JOURNAL OF BACTERIOLOGY, June 2009, p. 3526–3533 Vol. 191, No. 11
0021-9193/09/$08.00+0 doi:10.1128/JB.01812-08

In *Escherichia coli*, MreB and FtsZ Direct the Synthesis of Lateral Cell Wall via Independent Pathways That Require PBP 2[▽]

Archana Varma and Kevin D. Young*

Department of Microbiology and Immunology, University of North Dakota School of Medicine and Health Sciences, Grand Forks, North Dakota 58202-9037

Received 23 December 2008/Accepted 23 March 2009

In *Escherichia coli*, the cytoplasmic proteins MreB and FtsZ play crucial roles in ensuring that new muropeptide subunits are inserted into the cell wall in a spatially correct way during elongation and division. In particular, to retain a constant diameter and overall shape, new material must be inserted into the wall uniformly around the cell's perimeter. Current thinking is that MreB accomplishes this feat through intermediary proteins that tether peptidoglycan synthases to the outer face of the inner membrane. We tested this idea in *E. coli* by using a DD-carboxypeptidase mutant that accumulates pentapeptides in its peptidoglycan, allowing us to visualize new muropeptide incorporation. Surprisingly, inhibiting MreB with the antibiotic A22 did not result in uneven insertion of new wall, although the cells bulged and lost their rod shapes. Instead, uneven (clustered) incorporation occurred only if MreB and FtsZ were inactivated simultaneously, providing the first evidence in *E. coli* that FtsZ can direct murein incorporation into the lateral cell wall independently of MreB. Inhibiting penicillin binding protein 2 (PBP 2) alone produced the same clustered phenotype, implying that MreB and FtsZ tether peptidoglycan synthases via a common mechanism that includes PBP 2. However, cell shape was determined only by the presence or absence of MreB and not by the even distribution of new wall material as directed by FtsZ.

Bacteria apportion their components evenly between daughter cells at division, and cell wall metabolism is an integral part of accomplishing this task. The simplest view of how this occurs in rod-shaped cells is that the wall is created by two alternating processes. In the first, new peptidoglycan (PG) is inserted into the side wall so the cell can elongate and create the space in which cytoplasmic material is segregated. In the second stage, PG synthesis is concentrated at the leading edge of an invaginating ring that constricts and divides the cell, completing the process of segregation (17, 24, 38).

In *Escherichia coli*, the process of cell elongation is controlled by the actin-like cytoskeleton protein MreB, which is believed to localize components of the PG synthetic machinery along the lateral cell wall, thereby governing the geometry of wall growth (4, 5, 31). Cell division is initiated when the tubulin homologue FtsZ condenses on the inner membrane at the center of the cell and triggers invagination by attracting a set of proteins to form a septal ring (15). Circumferential invagination of this ring is accompanied by penicillin binding protein 3 (PBP 3; FtsI)-dependent PG synthesis to create the poles of two new daughter cells (2, 6, 15, 21, 22). Thus, MreB directs PG insertion into the side wall, and FtsZ directs PG insertion at the developing septum. The currently envisioned picture of these two activities is that they are separate and perhaps mutually exclusive (3, 16, 30). However, contrary to this expectation, FtsZ can also direct the incorporation of muropeptides into a segment of the side wall near the poles of *E. coli*, a function heretofore thought to be reserved for MreB (35).

MreB and FtsZ are located on the cytoplasmic side of the inner membrane, whereas the PG synthases are located in the periplasm. Since it is unlikely that either protein makes direct contact with the periplasm, MreB and FtsZ must interact with the PG synthases indirectly via one or more inner membrane proteins. We refer to this composite interaction as "tethering" of the PG synthases. Previously, we observed that simultaneous inhibition of MreB and FtsZ apparently releases ("untethers") the PG synthases, leaving them free to insert new PG into unevenly distributed clusters instead of uniformly in spirals spaced around the periphery of cell (35). This suggests that, at least in *E. coli*, localization of these synthases depends only on the activities of MreB and FtsZ and on no other cytoplasmic structures.

There is a growing consensus that MreB and its homologues tether PG synthases via an interaction with the inner membrane proteins MreC, MreD, and/or PBP 2 (10, 11, 20). Consistent with this interpretation, inhibiting MreB or PBP 2 produces similar FtsZ-dependent phenotypes and PG incorporation patterns (35). Here, we investigate this mechanism further and show that MreB and FtsZ tether the murein biosynthetic machinery via independent pathways that converge on PBP 2. Nonetheless, *E. coli* loses its rod shape only in the absence of fully functional MreB and not when FtsZ-directed PG incorporation is interrupted. Thus, MreB-directed PG synthesis must differ from that directed by FtsZ, either in the geometry of insertion or in some other way.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. The genes encoding PBPs 5 and 7 (*dacA* and *pbpG*, respectively) were deleted from *E. coli* MG1655 (*ilvG rfb-50 rph-1*) by P1 transduction. The antibiotic cassette within the *dacA* gene was removed as described previously (8). These procedures produced *E. coli* AV23-1 (MG1655 *dacA*::*res pbpG*::Kan *ilvG rfb-50 rph-1*) (35). Plasmid pFAD38 (pro-

* Corresponding author. Present address: Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, AR 72205-7199. Phone: (501) 526-6802. Fax: (501) 686-5359. E-mail: kdyoung@uams.edu.

[▽] Published ahead of print on 3 April 2009.

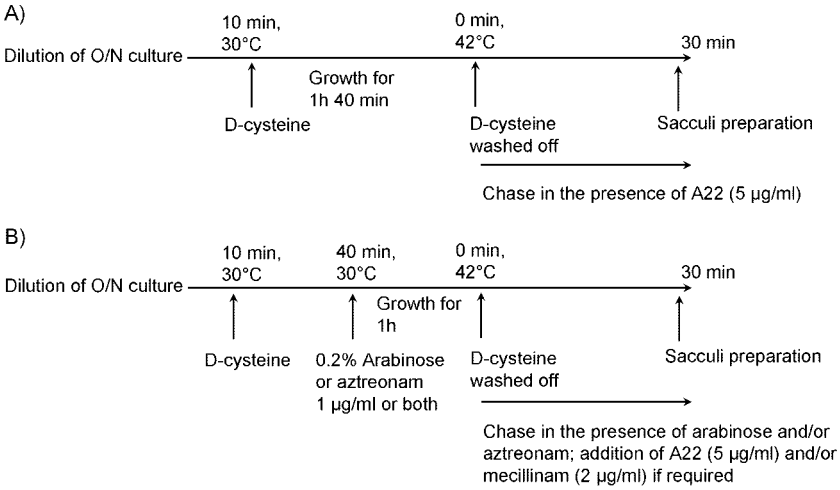

FIG. 1. Outlines of experimental procedures for D-cysteine labeling of *E. coli* peptidoglycan. The addition of arabinose (to 0.2% final concentration) induces expression of the *sulA* gene; A22 (5 μg/ml) inhibits the MreB protein; aztreonam (1 μg/ml) inhibits PBP 3; amdinocillin (mecillinam) (2 μg/ml) inhibits PBP 2. O/N, overnight.

