, 71). Therefore, we next assayed for PIPS in mutants impaired at different points in the divisome assembly pathway to determine how much of this structure was required. We first reconfirmed that PIPS bands appeared in sacculi from cells in which the temperature-sensitive FtsA12 protein had been inactivated (Fig. 1D and Table 2, row 4) (22). In contrast, PIPS was eliminated from about 90% of the cells in which the ZipA1 temperature-sensitive protein was inactivated (Fig. 1E and Table 2, row 5). As a further test, we assayed PIPS in a *zipA1* strain by localizing superfolder green fluorescent protein

TABLE 2 PIPS bands in sacculi from *E. coli* mutants

Row	Strain profile		Sacculi with PIPS (%)	Total no. of sacculi	No. of sacculi with indicated no. of PIPS bands			No. of experiments
	TS protein(s)[a]	Deleted protein(s)			1	2	3	
1	FtsI23		85.4	302	211	19	28	3
2	FtsZ84		1.0	478	5	0	0	3
3	FtsA12, ZipA1		6.8	880	51	8	1	5
4	FtsA12		80.1	594	350	52	74	3
5	ZipA1		9.6	1,620	136	10	9	5
6	FtsA12	ZapABC	75.5	367	16	28	126	3
7	FtsI23	FtsEX	87.0	231	14	29	72	2
8	FtsK44		92.8	783	672	27	28	2
9	FtsQ1		94.5	695	135	92	430	2
10	FtsA12	PBP1A	74.8	147	96	5	9	3
11	FtsI23	PBP1A	86.1	337	188	35	67	3
12	FtsA12	PBP1B	95.2	189	171	8	1	3
13	FtsI23	PBP1B	62.6	219	103	20	14	2
14		PBP1C, MtgA, YnhG, YcbB (+ aztreonam)	68.6	169	98	10	8	2
15	FtsA12	PBP1C, MtgA, YnhG, YcbB, PBP1A	71.2	340	99	26	117	3
16	FtsA12	PBP1C, MtgA, YnhG, YcbB, PBP1B	62.4	274	125	23	23	3
17		AmiC	83.9	280	202	15	6	3
18		EnvC	78.4	231	115	26	40	3
19		PBP1A, -4, -5, -6, -7, AmpC, AmpH	81.0	168	136	0	0	2
20		MreB	62.6	155	97	0	0	2
21		MreCD	45.2	93	42	0	0	2
22		MreBCD	76.1	197	144	6	0	3
23		RodZ	47.3	112	53	0	0	2
24		RodA	28.8	80	23	0	0	2
25		FtsA* ZapABC	85.8	260	118	22	22	3
26		FtsA*, ZipA	82.0	428	175	80	40	3
27		FtsA*, ZapABC, ZipA	81.0	247	101	10	24	3

[a] TS, temperature sensitive.

(sfGFP)-PBP5 in live cells, as described previously (52). No sfGFP-PBP5 rings were observed, confirming the absence of PIPS (not shown). The results indicated that in wild-type *E. coli*, preseptal peptidoglycan synthesis required an FtsZ ring anchored to the cytoplasmic membrane by ZipA.

Other downstream divisome proteins are not required for PIPS. Although Z rings can form in the presence of either FtsA or ZipA, in each case the resulting rings fail to recruit downstream divisome proteins, so that the cells do not divide (37, 50). The fact that ZipA was necessary strongly suggested that PIPS required only the earliest form of the Z ring without additional divisome proteins. However, to be certain, we assayed for PIPS in cells in which divisome assembly was impaired by the loss of other downstream proteins.

