Wag31, a homologue of the cell division protein DivIVA, regulates growth, morphology and polar cell wall synthesis in mycobacteria

Choong-Min Kang,¹ Seeta Nyayapathy,¹ Jung-Yeon Lee,² Joo-Won Suh² and Robert N. Husson³

Correspondence
Choong-Min Kang
cmkang@wayne.edu

¹Department of Biological Science, Wayne State University, 5047 Gullen Mall, Detroit, MI 48202, USA

²Department of Biological Science, Myongji University, San 38-2, Namdong, Yongin, Republic of Korea

³Division of Infectious Diseases, Children's Hospital Boston, Harvard Medical School, 300 Longwood Ave., Boston, MA 02115, USA

The *Mycobacterium tuberculosis* genome contains 11 serine/threonine kinase genes, and the products of two of these, PknA and PknB, are key components of a signal transduction pathway that regulates cell division and/or morphology. Previously, we have shown that one substrate of these kinases is Wag31, a homologue of the cell division protein DivIVA that is present, but not known to be phosphorylated, in other Gram-positive bacteria. Here, we investigate the localization and function of Wag31 and its phosphorylation. We demonstrate that Wag31 is localized to the cell poles. We further show that *wag31* is an essential gene and that depletion of its product causes a dramatic morphological change in which one end of the cell becomes round rather than rod-shaped. This abnormal morphology appears to be caused by a defect in polar peptidoglycan synthesis. Finally, expression of *M. tuberculosis wag31* in the *wag31* conditional mutant of *Mycobacterium smegmatis* altered the growth rate in a manner that depended on the phospho-acceptor residue encoded by the allele being expressed. Taken together, these results indicate that Wag31 regulates cell shape and cell wall synthesis in *M. tuberculosis* through a molecular mechanism by which the activity of Wag31 can be modulated in response to environmental signals.

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INTRODUCTION

Tuberculosis is a major and increasing health problem worldwide, not only because of current cases of active disease, which have a high mortality, but also because of the threat of latency (World Health Organization, 2007). The problem of latency, resulting in an enormous reservoir of infected individuals worldwide with the potential to develop active tuberculosis, is a major obstacle to tuberculosis control. It is not clear, however, how *M. tuberculosis* achieves or maintains a latent state *in vivo*. A key aspect of latency may be the ability of *M. tuberculosis* to regulate cell division in response to specific host signals in

making the transition between active replication and non-replicating persistence (Flynn & Ernst, 2000; Wayne & Hayes, 1996).

To alternate between the active and latent states, *M. tuberculosis* must be able to sense and respond to signals in the host environment. In both eukaryotic and prokaryotic cells, reversible protein phosphorylation is the most common mechanism by which environmental signals are transmitted to regulate gene expression. In bacteria, two-component systems transmit signals by phospho-transfer from a histidine residue on a sensor protein to an aspartyl residue on a response regulator protein (Magasanik, 1995). Many prokaryotes also possess eukaryotic-like protein kinases that function via reversible phosphorylation of serine, threonine or tyrosine residues; of these, the most widely distributed are serine/threonine protein kinases (STPKs) (Leonard *et al.*, 1998). *M. tuberculosis* has genes encoding 11 STPKs (Av-Gay & Everett, 2000). All but two of these kinases have predicted transmembrane domains with the kinase domain located intracellularly, suggesting

Abbreviations: DIC, differential interference contrast; Van-Alexa568, vancomycin–Alexa568 conjugate; Van-FL, vancomycin–fluorescein conjugate.

Two supplementary figures, showing the position of *wag31* in the *dcw* cluster in *M. tuberculosis* and *M. smegmatis* and the strategies for disruption of *M. smegmatis wag31* and confirmation of the *wag31* conditional mutant by PCR, and a supplementary table listing the primers used in this research, are available with the online version of this paper.

that these STPKs play an important role in signal transduction in *M. tuberculosis*.