vided by J. Lutkenhaus) carries the *sulA* gene under the arabinose promoter. Strains carrying pFAD38 were maintained in Luria-Bertani (LB) broth or LB agar plates supplemented with 50 μg/ml ampicillin and 0.4% glucose to inhibit *sulA* expression. Overnight cultures were washed to remove glucose and then diluted 1:50 into fresh LB medium containing 0.5% NaCl. Arabinose (0.2% final concentration) was added at specific time points to induce *sulA* expression. The compound A22 (5 μg/ml, final concentration) used to inhibit MreB (14) was synthesized and supplied by A. Novikov (University of North Dakota). Aztreonam (1 μg/ml; Sigma Chemical Co., St. Louis, MO) was added to inhibit PBP 3 to block cell division, and amdinocillin (2 μg/ml; Leo Pharmaceuticals, Denmark) was added to inhibit PBP 2 to block cell wall elongation. The antibiotics were added in different combinations and sequences, as outlined in Fig. 1.

Peptidoglycan labeling, preparation, and examination. Overnight cultures were washed once, diluted 1:50 in fresh medium, and grown as outlined in Fig. 1. Peptidoglycan was labeled with D-cysteine, sacculi were isolated after a 30-min chase period and prepared for confocal microscopy, and D-cysteine was detected by biotinylation and immunolabeling, as described previously (9, 35). In some experiments, the same sacculi were also incubated with vancomycin to label peptide side chains that terminate in D-alanine-D-alanine, and bound vancomycin was detected by immunolabeling, as described previously (35). Only those sacculi that were well isolated and evenly spread were included in the final observations. This is because not all sacculi that settle onto the surface are completely flat. Instead, many are bent or folded, which produces artifactual increases in fluorescence intensity in those areas where the sacculus overlaps itself. To guard against these artifacts, all sacculi were stained with an antibody directed against peptidoglycan and which stains sacculi uniformly (35). Only those sacculi that had no obvious bends or folds were selected for determining the distribution of newly inserted peptidoglycan.

Microscopy. Confocal microscopy was performed with a Zeiss META microscope, fitted with a Zeiss 63× 1.4 numerical aperture objective lens, using parameters appropriate for the Nyquist criteria for deconvolution. Images were deconvolved using Huygens software (Scientific Volume Imaging B.V., Hilversum, The Netherlands) and assembled with Adobe Photoshop and Image J.

RESULTS

Recently, we observed that simultaneous inhibition of FtsZ and MreB resulted in uneven incorporation of peptidoglycan precursors into the cell wall (35). In this situation, insertion of new material was unconstrained and accumulated in amorphous spots (clusters) rather than being spread uniformly around the cell periphery in spiral-like filaments. This implied that the loss of FtsZ and MreB produced an untethered PG biosynthetic machinery, in that cell wall synthesis continued but was not directed by underlying cytoskeleton factors. This result prompted us to devise an assay to test the possible pathways by which *E. coli* might tether and direct murein synthesis.

Either FtsZ or MreB can tether peptidoglycan biosynthesis. There are five possible pathways by which FtsZ and MreB might tether the murein biosynthetic machinery to the outer surface of the inner membrane and direct cell wall synthesis in the periplasm (Fig. 2). FtsZ (Fig. 2, path 1) or MreB (Fig. 2, path 5) may tether the PG synthases independently by interacting with proteins other than PBP 2. Alternatively, each of the two pathways might require PBP 2 as a common intermediary (Fig. 2, paths 2 and 4). Finally, it is theoretically possible that FtsZ and MreB interact with one another to tether via any of the other four paths (Fig. 2, path 3). To determine which of these pathways or interactions occur, we examined the incor-

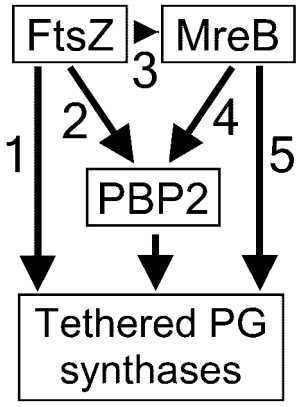

FIG. 2. Possible pathways by which the cytoskeleton proteins FtsZ and MreB may tether and direct the murein biosynthetic machinery. Path 1, FtsZ tethers independently, via an intermediary other than PBP 2; path 2, FtsZ tethers via PBP 2 but independently of MreB; path 3, FtsZ and MreB interact to form a tether; path 4, MreB tethers via PBP 2 but independently of FtsZ; path 5, MreB tethers independently, via an intermediary other than PBP 2.

TABLE 1. Cell shape and PG incorporation patterns in *E. coli* strains lacking FtsZ, MreB, or PBP 2

Strain no.[a]	Phenotype[b]			Cell shape		PG incorporation	
	FtsZ	MreB	PBP2	Rod	Sphere	Even	Clustered
1	+	+	+	+	−	+	−
2	−	+	+	+	−	+	−
3	+	−	+	−	+	+	−
4	−	−	+	−	+	−	+
5	+	+	−	−	+	−	+

[a] *E. coli* AV23-1 (*dacA pbpG*) was used in all experiments. The first strain (strain 1) was untreated. All the rest were treated as indicated to inactivate FtsZ, MreB, PBP 2, or combinations thereof.

[b] FtsZ was inactivated by endogenous expression of the SulA protein, MreB was inactivated by adding the antibiotic A22, and PBP 2 was inactivated by adding amdinocillin. +, active; −, inactive.

poration of new peptidoglycan into *E. coli* AV23-1 in the presence or absence of active FtsZ and/or MreB. Evenly distributed versus clustered (patchy) incorporation of peptidoglycan was visualized by labeling cells with D-cysteine and vancomycin and then observing the distribution of newly incorporated murein by noting the location of D-cysteine-unlabeled material and its overlap with vancomycin-labeled material.