The ZapABC proteins are added to the Z ring soon after FtsA and ZipA (17). Because PIPS occurs when FtsA is absent, we assayed for preseptal synthesis in a strain from which the *zapABC* genes were deleted and in which FtsA12 was heat inactivated at 42°C. Clear PIPS bands were formed under these conditions (Fig. 2A and Table 2, row 6). The next components to assemble onto the Z ring are the FtsEX proteins (17), and interestingly, association of FtsE with the ring requires ZipA but not FtsA (15). Because this behavior parallels the requirements for PIPS, it raised the possibility that FtsE might be involved. We deleted the *ftsEX* genes from a strain carrying the temperature-sensitive FtsI23 protein. Clear PIPS bands were observed in the absence of FtsE and FtsX (Fig. 2B and Table 2, row 7), and the same result was obtained when FtsI activity was inhibited by aztreonam (not shown). Similarly, PIPS bands formed in strains in which temperature-sensitive variants of FtsK (Fig. 2C and Table 2, row 8) or FtsQ (Fig. 2D and Table 2, row 9) were inactivated, indicating that neither of these divisome proteins were required for preseptal peptidoglycan synthesis. Thus, except for ZipA, PIPS required no divisome proteins associated with the first stage of Z-ring formation, nor did it require the first two proteins added during the second stage of divisome maturation.

Neither PBP1a nor PBP1b is a PIPS-specific synthase. Synthesis of preseptal peptidoglycan requires the activity of one or more peptidoglycan synthases. In *E. coli*, PBP1a and -1b possess both glycosyltransferase and transpeptidase activities, enabling them to polymerize and cross-link glycan chains (35). As far as is known, these two enzymes synthesize virtually all cellular peptidoglycan, and cells must retain one or the other to remain viable (20, 60). One difference between the two proteins is that PBP1b exhibits some preference for the septum (10), suggesting that it might be involved in preseptal synthesis. However, PBP1a and -1b behaved similarly in PIPS assays. Strains lacking PBP1a produced clear PIPS bands in either the *ftsA12* (Fig. 2E and Table 2, row 10) or *ftsI23* (Fig. 2F and Table 2, row 11) background, as did a strain lacking PBP1b in the *ftsA12* background (Fig. 2G and Table 2, row 12) or in the *ftsI23* background (Table 2, row 13; see also Fig. S1 in the supplemental material). Oddly, when sacculi were isolated