The kinase genes *pknA* and *pknB* are located near the origin of replication in an operon with genes predicted to function in cell wall synthesis and morphology. This operon contains a phosphatase gene (*pstP*) and two genes that are highly homologous to *rodA* and *pbpA*, which in both Gram-positive and Gram-negative bacteria have been shown to play a major role in cell wall synthesis and cell morphology (Henriques *et al.*, 1998; Rodriguez & de Pedro, 1990; Thibessard *et al.*, 2002). In our previous study, we have shown that *pknA* and *pknB* are primarily expressed during exponential growth, and that overexpression of these kinases impairs growth and alters cell morphology, suggesting that these kinases play an important role in a signal transduction pathway that regulates cell division/morphology in *M. tuberculosis*. Using peptide library screening and proteomics, we also identified three *in vivo* substrates of PknA and PknB. One substrate is PknB itself, the product of autophosphorylation. The others are Rv1422, a conserved hypothetical protein of unknown function, and Wag31, a homologue of DivIVA, a protein present during the early stages of cell division in Gram-positive bacteria. While Wag31 is highly phosphorylated by overexpression of either *pknA* or *pknB* in *M. tuberculosis*, only PknA can phosphorylate Wag31 *in vitro*, suggesting that PknA phosphorylates Wag31 directly and that PknB does so indirectly through PknA (Kang *et al.*, 2005).

In this work we focus on the function of Wag31. This protein was originally identified as an antigen of pathogenic mycobacteria that was recognized by serum from persons with leprosy and tuberculosis (Hermans *et al.*, 1995). Sequence analysis showed that Wag31 is a homologue of the Gram-positive cell division protein DivIVA (Cole *et al.*, 1998; Flardh, 2003). Although DivIVA is believed to function in cell division in all Gram-positive bacteria, the specific roles that it plays appear to vary in a species-specific manner. Originally identified as a mini-cell locus in *Bacillus subtilis*, DivIVA is known to have dual functions in the regulation of cell division in this organism. In vegetatively growing cells, it is required for appropriate septum placement through interacting with and confining the MinCD cell division inhibitory complex at the cell poles (Cha & Stewart, 1997; Marston & Errington, 1999). In sporulating cells, it facilitates chromosome segregation by interacting with the *oriC* complex (Thomaides *et al.*, 2001).

DivIVA in *B. subtilis* is not essential, but its inactivation produces mini-cells, and its overexpression produces filamentous cells (Cha & Stewart, 1997). In contrast, in *Streptomyces coelicolor*, DivIVA_{SC} is essential and is localized to the growing tips and lateral branches of hyphae where nascent peptidoglycan synthesis occurs (Flardh, 2003). Partial depletion of *divIVA*_{SC} in this species causes defects in hyphal growth, with normal septum formation. Overexpression produces hyperbranching, with

broad cells and abnormal tip growth, suggesting that DivIVA_{SC} is important for morphogenesis rather than for correct septum formation or nucleoid localization. DivIVA homologues in other Gram-positive bacteria, including *Brevibacterium lactofermentum* and *Enterococcus faecalis*, are essential, and overexpression of this gene in these species results in enlarged cells (Ramirez-Arcos *et al.*, 2005; Ramos *et al.*, 2003).

These studies on DivIVA homologues suggest that Wag31 may be involved in septum formation, cell wall synthesis and/or chromosome segregation in mycobacteria. Our previous work using phosphomimetic and non-phosphorylatable forms of Wag31 suggested that the activity of Wag31 is regulated by phosphorylation. Overexpression of wild-type *wag31*_{Mtb} or a *wag31* allele (*wag31T73E*_{Mtb}) encoding a phosphomimetic protein produced cells that were more rounded than control cells containing the vector alone or expressing a *wag31* allele (*wag31T73A*_{Mtb}) encoding a protein in which the phosphoacceptor residue was changed to alanine.

Here we investigate the localization and function of Wag31 in mycobacteria, and the role of phosphorylation in modulation of its function. We demonstrate that Wag31 is highly localized to the cell poles. We further show that *wag31* is essential in mycobacteria and that depletion of Wag31 causes a striking morphological change in which one end of the cell becomes round rather than rod-shaped. We demonstrate that this abnormal morphology appears to be caused by a defect in polar peptidoglycan synthesis. Finally, expression of *wag31*_{Mtb} in the *wag31* conditional mutant of *M. smegmatis* altered the growth rate in a manner dependent on the phospho-acceptor residue encoded by the allele being expressed. These results indicate that Wag31 regulates growth, cell shape and cell wall synthesis in mycobacteria. The involvement of phosphorylation has not been shown in DivIVA homologues in other prokaryotes. This novel finding suggests a molecular mechanism by which the function of Wag31 in regulating these processes in *M. tuberculosis* can be modulated in response to environmental signals.