We first determined whether FtsZ and MreB required one another to tether the murein biosynthetic machinery (Fig. 2, path 3). To do so, we inactivated FtsZ by overexpressing SulA and reconfirmed our previous observation (36), that MreB forms helices and directs the insertion of newly synthesized peptidoglycan in an evenly distributed manner in the absence of active FtsZ (Table 1, strain 2). This indicated that MreB could tether the PG synthesizing enzymes without having to interact with FtsZ. To determine if FtsZ could function as a general tether by itself, we inhibited MreB by adding the compound A22 (Table 1, strain 3). Although the cells began to round up due to disintegration of MreB helices, newly incorporated peptidoglycan was still distributed evenly in the wall (Fig. 3A). In Fig. 3A, the green signal represents newly inserted peptidoglycan stained with vancomycin, and this new material was spread evenly around the cells except for a bright band at sites of ongoing septation. The location of D-cysteine-labeled (i.e., prechase) peptidoglycan in these same sacculi is represented by a red signal (Fig. 3A), which indicates that new material was inserted over the surface of the cells in much the same way as observed in the vancomycin-labeled cells, with a dark band of new material at the septa of cells that were dividing at the time of labeling. Similar distributions were observed in 30 of 30 well-spread sacculi (100%). This outcome was surprising because FtsZ is thought to direct PG incorporation only during septation. Instead, the results suggest that in the absence of MreB, FtsZ can tether and direct a generalized and evenly dispersed PG incorporation into the side walls as the cells expand into spherical forms. Thus, FtsZ and MreB do not have to interact with one another to direct insertion of new peptidoglycan.

Although the preceding conclusion was the simplest interpretation, it was possible that the small size of the cells (and their sacculi) only made it appear that there was a uniform FtsZ-dependent incorporation of precursors into the side wall. This constraint might prevent the observation of patchy incorporation, or else brightly labeled septa may have obscured the presence of localized patches of vancomycin labeling. To rule out these possibilities, we added the antibiotic aztreonam to inactivate PBP 3 (FtsI) during the D-cysteine labeling and chase periods, thereby inhibiting septation and causing the cells to grow as filaments (Fig. 1B). This treatment enlarged the cells and made it easier to see the distribution of new cell wall material. As expected, older D-cysteine-labeled material was retained as bright areas at the poles (representing inert peptidoglycan) and a dark band of D-cysteine-free material was present at the center of each cell (representing new peptidoglycan inserted prior to septation) (Fig. 3B, center sections). This latter material is the product of PBP 3-independent PG synthesis (PIPS) (see below). Other D-cysteine-labeled peptidoglycan in the side walls was diluted in intensity and, other than at septa, did not show clear zones of clustered incorporation. This indicated that newly inserted material was distributed evenly. Vancomycin labeling corroborated this interpretation, in that new cell wall material was incorporated throughout the side walls (Fig. 3B, left-most sections). This distribution was observed in 20 of 20 well-spread sacculi (100%). These results proved that evenly dispersed incorporation of peptidoglycan in the absence of MreB was real and not an artifact created by inadequate resolution due to small cell size.

A second artifact that might have complicated interpretation of the above results was the formal possibility that, when PBP 3 was inactivated by aztreonam, an unknown pathway could have removed the pentapeptides from numerous glycan chains. The detection of newly synthesized wall depends on the presence of pentapeptides to which vancomycin can bind, and the

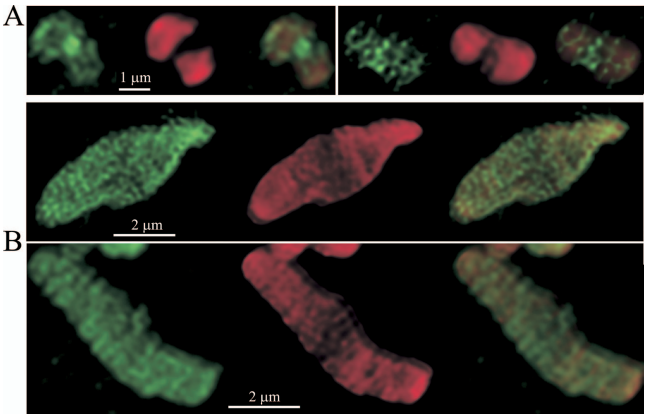

FIG. 3. Inhibiting MreB does not cause clustered incorporation of peptidoglycan into the cell wall. *E. coli* AV23-1 was labeled with D-cysteine for 100 min and then chased in the absence of D-cysteine for 30 min at 42°C in the presence of A22 (5 μg/ml), either without added aztreonam (A) or in the presence of aztreonam (1 μg/ml) (B). Sacculi were prepared and incubated with vancomycin, followed by immunolabeling to detect bound vancomycin. The left-most image shows the location of vancomycin-labeled peptidoglycan (green); the middle image shows the location of older D-cysteine-labeled peptidoglycan (red); the right-most image merges the two. Note that the location of vancomycin-labeled material is evenly distributed in the side walls in the absence of aztreonam, but there are zones of high incorporation (bright green) (A, left) that correspond to the D-cysteine-free (dark) zones of septal incorporation (A, middle). In the presence of aztreonam there is no heightened labeling by vancomycin (B, left) in the central D-cysteine-free zones (B, middle). This latter represents preseptal PIPS, as discussed in the text.