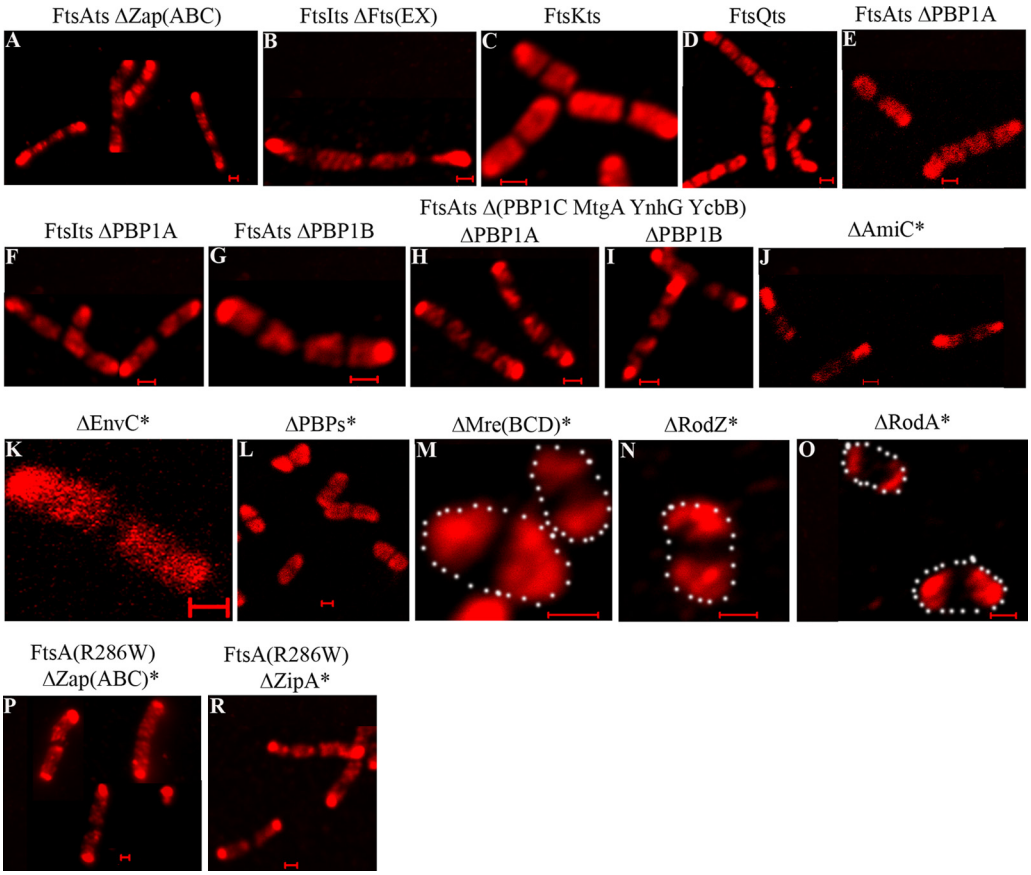

FIG 2 PIPS does not require downstream divisome proteins, peptidoglycan synthases, hydrolases, or low-molecular-weight PBPs. Sacculi from *E. coli* carrying the indicated mutations were prepared and labeled as described in the legend to Fig. 1. Only the α-biotin labeling is presented because, with one exception as noted in the text, all sacculi were stained uniformly with antipeptidoglycan antibody (not shown). The dashed lines in panels M, N, and O outline sacculi from individual cells. Images were obtained with a Zeiss bright-field microscope (A and P) or a Zeiss confocal microscope (all other images). The asterisk (*) indicates sacculi prepared from strains that were treated with aztreonam (2 μg/ml) to inhibit PBP3 (FtsI) to observe PIPS bands. The following *E. coli* strains were processed: SKW49-1 (A), SKW36-1 (B), LP22-1 (C), SKW33-1 (D), LP8-1K (E), SKW20-1 (F), LP6-1K (G), SKW41-1, also including a deletion that eliminates PBP1A expression (H), SKW42-1, including a deletion that eliminates PBP1B expression (I), RP182 (J), LP3 (K), CS703-1 (L), LP21-1 (M), SKW30-1 (pCX16) (N), LP35-1K (pCX16) (O), SKW27-1 (P), and WM1657 (R). In each panel, bar = 1 μm.

from a mutant lacking PBP1b but carrying the *ftsI23* allele, at 55% of the potential septa ($n = 55$), sacculi labeled with antibodies directed against total murein exhibited a narrow band where the peptidoglycan was stained less intensely (see Fig. S1A in the supplemental material, α-PG). This seemed to indicate that there was a defect in making peptidoglycan at these sites, which might account for at least part of the absence of D-cysteine labeling. However, even at these sites, the associated PIPS bands were clear and wider than the putative peptidoglycan defects (see Fig. S1, α-biotin). In addition, the defects were observed in this particular parent strain even when FtsI23 was inactivated at 42°C in the presence of active PBP1a and PBP1b (see Fig. S1B, α-PG), indicating that the phenomenon was not related to the deletion of PBP1b. Finally, even though about half of the observed PIPS bands might have been artifacts caused by this defect, clear preseptal bands were present at all other sites where the peptidoglycan remained intact, further confirming that PBP1b was not absolutely required for PIPS as long as PBP1a was present. Thus, neither PBP1a nor PBP1b, individually or specifically, was required for preseptal peptidoglycan synthesis. Since mutants lacking both proteins are not viable, it is likely that PBP1a and -1b can substitute for one another in synthesizing peptidoglycan during this stage of division.

