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NOTES

The Monofunctional Glycosyltransferase of *Escherichia coli* Localizes to the Cell Division Site and Interacts with Penicillin-Binding Protein 3, FtsW, and FtsN▽‡

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The monofunctional peptidoglycan glycosyltransferase (MtgA) catalyzes glycan chain elongation of the bacterial cell wall. Here we show that MtgA localizes at the division site of *Escherichia coli* cells that are deficient in PBP1b and produce a thermosensitive PBP1a and is able to interact with three constituents of the divisome, PBP3, FtsW, and FtsN, suggesting that MtgA may play a role in peptidoglycan assembly during the cell cycle in collaboration with other proteins.

Bacterial cell morphogenesis and cytokinesis are closely related to peptidoglycan metabolism (25). The two final periplasmic steps of peptidoglycan assembly are essential for cell integrity and shape (13, 34). They are catalyzed by membrane-bound penicillin-binding proteins (PBPs) that belong to either class A or class B (13). The C-terminal penicillin-binding module of both classes has transpeptidase activity. In class A, the N-terminal module has glycosyltransferase (GT) activity, whereas in class B, the N-terminal module is devoid of any known catalytic activity. *Escherichia coli* possesses three class A PBPs (PBP1a, -1b, and -1c) and two class B PBPs (PBP2 and -3) involved in cell morphogenesis. The presence of at least one class A PBP (PBP1a or PBP1b) is required for cell viability (29), whereas PBP1c is dispensable (27). A monofunctional GT (MtgA) capable of catalyzing un-cross-linked glycan chain formation in vitro is also present in *E. coli* membranes and is dispensable (9, 27, 28). The GT domain of class A PBPs and the monofunctional GTs show high sequence similarity and belong to the GT51 family (http://www.cazy.org/). Their structure has some resemblance to the lysozyme fold (19, 37). The role of class A PBPs and MtgA in morphogenesis is not well known. During the cell cycle, peptidoglycan synthesis is performed by two dynamic protein complexes: the elongase during cell elongation and the divisome during septation (8). These machineries share portions of the peptidoglycan-synthesizing proteins. PBP1b, the main peptidoglycan synthetase, localizes to the lateral cell wall and division site, indicating that it is involved in both lateral and septum wall peptidoglycan synthesis (4). In *E. coli*, the division machinery includes at least 15 proteins: FtsZ, FtsA, ZipA, ZapA, FtsE, FtsX, FtsK, FtsQ, FtsL, FtsB, FtsW, PBP3, PBP1b, FtsN, and AmiC (8, 11). Division is initiated by the polymerization of the FtsZ ring at midcell underneath the plasma membrane, which subsequently functions to recruit the downstream components in hierarchical and interdependent orders (11, 35), either as single molecules or as preformed subgroups (e.g., FtsQ-FtsB-FtsL and FtsW-PBP3) (6, 12). PBP1b interacts directly with PBP3 (4), which is specifically involved in septal peptidoglycan synthesis (23), and FtsW (our unpublished data). The septal localization of PBP1b depends on the presence of PBP3. These results indicate that these proteins may act together within the divisome to form the peptidoglycan of the new poles during cell division. FtsN interacts with PBP3 and PBP1b and stimulates the in vitro peptidoglycan synthesis activities of PBP1b (21). Thus, FtsN might have a role in coordinating the peptidoglycan synthases during cell division (21). In order to understand the role of MtgA in peptidoglycan metabolism during the cell cycle, we have analyzed the localization of the protein fused to the green fluorescent protein (GFP) and its interaction with the divisome proteins using a bacterial two-hybrid system.

MtgA localizes at the division site of a *ponA*(Ts) *ponB E. coli* strain. *E. coli* strains were transformed with the plasmid pDML2005 (derived from pDSW207) (36), which allowed the expression of MtgA fused to the C-terminal end of GFP (see the supplemental material). Transformants were grown at 28°C in Luria-Bertani medium, harvested at exponential growth phase (A_{450} of 0.2 to 0.4), fixed on glass slabs coated with 1% agarose, and examined by fluorescent microscopy (18, 24) (Fig. 1). The localization of GFP-MtgA was first examined in *E. coli* LMC500 cells (31) and *E. coli* JW3359*ponA*, JW0145*ponB*,