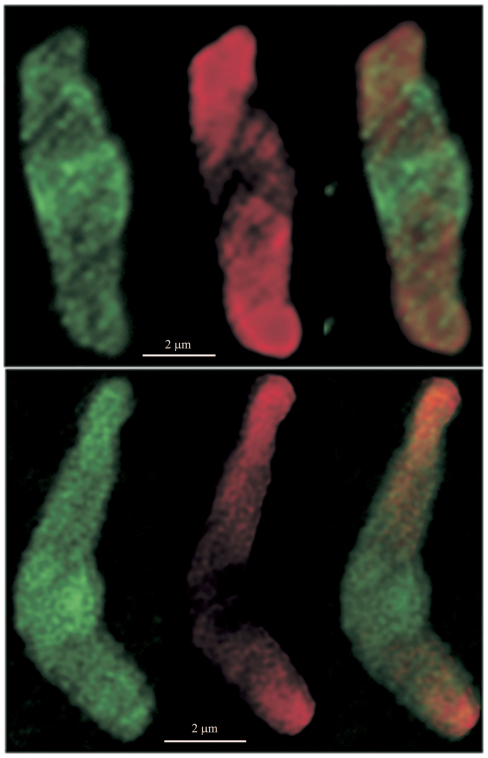

FIG. 4. PBP 3 does not interfere with FtsZ-directed insertion of peptidoglycan into the side wall. *E. coli* AV23-1 was labeled with D-cysteine in the presence of arabinose (to induce production of SulA protein, which inhibits FtsZ) and aztreonam (to inhibit PBP 3), as outlined in Fig. 1B. The cells were chased in the absence of D-cysteine at 42°C for 30 min in the presence of arabinose, aztreonam, or A22 (to inhibit MreB). Sacculi were prepared and incubated with vancomycin, followed by immunolabeling to detect bound vancomycin. The leftmost images show sacculi labeled with vancomycin (green); the middle images show the same sacculi labeled with D-cysteine (red); the rightmost images are superimposed versions of the first two. Note that the clustered labeling by vancomycin (left, green) coincides with the dark regions of D-cysteine-unlabeled, newly inserted peptidoglycan (center images).

absence of terminal D-alanines would make it impossible to detect localized incorporation of new material with this technique. Therefore, to exclude this possibility, we inhibited FtsZ, MreB, and PBP 3 simultaneously in *E. coli* AV23-1 during the chase period. We already established that simultaneous inactivation of FtsZ and MreB results in clustered incorporation of murein precursors (35). Therefore, if inactivation of PBP 3 triggers the cleavage of pentapeptides, then clustered incorporation should disappear from sacculi of cells treated in this way. Instead, we found that unstained regions of D-cysteine-labeled cells (Fig. 4, center sections) coincided with bright patches of newly inserted pentapeptides detected with vancomycin labeling (Fig. 4, left sections), as was clearly observed in superimposed images (Fig. 4, right-most photos). This clustered insertion pattern was observed in 14 of 18 well-spread sacculi (78%). The existence of these clustered patches indicated that inactivation of PBP 3 did not interfere with visualization of newly incorporated murein. This observation also confirmed that FtsZ-directed insertion of new material into the side wall was PBP 3 independent, highlighting the fact that this reaction differs from PG incorporated during septation.

Overall, the results indicate that the disintegration of MreB helices is not sufficient to untether the PG biosynthetic machinery. Instead, FtsZ can tether the PG synthases and distribute their activity around the cell without the aid of MreB. Incorporation of precursors into the cell wall continued to be evenly dispersed when either FtsZ or MreB was active, indicating that FtsZ and MreB can tether the PG synthases independently of one another. This rules out the possibility that the two proteins must interact (Fig. 2, path 3), although they may do so in other situations.

Tethering of PG synthases by MreB requires PBP 2. PBP 2 is a periplasmic transpeptidase required for lateral cell wall synthesis (17, 29, 32), and inactivating PBP 2 produces the same spherical growth phenotype as does inhibition of MreB. Similarly, inhibiting FtsZ and PBP 2 produces the same morphological phenotype as does inhibiting FtsZ and MreB; in both cases the cells become lemon-shaped with tubular polar extensions (35). These results suggest that PBP 2 is part of the MreB-based tethering pathway (Fig. 2, path 4), which is the currently favored mechanism of action for MreB in *E. coli* (19).

To confirm that PBP 2 was required in the MreB-directed pathway of peptidoglycan insertion in *E. coli*, we inhibited FtsZ by expressing SulA during the D-cysteine-labeling stage and then inactivated PBP 2 by adding amdinocillin during the chase period (Fig. 1B). Inhibiting FtsZ and PBP 2 produced results consistent with untethering of the PG biosynthetic machinery (Fig. 5). That is, D-cysteine labeling indicated that new murein (unlabeled material) was incorporated unevenly in large patches in the bacterial side walls (Fig. 5, center sections). These same areas were filled with newly inserted murein as determined by vancomycin labeling (brightly labeled green clusters) (Fig. 5, left sections). This distribution was observed in 14 of 16 well-spread sacculi (88%). The results support the hypothesis that PBP 2 functions in the MreB pathway to tether the murein biosynthetic machinery (Fig. 2, path 4).

Tethering of PG synthases by FtsZ requires PBP 2. To determine if FtsZ-directed side wall synthesis also requires PBP 2, we inhibited MreB and PBP 2 simultaneously during the chase period in D-cysteine-labeled cells (Fig. 1B). To observe the positioning of new peptidoglycan more clearly, the cells were filamented slightly by adding aztreonam to inhibit PBP 3 during the chase period (Fig. 1B). However, because simultaneous inhibition of PBPs 2 and 3 leads to cell lysis (13), we grew the cells in the presence of aztreonam and amdinocillin for only 30 min. Sacculi treated this way exhibited clustered incorporation of new murein into the side walls (Fig. 6). Vancomycin-labeled material was concentrated primarily in the region of the enlarged portions of the cells (Fig. 6, left sections), and these incorporation sites coincided with the insertion of new material as determined by D-cysteine labeling (Fig. 6, center sections). This distribution was observed in 4 of 12 well-spread sacculi (33%). The percentage of sacculi exhibiting clustered incorporation was lower than that observed for conditions in which only PBP 2 or PBP 3 was inhibited (see above). Because inhibiting PBPs 2 and 3 caused the cells to lyse during the chase period, it was difficult to identify suitably intact sacculi. Clustered incorporation followed by lysis evidently decreased the number of this class of sacculi available