METHODS

Bacterial strains, media, and growth conditions. *M. smegmatis* and *Mycobacterium bovis* BCG cultures were grown at 37 °C in Middlebrook 7H9 liquid medium (Difco) supplemented with 10 % ADC [ADC=5 % (w/v) BSA fraction V, 2 % (w/v) glucose and 0.85 % (w/v) NaCl], and 0.05 % (w/v) Tween-80 (7H9-ADC-Tw), or on Middlebrook 7H9-ADC agar plates. Kanamycin (50 µg ml⁻¹), hygromycin (50 µg ml⁻¹) or apramycin (50 µg ml⁻¹) was added to culture media as indicated. For growth analysis of the *wag31* conditional mutant of *M. smegmatis*, cells were incubated on 7H9-ADC agar plates containing 0.1 % (w/v) acetamide, then reinoculated onto fresh 7H9-ADC agar plates with or without 0.1 % acetamide. For growth analysis in liquid medium, the *wag31* conditional mutant cells grown on 7H9-ADC agar plates containing 0.1 % acetamide or 5 ng tetracycline ml⁻¹ were inoculated into fresh 7H9-ADC liquid medium without inducer and cultured overnight to deplete the

inducer and functional Wag31 protein inside the cell. Then, cells were reinoculated into 7H9–ADC liquid medium with or without inducer (0.1 % acetamide or 2 ng tetracycline ml⁻¹), followed by OD₆₀₀ measurement. *Escherichia coli* DH5 α (Life Technologies) was used as a host strain for cloning experiments, and was grown in LB broth or solid medium with kanamycin (25 μ g ml⁻¹) or apramycin (50 μ g ml⁻¹) added where appropriate.

Plasmid construction. For localization of Wag31 in wild-type *M. smegmatis*, the *gfp-wag31*_{Msmeg} construct was subcloned behind the acetamide-inducible promoter in an integrating plasmid pMV306 (Km^R), which contains the attachment site (*attP*) and integrase (*int*) of mycobacteriophage L5 and integrates into the chromosomal *attB* attachment site (Hess *et al.*, 1998; Lee *et al.*, 1991; Parish *et al.*, 1997). Two-step PCR was conducted to make *gfp-wag31*_{Msmeg}. First, the *gfp* gene was amplified by PCR using pTracerCMV (Invitrogen) as the template and the Ngfp-wag-1 and Ngfp-wag-2 primers. All primer sequences are shown in Supplementary Table S1. All PCR products were sequenced to verify the absence of mutations. The *wag31* gene from *M. smegmatis* was also amplified using chromosomal DNA from wild-type *M. smegmatis* and the Ngfp-wag-3 and Ngfp-wag-4 primers. Using the above two PCR products and Ngfp-wag-1 and Ngfp-wag-4 primers, the second overlap PCR was conducted to produce *gfp-wag31*_{Msmeg}. The PCR product was then subcloned into pMV306, which contained the acetamidase promoter. As a control, the *gfp* gene alone was cloned into pMV306 using Ngfp-wag-1 and Ngfp-wag-2 primers. In addition, to examine the localization of Wag31_{Mtb}, *gfp-wag31*_{Mtb} was similarly cloned into pMV306 using Ngfp-wag-1, Ngfp-TBwag-2, Ngfp-TBwag-3 and Ngfp-TBwag-4 primers. pMV306-P_{acet}-*gfp-wag31*_{Msmeg} was transformed into wild-type *M. smegmatis*, and pMV306-P_{acet}-*gfp-wag31*_{Mtb} was transformed into *M. bovis* BCG. To examine the localization of GFP-Wag31_{Msmeg} in the *wag31* conditional mutant, the P_{acet}-*gfp-wag31*_{Msmeg} construct from pMV306-P_{acet}-*gfp-wag31*_{Msmeg} was subcloned into the replicating plasmid pMV261 (Km^R). This construct was then introduced into the *wag31* conditional mutant containing wild-type *wag31*_{Msmeg} under the tetracycline-inducible promoter (see below).

The deletion construct for *wag31* in *M. smegmatis* was made in the integrating plasmid pRH1351 that contains a *sacB* counterselection marker, a gentamicin-resistance gene, and a temperature-sensitive mycobacterial replication origin (Pelicic *et al.*, 1997; Raman *et al.*, 2001). The upstream 971 bp region of the *wag31* gene, including the first 28 codons of Wag31, was amplified by PCR from *M. smegmatis* chromosomal DNA using Ms-wag31-cond-1 and Ms-wag31-cond-2 primers. The downstream 842 bp of *wag31*, which includes the final 43 codons, was amplified using Ms-wag31-cond-3 and Ms-wag31-cond-4 primers. The second PCR was conducted using the two PCR products and Ms-wag31-cond-1 and Ms-wag31-cond-4 primers. This construct was subcloned into pRH1351. Finally, a hygromycin-resistance gene was inserted between two *wag31* flanking regions.