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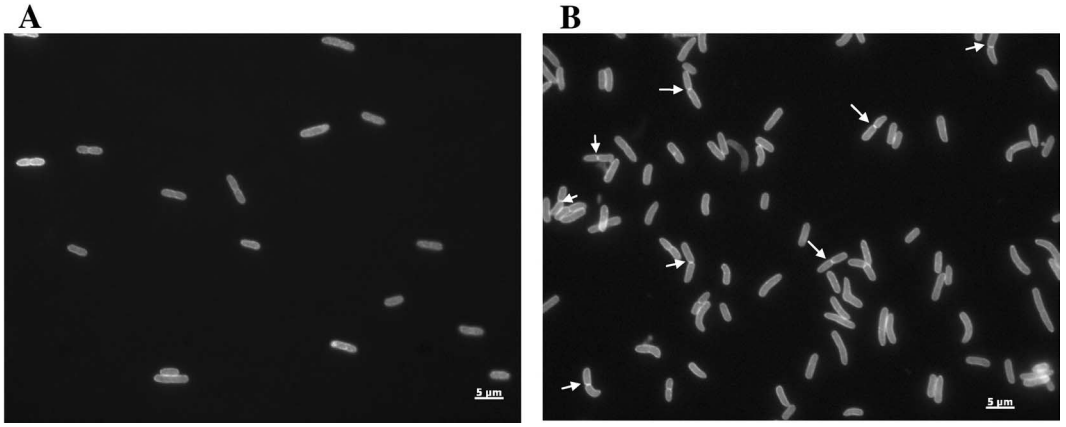

FIG. 1. Localization of GFP-MtgA in wild-type *ponA*$^+$ *ponB*$^+$ strain LMC500 (A) and *ponA*(ts) *ponB* strain EJ801 (B) grown in LB medium at 28°C, both harboring pDML2005. The arrows point to cells with fluorescent signals at the division site. Photographs were taken with a cooled AxioCam MRm (Zeiss) mounted on an Zeiss Axio Imager.Z1 fluorescent microscope through a EC Plan-Neofluar 100 × 1.3 oil immersion objective in bright field and fluorescence using filter set 37 (400- to 500-nm band pass excitation and 460- to 560-nm band pass emission; Zeiss). Images were analyzed using the AxioVision Rel. 4.5 (Zeiss) software as previously described (24). Bar, 5 μm.

JW2503*pbpC*, or JW3175*mtgA* single null mutant strains, which are derived from strain BW25113 (2). In the presence of all PBPs (Fig. 1A) or in cells deleted of one class A PBPs or MtgA (data not shown), the GFP-MtgA fusion was localized around the cell periphery, and no specific localization of fluorescence was observed. The localization of GFP-MtgA was then examined in *E. coli* EJ801, which is deficient in PBP1b and produces a thermosensitive PBP1a, which binds penicillin at 28°C but not at 42°C (14, 30). Consequently, this strain grows at 28°C in LB medium with a mass doubling time of 120 min and lyses at 42°C. At the permissive temperature, the GFP-MtgA fluorescence signal was observed at the division site in 23% ± 2% of the cells using three series of 200 cells each from three cultures (Fig. 1B). For comparison, it has been reported that in *E. coli* LMC500, which has a mass doubling time of 85 or 140 min, the proportions of cells that contains a PBP3 signal at midcell are 33 and 28%, respectively (1). PBP1b localizes to the lateral cell wall and division site (4). As PBP1a is able to replace PBP1b, it is expected to localize at the same sites. Thus, one explanation for the different localization patterns of MtgA is that the thermosensitive PBP1a, which is active and produced at the same level as the native one at 28°C (30), may have a reduced capacity to interact with cell division proteins at the division site and was replaced by MtgA in the absence of PBP1b, indicating a hierarchical competition between these proteins for the same site.

As the *ponA*(ts) *ponB* strain EJ801 also carries *dacA* and *dacB* mutations, which are related to peptidoglycan metabolism and which are not present in BW25113-derived strains, this difference in the genetic background could possibly explain the observed differences of MtgA localization between the strains. To eliminate this possibility, the MtgA localization was examined in EJ801 *ponA*(ts) *ponB* transformed with plasmid pDML924, expressing PBP1b (32). In this case, no localization of MtgA at midcells was observed (data not shown), corroborating the fact that MtgA localization is allowed because competition with class A PBP1a for the division site is reduced in strain EJ801. Thus, MtgA might compensate for the absence of PBP1b and the expression of a mutated form of PBP1a.