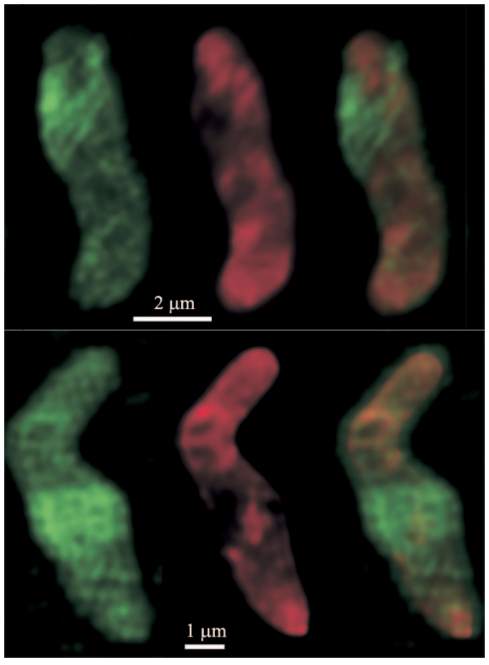

FIG. 5. PBP 2 inhibition produces clustered insertion of peptidoglycan in the absence of functional FtsZ. *E. coli* AV23-1 was labeled with D-cysteine in the presence of arabinose (to induce SulA production and inhibit FtsZ). The cells were chased in the absence of D-cysteine at 42°C for 30 min, and arabinose (to inhibit FtsZ) and amdinocillin (to inhibit PBP 2) were present during this time. The left-most images show sacculi stained with vancomycin (green); the central images show the same sacculi stained with D-cysteine (red); the right-most images are superimposed versions of the first two.

for observation, which may explain the lower percentage of sacculi with this type of peptidoglycan incorporation. Even so, the existence of a substantial fraction of sacculi with clustered incorporation supports the hypothesis that FtsZ requires PBP 2 to tether the side wall synthesis machinery (Fig. 2, path 2).

As noted, it was difficult to see clustered patches of newly inserted murein in sacculi from cells in which MreB, PBP 2, and PBP 3 were all inhibited. Other than simply being a matter of the cells being difficult to isolate and observe, two other possibilities may have been at play. First, because the cells were beginning to lyse, PG incorporation could have been affected in some fraction of the cells from which sacculi were prepared. It was not possible to determine whether a particular sacculus was actively synthesizing murein at the time of harvest or how close to death the intact cell might have been. However, a dark unstained band was visible at the septum in some sacculi previously labeled with D-cysteine (Fig. 6, center sections). This indicated that these cells were growing and incorporating PG during the chase period, resulting in the classical banding pattern known as PIPS. A second possible reason for the difficulty of observing patchy insertion is that there may have been a general reduction in the amount of peptidoglycan incorporation under these conditions. This would occur if the enzymatic activity of these enzymes decreases when they are not tethered, as suggested by Uehara and Park (34). If the activity of peptidoglycan polymerases is altered when they interact with the FtsZ or MreB complexes, this would explain the poor labeling observed in sacculi prepared from cells treated in the above manner.

Inactivating PBP 2 untethers peptidoglycan incorporation. If PBP 2 is a member of both the FtsZ- and MreB-directed pathways, then inactivating PBP 2 by itself should be sufficient to produce a clustered pattern of murein incorporation similar to that caused by inhibiting MreB and PBP 2 or by inhibiting FtsZ and MreB. To test this, we inhibited PBP 2 by adding amdinocillin during the chase period and observed the distribution of newly inserted peptidoglycan (Fig. 1B). In some cases we also inhibited PBP 3 during D-cysteine labeling to filament cells containing active FtsZ and MreB, thereby enlarging the cells to simplify the observations.

When PBP 2 was inhibited, peptidoglycan incorporation was not even but was inserted into one or more large patches in the enlarged portion of the side walls of these cells (Fig. 7; Table 1, strain 5). Newly inserted cell wall material was clustered into

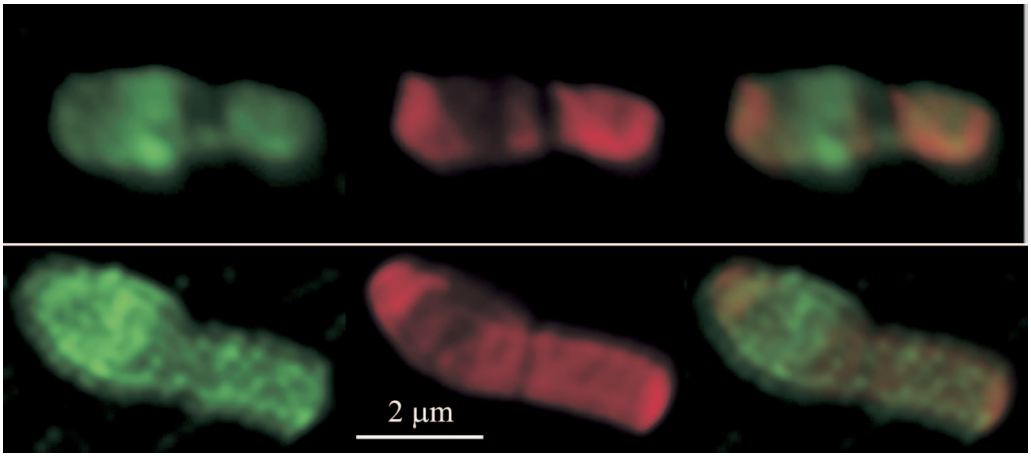

FIG. 6. In the absence of functional MreB, tethering by FtsZ requires PBP 2. *E. coli* AV23-1 was labeled with D-cysteine in the presence of aztreonam. The cells were chased at 42°C for 30 min in the presence of aztreonam, A22 (to inhibit MreB), or amdinocillin (to inhibit PBP 2). The left-most images show sacculi stained with vancomycin (green). The clustered labeling by vancomycin (left, green) coincides with the dark regions of D-cysteine-unlabeled, newly inserted peptidoglycan (center images). The right-most images are superimposed versions of the first two.

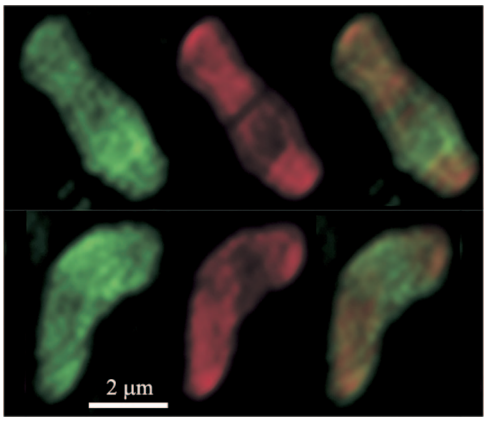

FIG. 7. Inactivation of PBP 2 untethers peptidoglycan incorporation. *E. coli* AV23-1 was labeled with D-cysteine in the presence of aztreonam. The cells were chased at 42°C for 30 min in the presence of aztreonam and amdinocillin (to inhibit PBP 2). Sacculi were prepared and incubated with vancomycin, followed by immunolabeling to detect bound vancomycin. The left-most images are sacculi labeled with vancomycin (green). The clustered labeling by vancomycin (left, green) coincides with the dark regions of D-cysteine-unlabeled, newly inserted peptidoglycan (center images). The right-most images are superimposed versions of the first two.