Alternate peptidoglycan synthases and L,D-transpeptidases are not required for PIPS. It was possible, as was suggested by Nanninga (48), that preseptal peptidoglycan synthesis might require one or more penicillin-insensitive peptidoglycan synthases instead of PBP1a and -1b. The known candidates for such an activity in *E. coli* are PBP1c (56, 69) and MtgA (4, 24, 25). Because these enzymes exhibit only glycosyltransferase activity, they can polymerize but not cross-link glycan strands. Yet another possibility was that unlabeled PIPS bands might appear because D-cysteine in the fourth position in the peptide side chain had been removed, resulting in zones of D-cysteine-free peptidoglycan. *E. coli* encodes two penicillin-insensitive L,D-transpeptidases, YnhG and YcbB, that might produce this effect (40, 44, 45, 69). To test both these alternatives, we assayed for PIPS in a strain that lacked these four genes (*pbpC*, *mtgA*, *ynhG*, and *ycbB*) (SKW32-1; see Table S1 in the supplemental material). When PBP3 was inhibited by adding aztreonam, clear PIPS bands appeared (Table 2, row 14). When the temperature-sensitive *ftsA12* allele was introduced into this quadruple mutant (SKW35-1), clear PIPS bands also

formed at 42°C (not shown). PIPS bands were also observed in *ftsA12* strains lacking these same four proteins but also missing either PBP1a (Fig. 2H and Table 2, row 15) or PBP1b (Fig. 2I and Table 2, row 16). Thus, the two alternate peptidoglycan synthases and the two L,D-carboxypeptidases were not required to produce PIPS.

AmiC, EnvC, and the hydrolytic PBPs are not required for PIPS. The absence of D-cysteine label from PIPS bands might also be explained by the complete removal of peptide side chains from the peptidoglycan backbone. In particular, several peptidoglycan hydrolases remove peptide side chains during daughter cell separation (8, 9, 39, 54, 62), so it was possible that D-cysteine tags might be removed at this time. To determine if PIPS bands were created in this way, we tested mutants lacking the major septal hydrolase AmiC (8, 62), the amidase activator EnvC (62), or six low-molecular-weight PBPs that might remove D-cysteine from peptidoglycan side chains (20). PIPS bands were present in sacculi isolated from aztreonam-treated strains lacking AmiC (Fig. 2J and Table 2, row 17) or EnvC (Fig. 2K and Table 2, row 18). PIPS bands were also present in a mutant lacking seven PBPs, including PBP1a and six low-molecular-weight PBPs (Fig. 2L and Table 2, row 19). Thus, none of these peptidoglycan-modifying proteins were required for PIPS, providing further support for the interpretation that unlabeled regions in this assay represent the insertion of new material.

MreBCD and associated cell wall elongation proteins are not required for PIPS. The MreBCD proteins help maintain the rod shape of *E. coli* and other bacteria by orienting and perhaps guiding peptidoglycan synthesis during cell elongation (19, 27, 30, 63, 69). A hint that MreBCD might be involved in preseptal peptidoglycan synthesis comes from the fact that these proteins form paired rings on either side of nascent Z rings at a time when peptidoglycan synthases might be transferred from MreB to FtsZ control (67, 68). In addition, other proteins are associated with this MreB-directed complex, including RodZ and RodA, and either of these might also be involved in PIPS. We assayed for PIPS in the absence of these proteins by taking advantage of the fact that *mre* and similar mutants survive in the presence of excess FtsZ (6). After inhibiting PBP3 by adding aztreonam, sacculi from cells lacking MreB (strain LP19-1; Table 2, row 20), MreCD (strain LP20-1; Table 2, row 21), MreBCD (Fig. 2M and Table 2, row 22), RodZ (Fig. 2N and Table 2, row 23), or RodA (Fig. 2O and Table 2, row 24) all had clear PIPS bands at predivision sites. Many of these bands were uneven or slightly V shaped, being wider on one side of the cell than on the other (e.g., Fig. 2M and O). This pattern was expected because such spherical *E. coli* bacteria frequently divide by initiating invagination on one side of the cell before the other (5, 6). Importantly, not only was MreB not required for PIPS but neither were MreC or MreD, which connect cytoplasmic MreB to the periplasmic peptidoglycan synthases. We note that in three of these strains fewer than half the sacculi exhibited PIPS. However, strains lacking PBP2 lyse after PBP3 is inhibited by aztreonam, making it difficult to isolate intact sacculi from cells treated this way (66). A similar situation may have arisen in the current mutants because MreCD, RodA, and RodZ collaborate with PBP2 to synthesize the lateral cell wall. Thus, sacculi with PIPS bands may be underrepresented under these conditions. In any case, the results indicate that none of the major proteins in the peptidoglycan elongation complex are required for this preseptal synthesis, extending our previous observations that PIPS occurs in cells in which either MreB or PBP2 is inactivated by antibiotics (66).