To verify the GT activity of the GFP-MtgA, the fusion protein was expressed at 28°C in the *E. coli ponA*(ts) *ponB* double mutant EJ801 harboring pDML2005 in the presence of 0.5 mM IPTG for 2 h. As a control, the same strain was transformed with the empty plasmid (pDSW207). Membrane fractions were prepared from the two transformants and suspended in 25 mM Tris-HCl (pH 7.5), and the expression of the GFP-MtgA fusion was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The membrane preparations (equivalent of 100 μl of cell culture with an A_{600} of 1.0) were incubated in 50 μl GT reaction mixture containing 12 μM [^{14}C]GlcNAc-labeled lipid II (9,180 dpm/nmol) (3), 15% dimethyl sulfoxide, 10% octanol, 50 mM HEPES (pH 7.0), 0.5% decyl-polyethylene glycol, and 10 mM $CaCl_2$. The products were separated and analyzed as previously described (33). A 2.4-fold increase in peptidoglycan polymerization occurred when the GFP-MtgA was overexpressed compared to the control (26% versus 11% of lipid II used), showing that the fusion protein has GT activity in vitro. Addition of lysozyme to the reaction products resulted in complete digestion of the polymerized material.

MtgA interacts with PBP3, FtsW, and FtsN in a bacterial two-hybrid system. The localization of the GFP-MtgA to the division site suggested that MtgA might interact with the divisome proteins. To test this hypothesis, we used the bacterial Cya two-hybrid system (16) and analyzed the interaction of MtgA with PBP3, FtsW, and FtsN. The CyaT18 and CyaT25 fragments of the adenylate cyclase (Cya) were fused to the N-terminal ends of MtgA, PBP3, FtsW, FtsN, and Lpp-PBP3 through a $(G_4S)_3$ linker. Lpp-PBP3, which was obtained by substitution of the uncleavable Lpp signal peptide for the M1-G40 segment of PBP3, was able to bind penicillin but was not functional in vivo, could not localize at the division site, and did not interact with FtsW (20). It was used as a negative control. Plasmids (see the supplemental material) allowing the coexpression of T18-$(G_4S)_3$-MtgA or T25-$(G_4S)_3$-MtgA with T25-$(G_4S)_3$-X or T18-$(G_4S)_3$-X, respectively (where X is PBP3, FtsW, FtsN, MtgA, or Lpp-PBP3), were introduced into the *cya*-deficient *E. coli* strain

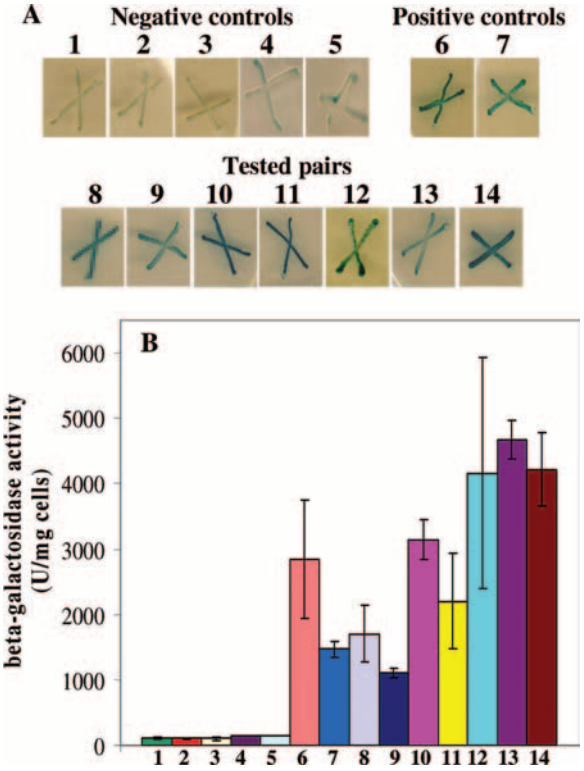

FIG. 2. Interaction between MtgA and PBP3, FtsW, FtsN, or itself by the Cya bacterial two-hybrid assay. DHM1 transformants producing T18–T25 (1), T18-MtgA–T25 (2), T18-T25-MtgA (3), T18-MtgA–T25-Lpp-PBP3 (4), T18-Lpp-PBP3–T25-MtgA (5), T18-PBP1B–T25-PBP3 (6), T18-PBP3–T25-PBP1B (7), T18-MtgA–T25-PBP3 (8), T18-PBP3–T25-MtgA (9), T18-MtgA–T25-FtsN (10), T18-FstN–T25-MtgA (11), T18-MtgA–T25-FstW (12), T18-FtsW–T25-MtgA (13), and T18-MtgA–T25-MtgA (14) were used. (A) Transformants were grown at 30°C for 30 h on LB agar plates containing 0.5 mM IPTG, 40 μg/ml X-Gal, 50 μg/ml ampicillin, and 20 μg/ml chloramphenicol. (B) Quantitative analysis of the β-galactosidase activity from transformants grown in LB medium in the presence of 0.4 mM IPTG for 16 h at 30°C was carried out as described previously (17). Data are averages of three independent experiments.