pentapeptide-rich areas, as determined by vancomycin labeling (Fig. 7, left sections). D-Cysteine labeling corroborated this finding in that newly inserted (unlabeled) material accumulated in these same areas (Fig. 7, center sections). These distributions were observed in 5 of 10 well-spread sacculi (50%). If either FtsZ or MreB had been able to tether the murein synthases independently of PBP 2, then the insertion patterns would have been evenly dispersed in all sacculi. The fact that we observed clustered insertion in half the cells indicates that tethering of the murein synthesizing machinery did not occur via pathways 1 or 5 (Fig. 2) but depended on the presence of active PBP 2. This result, combined with the finding that FtsZ and MreB need not interact, confirms that both FtsZ and MreB can tether PG synthases via a pathway that includes PBP 2 (Fig. 2, paths 2 and 4, respectively). Therefore, generalized insertion of new murein requires an active PBP 2 and either FtsZ or MreB.

Spherical cell shape is not correlated with evenly distributed PG insertion. When MreB helices are disrupted, *E. coli* cells swell and grow as spheres (19, 35, 37). Initially, we assumed that the change from rod-like to spherical growth would be caused by a change in the way peptidoglycan was inserted, i.e., by switching from evenly dispersed to clustered insertion. As expected, when FtsZ and MreB were inhibited simultaneously, the cells became spherical and exhibited clustered insertion of new murein precursors (Table 1, strain 4). Surprisingly, the insertion of precursors in cells in which only FtsZ or MreB was inhibited did not follow this simple pattern. Instead, spherical cells were formed when MreB was inactivated (Table 1, strain 3) and when FtsZ and MreB were both inactivated (Table 1, strain 4). However, murein incorporation was clustered only when both FtsZ and MreB were inactive (Fig. 4 and Table 1, strain 4) (35). When MreB was inhibited while FtsZ remained active, new murein was incorporated evenly but the cells still grew as spheres (Table 1, strain 3). Thus, spherical growth of *E. coli* did not correlate with the general manner of murein insertion but was determined only by the absence of MreB. Although either FtsZ or MreB could direct evenly distributed peptidoglycan incorporation, only MreB-directed PG insertion produced rod-shaped cells.

DISCUSSION

In *E. coli*, cell wall synthesis is dispersed, and so new material is incorporated evenly around the periphery of the cell (9, 25). The mechanism by which this occurs was once assumed to be simple: PG synthases diffuse randomly throughout the periplasm and newly inserted strands of PG are distributed homogeneously throughout the wall. However, a perfectly dispersed and uniform pattern of insertion is, by definition, the least random outcome of unfettered diffusion. Absent any external direction, diffusion should move PG synthases in a random walk that is more likely to produce nonuniform incorporation, with zones of concentrated insertion separated by areas of less incorporation. This conundrum was solved by the discovery of cytoplasmic proteins related to actin and tubulin (MreB and FtsZ, respectively, in *E. coli*), which organize and direct cell wall synthesis in the periplasm. Currently, MreB is thought to direct PG incorporation to the side walls, while FtsZ takes over and performs the same role during septation (4, 5, 31). Previously, we found that in certain PBP mutants FtsZ directs cell wall synthesis to positions other than at the septum, most noticeably into side walls near the poles (35). A similar phenomenon occurs in *Caulobacter crescentus* during at least part of the cell cycle in which the cells are not actively dividing (1). Here we show that both MreB and FtsZ can act independently to govern insertion of PG into the side walls of *E. coli* and that each pathway requires the help of PBP 2.

Tethering and distributing peptidoglycan synthesis. We expected that inactivating MreB would force the PG synthetic apparatus to insert muropeptides in a nondirected, uneven manner. Instead, irregular and clustered incorporation occurred only when FtsZ and MreB were inactivated simultaneously, indicating that FtsZ can act as a substitute to distribute PG synthesis evenly (reference 35 and our results reported here). Although this activity is surprising, observations in other organisms provide precedents for FtsZ playing such a role. In *Staphylococcus aureus*, the loss of FtsZ causes one PG-related protein, PBP 2, to relocalize from a general distribution to a few discrete clusters, and electron micrographs indicate that PG is incorporated as large patches instead of being evenly dispersed (28). Pinho and Errington surmised that insertion becomes undirected because FtsZ no longer anchors the PG synthetic machinery (28). *S. aureus* does not contain a homologue of MreB (18), so FtsZ may be the only tether for periplasmic synthases in this organism. In another example, polymeric strands of Mbl (not MreB) are required for proper localization of PG synthesis in the side walls of *Bacillus subtilis* (7, 12). New PG is inserted in evenly distributed helical ribbons, which presumably track along underlying Mbl protein cables (7). This well-distributed incorporation is eliminated in strains lacking Mbl, so that PG is inserted only in septal regions (7). Continued growth of these mutants depends on the presence of active FtsZ, and Daniel and Errington surmised that FtsZ directs cell elongation from circumscribed growth zones

(7). Finally, depleting MreC or MreD from *B. subtilis* causes cells to grow as spheres, and a further depletion of FtsZ reduces growth to a minimum, with the resulting cells having aberrant morphologies and uneven peptidoglycan incorporation (20). Leaver and Errington concluded that cell division is required to maintain exponential growth (20), but we suggest that an alternative interpretation is worth considering. FtsZ may be required not because cell division per se is required but because FtsZ acts as an alternative tether for directing generalized PG incorporation.

The fact that FtsZ can direct the insertion of precursors over the entire surface of *E. coli* in the absence of MreB suggests that FtsZ need not coalesce into a defined ring before it directs PG insertion. This is not as outlandish as it seems. Not only are FtsZ polymers highly dynamic (33) but they move in helical paths along the interior surface of the inner membrane in *E. coli* (33) and *B. subtilis* (23, 26). It was once assumed that this behavior represented only an intermediate stage of FtsZ as it disassembled from one ring and moved into position to form another (33). However, it appears likely that short FtsZ oligomers are spread around the inner membrane at all times during the cell cycle (27). Our current results imply that these nonring forms of FtsZ may be active and have the potential to direct PG insertion at sites other than at the septum.