The FtsA* protein bypasses the requirement for ZipA. ZipA is essential for the growth of wild-type *E. coli*. However, a single amino acid change in FtsA creates a protein, FtsA* ($FtsA^{R286W}$), that allows *E. coli* to divide and survive in the absence of ZipA or the normally essential cell division protein FtsK or FtsN (7, 31–33). Therefore, we determined if cells expressing FtsA* could also produce preseptal peptidoglycan in the absence of ZipA. PBP3 was inhibited by adding aztreonam to strains carrying FtsA* and lacking either the ZapABC proteins (Fig. 2P and Table 2, row 25), the ZipA protein (Fig. 2R and Table 2, row 26), or the ZapABC and ZipA proteins (Table 2, row 27). PIPS bands formed in all three cases. The same result was observed when FtsA* was moved into the W3110 background (not shown). Thus, under special circumstances, preseptal peptidoglycan could be synthesized in the absence of ZipA, though this protein is normally essential for the viability of wild-type *E. coli*. Of special note, though, is that PIPS bands appeared even in cells carrying the bypass FtsA* protein, suggesting that cell division may require this peculiar stage of cell wall synthesis.

DISCUSSION

Synthesis of the peptidoglycan cell wall is driven by two apparently independent complexes: cell elongation is directed by MreBCD plus PBP2 and associated proteins, whereas cell division is directed by FtsZ plus PBP3 and associated proteins (19, 46). Surprisingly little is known about what happens during the transition between these two activities, but at least one event occurs after elongation ends and just before division commences. This event, the FtsZ-dependent synthesis of preseptal peptidoglycan (PIPS), introduces a circumferential band of new peptidoglycan at the midcell where septation will eventually take place (22, 48, 49, 72). This phenomenon may be a bona fide transitional stage between elongation and division, because it occurs at a defined time during each cell cycle and because its formation is tightly regulated so that sacculi of nondividing cell filaments contain multiple, evenly spaced PIPS bands, each with similar dimensions and located at potential septal sites (22). Here we report that of the major known division and elongation proteins in wild-type *E. coli*, PIPS depends only on the presence of FtsZ and ZipA. This requirement may explain why ZipA is essential in the gammaproteobacteria (17), and the results narrow the possibilities for what PIPS is and does. In particular, the results rule out the following as being responsible for this intermediate stage of cell division: any currently known divisome structure beyond that containing ZipA, the cell wall elongation complex built around MreB, any of the known alternate peptidoglycan synthases, and any of several peptidoglycan-modifying enzymes (the major amidases, known and predicted L,D-carboxypeptidases, or the endopeptidase and D,D-carboxypeptidase PBPs).