DHM1 (17). Transformants producing the fusion protein pair T18-$(G_4S)_3$-MtgA and T25-$(G_4S)_3$-PBP3, T25-$(G_4S)_3$-FtsW, T25-$(G_4S)_3$-FtsN, or T25-$(G_4S)_3$-MtgA formed blue colonies on LB–X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)–IPTG (isopropyl-β-D-thiogalactopyranoside) plates and red colonies on MacConkey-maltose plates, indicating that functional adenylate cyclase was produced. In contrast, the negative control colonies expressing the couples T18-T25, T18–T25-X, and T25–T18-X (where X is any of the studied proteins: MtgA, PBP3, Lpp-PBP3, FtsW, or FtsN) or the Cya fusion MtgA–Lpp-PBP3 remained white (Fig. 2A). The level of β-galactosidase activity due to the complementation by the Cya fusion pairs MtgA-PBP3, MtgA-FtsN, and MtgA-FtsW in permeabilized cells was at least 10-, 20-, and 37-fold higher, respectively, than that of the controls (~100 U/mg), and that of the Cya fusion MtgA-MtgA was 37-fold higher (Fig. 2B). Similarly, coexpression of the opposite constructs T25-$(G_4S)_3$-MtgA and PBP3, FtsW, FtsN, or MtgA fused to the T18 fragment allowed the complementation and reconstitution of the *cya*$^+$ phenotype of DHM1 strain except for CyaT18-Lpp-PBP3 (Fig. 2B). For the sake of comparison, the value for PBP1b-PBP3 was 13-fold higher than that in the negative control and was in agreement with the previously reported results (4). Note that the strain used for these experiments has wild-type PBPs and the studied proteins are overproduced, and therefore the observed interactions may also occur outside the septal region. These results show that MtgA interacts specifically in vivo with PBP3, FtsW, and FtsN and that the transmembrane segment of PBP3 is required for the interaction. Interestingly, coexpression of T18-$(G_4S)_3$-MtgA and T25-$(G_4S)_3$-MtgA leads to positive response, which indicates that MtgA interacts with itself in vivo (Fig. 2). Therefore, these data suggest that MtgA may collaborate with PBP3 to synthesize at least a part of the peptidoglycan of the new poles during cell division.

Septal peptidoglycan synthesis can be divided into two steps: an early step, which requires the Z-ring assembly, and a later step, which requires the mature divisome (1). It has been shown that the initiation of division, which is independent of PBP3, requires penicillin-insensitive peptidoglycan synthesis before constriction (22). Both PBP1c and MtgA are insensitive to penicillin (27) and may be responsible for this activity, which is taken over by penicillin-sensitive proteins later on. However, single PBP1c or MtgA mutants and a double mutant do not show any obvious phenotype change (27) but have a 5- to 10-fold increase in tetra-pentamuropeptide. PBP1c interacts with PBP1b, PBP2, PBP3, and MltA (27). All these observations tend to indicate the involvement of MtgA and PBP1c in the formation or discrete modification of the septal and the lateral peptidoglycan wall of *E. coli*. In *Pasteurella multocida* and *Brucella abortus*, PBP1c and Mgt may play a role in mediating bacterium-host interaction, as *P. multocida* depleted of class A PBP1c (15) (homologue of *E. coli* PBP1c) and *B. abortus* depleted of Mtg (7) grow similarly to the wild type in broth medium but show significant attenuation of pathogenicity in mice.

Concluding remarks. Although PBP1a and PBP1b have common features, several of their in vitro and in vivo properties are different (3, 5, 10, 26, 38). Therefore, it seems that the peptidoglycan GTs, although catalyzing the same type of reaction, have different specificities and may fulfill various and hierarchical functions; some of them are essential for the formation and division of the bacterial sacculus, and others may play a yet-unknown secondary role in the synthesis or remodeling of peptidoglycan, which in addition is modulated by other proteins.

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