Finally, along this line we urge a note of interpretative caution. One way to describe the ability of FtsZ to govern PG insertion into the bacterial side wall is to say that the new wall is composed entirely of "septal peptidoglycan," because the reaction depends on FtsZ (i.e., by reasoning from the classical role of FtsZ during cell division). However, we showed that FtsZ continues to direct an evenly distributed incorporation of peptidoglycan even when MreB and PBP 3 are inhibited simultaneously. Since PBP 3 is not involved with this new form of FtsZ-directed wall growth, the material is not septal. There is a difference in saying that *mreB* mutants produce only septal PG and saying that new PG is incorporated in a FtsZ-dependent manner, the latter statement being more accurate.

Potential competition between FtsZ- and MreB-directed side wall insertion. An important question is whether FtsZ and MreB direct cell wall synthesis by using a common mechanism, in which case the two pathways might compete with one another for proteins or products, or whether they tether the PG synthases by independent means, in which case the two processes might complement one another without competing. The most straightforward interpretation of the present results is that FtsZ and MreB both tether through PBP 2, the implication being that the two processes may compete for PBP 2 or other components. This potentially competitive situation has a parallel in *C. crescentus*, where FtsZ draws PBP 2 away from the side walls and to the septum during cell division or in the absence of MreB or MreC (10, 11).

PIPS. In addition to its classical role in governing cell wall synthesis during division, FtsZ also directs a brief period of PG synthesis just prior to septal invagination in a reaction that does not require PBP 3 (FtsI), called PIPS (9, 38). The requirements and nature of this reaction are unknown. An important question is whether the FtsZ-directed side wall synthesis we have reported here is simply a more generalized version of PIPS that is spread over a greater surface area. The answer appears to be "no." The side wall synthesis described in this work is not a diffuse version of PIPS, because this new FtsZ-dependent reaction proceeds by a slightly different route and produces a different end product. First, PBP 2 is required for evenly distributed PG incorporation into the side walls (this work) but is not required for PIPS (A. Varma, L. Potluri, and K. Young, unpublished results). Second, the two cases produce PGs with different compositions. PG incorporated into lateral walls is rich in pentapeptide side chains, as evidenced by vancomycin labeling, but PG incorporated during PIPS is not stained by vancomycin, indicating the absence of pentapeptides during this process. Thus, pentapeptide-rich PG is inserted when PBP 2 is active (during side wall synthesis) or when PBP 3 is active (during septation), but PIPS requires neither PBP 2 nor PBP 3 and its product contains few or no pentapeptide side chains. Although all three reactions depend on FtsZ, their timing, extent, and end products appear to be determined by other factors.

Physiological roles of PBPs 2 and 3. The exact roles of PBPs 2 and 3 are not clear. They are assumed to be transpeptidases required for cross-linking glycan strands during PG synthesis (17, 24). However, these PBPs may also contribute a structural function in drawing other proteins into complexes that build up around FtsZ or MreB. One indication that this may be true is that cell wall synthesis in *E. coli* proceeds for a short time in the absence of active PBP 2 and PBP 3, after which the cells lyse rapidly and completely. It is possible that inhibiting these two proteins causes lysis because the major glycan chain polymerases become completely untethered, thereby restricting the insertion of new peptidoglycan to abnormally confined areas. Either the newly inserted PG or the older portions of the cell wall may be less stable and prone to breakage.

Cellular morphology. We had imagined that MreB maintained a cell's rod shape by directing cell wall growth such that new material was inserted evenly over the entire surface. In this view, spherical growth would be the consequence of randomized, untethered PG synthesis. Instead, in the absence of MreB *E. coli* becomes spherical even though FtsZ continues to direct PG incorporation that is evenly distributed around the wall. Thus, PG incorporation directed by MreB produces a rod-shaped cell, whereas that directed by FtsZ produces a rounded cell. Two extremes could explain this result. The first and simplest alternative is that MreB forces PG incorporation to follow a specific geometry, which is in turn responsible for creating a rod-shaped sacculus. In this scenario, the geometry of FtsZ-mediated PG insertion may be incompatible with forming a rod. The other alternative is that shape is not determined by differences in the spatial organization for new cell wall synthesis. Instead, PG created by MreB-directed synthesis might have a different structure from PG synthesized via FtsZ, although what this difference might be is unclear.

Summary. Both FtsZ and MreB tether peptidoglycan synthases independently of one another and do so by a mechanism that relies on PBP 2. In addition, in the absence of MreB, FtsZ can direct peptidoglycan incorporation into the lateral walls of *E. coli*, which may indicate a more general role for this protein. FtsZ may drive a general form of peptidoglycan synthesis while taking on its classic role in cell division at a particular time and place. Finally, the genesis of cell shape may depend on a specific geometry of cell wall growth that is directed only by MreB.

ACKNOWLEDGMENTS

This work was supported by grant R01-GM61019 from the National Institutes of Health.

We thank the anonymous reviewers for suggestions that clarified the results and interpretations.