Explaining PIPS. Before this work, three possibilities could be advanced to explain the synthesis of preseptal peptidoglycan synthesis. PIPS could represent the following: (i) the last synthetic activity of cell wall elongation complexes drawn to the midcell by the nascent FtsZ ring, (ii) the first synthetic activity of an incomplete early-stage Z ring, or (iii) an intermediate stage of cell wall synthesis or modification mediated by proteins different from those involved in elongation or division. In the first scenario, FtsZ would attract the MreB elongation complex to the midcell, and

PIPS would represent a period of MreB-directed peptidoglycan synthesis in which all activity was concentrated at the preseptal site. Such a possibility was suggested by the fact that rings of MreBCD, PBP2, and RodA assemble on either side of the nascent FtsZ ring, placing them at midcell at the time when PIPS occurs (67, 68). In fact, it seems quite logical that the elongation and division complexes should be brought into close proximity to spur the transition from sidewall to septal synthesis, and PIPS might be the visible signature of this event (67, 68). Also, the MreBCD-PBP2-RodA complex spans the inner membrane into the periplasm (19, 43), making these proteins prime candidates for connecting FtsZ-ZipA to the periplasmic peptidoglycan synthases. And finally, formation of the paired MreB rings requires FtsZ but not FtsA, FtsQ, or PBP3, requirements that are identical to those for PIPS (67). However, two pieces of evidence reported here argue that the cell wall elongation complex does not generate PIPS. First, MreB rings assemble near FtsZ rings that are anchored to the membrane by either FtsA or ZipA (67, 68), but PIPS occurs only when FtsZ is anchored by ZipA. This indicates that the presence of periseptal MreB rings is not sufficient to account for preseptal peptidoglycan synthesis. Second, PIPS bands appear during the growth of spherical cells lacking the MreBCD proteins, indicating that PIPS is not simply a by-product created by concentrating the normal synthetic activity of the MreB elongation complex at midcell.

The second explanation for PIPS is that it could represent an early FtsZ-directed stage of septal synthesis, perhaps immediately after peptidoglycan synthases are transferred from MreB to FtsZ. The divisome itself matures in two stages: an early stage in which FtsZ assembles with FtsA, ZipA, and ZapABC (and perhaps with FtsEX), followed several minutes later by a second stage in which the FtsKQBLWIN proteins complete the ring and initiate constriction (2, 19). With the exception of the requirement for FtsZ and ZipA, PIPS bands appear in the absence of each of the other first-stage components. Therefore, PIPS must occur during the earliest stage of divisome assembly, well before FtsK, FtsQ, and PBP3 are added. In this scenario, FtsZ-ZipA would receive the peptidoglycan synthases (e.g., either PBP1a or -1b) from MreB and redirect their activities to produce PIPS. Supporting this possibility are observations that under certain circumstances, FtsZ can direct the synthesis of the lateral cell wall in the absence of invagination (i.e., during the first stage of Z-ring formation) and can do so in conjunction with PBP2 (1, 64, 66). Though PBP2 normally works with MreB to synthesize the sidewall, it is also found at the division site (18), raising the possibility that a transient FtsZ-PBP2 collaboration could direct PIPS. However, PIPS bands also appear when PBP2 is inactivated (66), so some type of non-PBP2-dependent scenario must be at work. In any case, PIPS may represent a division stage that accompanies or occurs immediately after the transfer of peptidoglycan synthases from MreB to FtsZ (61). This is the simplest alternative consistent with current data.

The third possibility is that PIPS is produced by cell wall synthases or accessory enzymes that differ from those involved in sidewall synthesis or in PBP3-dependent invagination. If so, then one or more peptidoglycan synthases would be recruited to the FtsZ-ZipA ring and become active before the addition of PBP3. There is as yet no evidence to support this alternative, and our current results rule out many known and predicted alternate peptidoglycan synthases and several hydrolases as being involved. However, due to the current dearth of experimentation on the subject, this option cannot be ruled out.