REFERENCES

1. **Aaron, M., G. Charbon, H. Lam, H. Schwarz, W. Vollmer, and C. Jacobs-Wagner.** 2007. The tubulin homologue FtsZ contributes to cell elongation by guiding cell wall precursor synthesis in *Caulobacter crescentus*. Mol. Microbiol. **64:**938–952.
2. **Buddelmeijer, N., and J. Beckwith.** 2002. Assembly of cell division proteins at the *E. coli* cell center. Curr. Opin. Microbiol. **5:**553–557.
3. **Canepari, P., C. Signoretto, M. Boaretti, and M. M. Lleo.** 1997. Cell elongation and septation are two mutually exclusive processes in *Escherichia coli*. Arch. Microbiol. **168:**152–159.
4. **Carballido-Lopez, R.** 2006. The bacterial actin-like cytoskeleton. Microbiol. Mol. Biol. Rev. **70:**888–909.
5. **Carballido-Lopez, R.** 2006. Orchestrating bacterial cell morphogenesis. Mol. Microbiol. **60:**815–819.
6. **Dajkovic, A., and J. Lutkenhaus.** 2006. Z ring as executor of bacterial cell division. J. Mol. Microbiol. Biotechnol. **11:**140–151.
7. **Daniel, R. A., and J. Errington.** 2003. Control of cell morphogenesis in bacteria. Two distinct ways to make a rod-shaped cell. Cell **113:**767–776.
8. **Denome, S. A., P. K. Elf, T. A. Henderson, D. E. Nelson, and K. D. Young.** 1999. *Escherichia coli* mutants lacking all possible combinations of eight penicillin binding proteins: viability, characteristics, and implications for peptidoglycan synthesis. J. Bacteriol. **181:**3981–3993.
9. **de Pedro, M. A., J. C. Quintela, J. V. Höltje, and H. Schwarz.** 1997. Murein segregation in *Escherichia coli*. J. Bacteriol. **179:**2823–2834.
10. **Divakaruni, A. V., C. Baida, C. L. White, and J. W. Gober.** 2007. The cell shape proteins MreB and MreC control cell morphogenesis by positioning cell wall synthetic complexes. Mol. Microbiol. **66:**174–188.
11. **Dye, N. A., Z. Pincus, J. A. Theriot, L. Shapiro, and Z. Gitai.** 2005. Two independent spiral structures control cell shape in *Caulobacter*. Proc. Natl. Acad. Sci. USA **102:**18608–18613.
12. **Formstone, A., and J. Errington.** 2005. A magnesium-dependent *mreB* null mutant: implications for the role of *mreB* in *Bacillus subtilis*. Mol. Microbiol. **55:**1646–1657.
13. **Garcia del Portillo, F., and M. A. de Pedro.** 1990. Differential effect of mutational impairment of penicillin-binding proteins 1A and 1B on *Escherichia coli* strains harboring thermosensitive mutations in the cell division genes *ftsA*, *ftsQ*, *ftsZ*, and *pbpB*. J. Bacteriol. **172:**5863–5870.
14. **Gitai, Z., N. A. Dye, A. Reisenauer, M. Wachi, and L. Shapiro.** 2005. MreB actin-mediated segregation of a specific region of a bacterial chromosome. Cell **120:**329–341.
15. **Goehring, N. W., and J. Beckwith.** 2005. Diverse paths to midcell: assembly of the bacterial cell division machinery. Curr. Biol. **15:**R514–R26.
16. **Higgins, M. L., and G. D. Shockman.** 1971. Procaryotic cell division with respect to wall and membranes. CRC Crit. Rev. Microbiol. **1:**29–72.
17. **Höltje, J.-V.** 1998. Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. Microbiol. Mol. Biol. Rev. **62:**181–203.
18. **Jones, L. J., R. Carballido-Lopez, and J. Errington.** 2001. Control of cell shape in bacteria: helical, actin-like filaments in *Bacillus subtilis*. Cell **104:**913–922.
19. **Kruse, T., J. Bork-Jensen, and K. Gerdes.** 2005. The morphogenetic MreBCD proteins of *Escherichia coli* form an essential membrane-bound complex. Mol. Microbiol. **55:**78–89.
20. **Leaver, M., and J. Errington.** 2005. Roles for MreC and MreD proteins in helical growth of the cylindrical cell wall in *Bacillus subtilis*. Mol. Microbiol. **57:**1196–1209.
21. **Lutkenhaus, J., and S. G. Addinall.** 1997. Bacterial cell division and the Z ring. Annu. Rev. Biochem. **66:**93–116.
22. **Margolin, W.** 2000. Themes and variations in prokaryotic cell division. FEMS Microbiol. Rev. **24:**531–548.
23. **Michie, K. A., L. G. Monahan, P. L. Beech, and E. J. Harry.** 2006. Trapping of a spiral-like intermediate of the bacterial cytokinetic protein FtsZ. J. Bacteriol. **188:**1680–1690.
24. **Nanninga, N.** 1998. Morphogenesis of *Escherichia coli*. Microbiol. Mol. Biol. Rev. **62:**110–129.
25. **Park, J. T.** 1996. The murein sacculus, p. 48–57. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, DC.
26. **Peters, P. C., M. D. Migocki, C. Thoni, and E. J. Harry.** 2007. A new assembly pathway for the cytokinetic Z ring from a dynamic helical structure in vegetatively growing cells of *Bacillus subtilis*. Mol. Microbiol. **64:**487–499.
27. **Pichoff, S., and J. Lutkenhaus.** 2005. Tethering the Z ring to the membrane through a conserved membrane targeting sequence in FtsA. Mol. Microbiol. **55:**1722–1734.
28. **Pinho, M. G., and J. Errington.** 2003. Dispersed mode of *Staphylococcus aureus* cell wall synthesis in the absence of the division machinery. Mol. Microbiol. **50:**871–881.
29. **Popham, D. L., and K. D. Young.** 2003. Role of penicillin-binding proteins in bacterial cell morphogenesis. Curr. Opin. Microbiol. **6:**594–599.
30. **Satta, G., R. Fontana, and P. Canepari.** 1994. The two-competing site (TCS) model for cell shape regulation in bacteria: the envelope as an integration point for the regulatory circuits of essential physiological events. Adv. Microb. Physiol. **36:**181–245.
31. **Shih, Y. L., and L. Rothfield.** 2006. The bacterial cytoskeleton. Microbiol. Mol. Biol. Rev. **70:**729–754.
32. **Spratt, B. G.** 1978. *Escherichia coli* resistance to beta-lactam antibiotics through a decrease in the affinity of a target for lethality. Nature **274:**713–715.
33. **Thanedar, S., and W. Margolin.** 2004. FtsZ exhibits rapid movement and oscillation waves in helix-like patterns in *Escherichia coli*. Curr. Biol. **14:**1167–1173.
34. **Uehara, T., and J. T. Park.** 2008. Growth of *Escherichia coli*: significance of peptidoglycan degradation during elongation and septation. J. Bacteriol. **190:**3914–3922.
35. **Varma, A., M. A. de Pedro, and K. D. Young.** 2007. FtsZ directs a second mode of peptidoglycan synthesis in *Escherichia coli*. J. Bacteriol. **189:**5692–5704.
36. **Varma, A., and K. D. Young.** 2004. FtsZ collaborates with penicillin binding proteins to generate bacterial cell shape in *Escherichia coli*. J. Bacteriol. **186:**6768–6774.
37. **Wachi, M., M. Doi, Y. Okada, and M. Matsuhashi.** 1989. New *mre* genes *mreC* and *mreD*, responsible for formation of the rod shape of *Escherichia coli* cells. J. Bacteriol. **171:**6511–6516.
38. **Wientjes, F. B., and N. Nanninga.** 1989. Rate and topography of peptidoglycan synthesis during cell division in *Escherichia coli*: concept of a leading edge. J. Bacteriol. **171:**3412–3419.