How might ZipA direct or influence PIPS? FtsZ is cytoplasmic, and PIPS occurs in the periplasm, so it seems reasonable to assume that at least one integral or transmembrane protein connects FtsZ to the periplasmic peptidoglycan synthases (as suggested by Nanninga more than 20 years ago) (48). One possibility is that ZipA makes such a connection via its short N-terminal integral membrane domain. Notably, when this segment is replaced with a related transmembrane domain, the hybrid protein still localizes to the Z ring but does not restore division to a ZipA-depleted strain (38). Thus, the ZipA N-terminal domain has an unknown function in addition to its role as a membrane anchor, though whether this includes making contact with another protein is unknown. One option is that this domain might interact with the transmembrane domains of PBP1a or -1b. Another possibility is that the ZipA-to-periplasm connection is indirect; i.e., ZipA may bind a second protein that is the direct (or indirect) connection to the periplasm. To our knowledge, ^{D}MinC/DicB is the sole known protein complex that requires only ZipA for binding to the Z ring (42). Although these two proteins inhibit Z ring formation and are not candidates for being the PIPS connector, the existence of such an interaction argues that an indirect ZipA-to-periplasm linkage is possible. The results reported here rule out the most obvious candidates as being the transmembrane connector, so the communication between FtsZ and PIPS enzymes may be novel.

Based on sequence homology alone, ZipA is not a highly conserved protein, being found only in the Gram-negative gammaproteobacteria (17, 47). However, orthologues or functional homologues may be expressed in *Mycobacterium tuberculosis* (59), *Bacillus subtilis* (58), and *Neisseria gonorrhoeae* (28). The *B. subtilis* protein SepF may bundle FtsZ polymers and tether them to the membrane in a manner similar to that of ZipA (36). Moreover, the *N. gonorrhoeae* protein SLO7ORF3, although sharing no sequence similarity with *E. coli* ZipA, is structurally homologous and complements an *E. coli zipA* null mutant (28). Thus, other proteins may fill the physiological role of ZipA across many genera, making the observations we report here of general import.

Bypassing ZipA. Finally, although wild-type *E. coli* requires ZipA to form PIPS bands, this requirement can be waived, at least in part, in a strain containing FtsA* (FtsA^{R286W}). We note, however, that although this mutant can bypass the requirement for ZipA, the PIPS phenomenon itself was recreated in FtsA* mutants, implying that preseptal peptidoglycan synthesis may represent a distinct and necessary stage of bacterial division. In any event, the ability of FtsA* to bypass the need for ZipA has at least two possible implications. First, if PIPS is synthesized by the same mechanism in both wild-type and ZipA^{-} FtsA* cells, then PIPS cannot be mediated by an interaction that requires a specific ZipA-to-transmembrane protein link (because the reaction continues to occur in the absence of ZipA). What this alternate mechanism might be is hard to fathom because it implies that an unknown protein binds FtsZ and communicates with the periplasm. Second, PIPS bands in FtsA* cells might be created by a different, ZipA-independent mechanism. The working definition of PIPS is simply that these preseptal zones represent peptidoglycan incorporation in the absence of functional PBP3 (FtsI), which means that any such activity may give the same visual signature. For example, if PIPS represents an intermediate stage in which the peptidoglycan synthases are transferred from the MreB apparatus

to the FtsZ ring, then cells carrying FtsA* may have developed a way to do this in the absence of ZipA. This is certainly possible, because FtsA* recruits downstream divisome proteins in the absence of ZipA (31, 33), and several newly identified FtsA mutants behave similarly (51). Also, some bacteria seem to lack ZipA altogether (17, 47), so other avenues that perform the same function must exist. Intriguingly, mutations in genes other than *ftsA* can also bypass the requirement for ZipA, but these suppressor mutations have not yet been identified (51). In short, we do not yet know whether these alternate pathways for generating PIPS bands are mechanistically the same, aside from being ZipA independent. This question can be answered only after the specific requirements for ZipA-mediated PIPS formation have been defined.

In summary, ZipA orchestrates a distinctive type of FtsZ-dependent cell wall synthesis at the transition between cell wall elongation and invagination, thus opening the study of a new sequence of events during this phase of cell division.

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