Construction of the *wag31* conditional mutant strains. First, the plasmid that contains the wild-type *wag31* gene under the control of the acetamide-inducible promoter (pMV306-P_{acet}-*wag31*_{Msmeg}) was integrated into the L5 *attB* locus of wild-type *M. smegmatis* (Kang *et al.*, 2005). The *wag31* deletion construct described above was transformed into this strain. To facilitate homologous recombination, transformant cells were cultured in 7H9 liquid medium containing hygromycin at 30 °C for 3 days, then plated onto 7H9 agar plates with hygromycin and 10 % sucrose and incubated at 39 °C. Thirty colonies were picked and genomic DNA was purified from these. Four out of 30 candidates produced the correct size of PCR product (2.15 kb) for the *wag31* insertion/deletion; wild-type control DNA produced a 1.55 kb PCR product when Ms-wag31-cond-1 and Ms-wag31-2 primers were used.

To express *gfp-wag31* or wild-type *wag31* in a *wag31* conditional mutant, we constructed a new conditional mutant strain that contained the *wag31*_{Msmeg} gene under the control of a tetracycline-inducible promoter. To do this, we subcloned the *wag31*_{Msmeg} gene behind P_{tet} of the pMind plasmid (Blokpoel *et al.*, 2005). The P_{tet}-*wag31*_{Msmeg} region was then subcloned into an integration vector pMH94A (Apra^R). The resulting construct was then transformed into the previous *wag31* conditional mutant to replace the P_{acet}-*wag31*_{Msmeg} with P_{tet}-*wag31*_{Msmeg} at the L5 *attB* locus (Pashley & Parish, 2003). Approximately half of the Tet^R, Apra^R transformants could not grow on 7H9 agar medium containing kanamycin and 0.1 % acetamide, confirming the replacement of pMV306-P_{acet}-*wag31*_{Msmeg} (Km^R) with pMH94-P_{tet}-*wag31*_{Msmeg} (Apra^R). We then electroporated pMV261-P_{acet}-*gfp-wag31*_{Msmeg} (Km^R) into this conditional mutant of *wag31*. Construction of the additional conditional mutants of *wag31* in which P_{acet}-*wag31*_{Msmeg} was replaced with P_{tet}-*wag31*_{Mtb}, P_{tet}-*wag31*_{T73A}_{Mtb} or P_{tet}-*wag31*_{T73E}_{Mtb} was performed similarly.

Microscopic analysis of Wag31 localization, cell morphology, nascent peptidoglycan synthesis and chromosome segregation. For cellular localization of Wag31, pMV306-P_{acet}-*gfp-wag31*_{Msmeg} or pMV306-P_{acet}-*gfp* was electroporated into wild-type *M. smegmatis*. The resulting strains were grown in 7H9 liquid medium containing 0.1 % acetamide for 7 h, transferred onto glass slides and observed using an Olympus BX51 fluorescence microscope equipped with a $\times 100$ differential interference contrast (DIC) oil UPLAPO objective and fluorescence filter set. Photographs were taken with an Olympus DP30BW high-sensitivity cooled charge-coupled device (CCD) camera, acquired with DP-BSW software and processed with Adobe Photoshop CS2. For the localization of GFP-Wag31_{Mtb} in *M. bovis* BCG, transformant cells were induced with 0.1 % acetamide for 16 h before examination by microscopy.

For observation of morphological changes and nascent peptidoglycan synthesis in the *wag31* conditional mutant during gradual depletion of Wag31, cells grown on 7H9 agar medium with 0.1 % acetamide were inoculated into fresh 7H9 liquid medium without inducer. At 5, 22 and 46 h of incubation with shaking, 1 ml of cells was treated with 5 μ g Van-Alexa568, and incubated for a further 2 h. A 5 mg ml⁻¹ stock of Van-Alexa568 (vancomycin–Alexa568 conjugate) was synthesized according to the manufacturer's manual (Alexa Fluor 568 protein labelling kit, Molecular Probes). Cells were then washed with 7H9 liquid medium and examined by microscopy. To study nascent peptidoglycan synthesis in wild-type *M. smegmatis*, exponentially growing cells were incubated with 1 μ g Van-Alexa568 ml⁻¹ for 7 h and examined by fluorescence microscopy.

For co-localization of GFP-Wag31 and nascent peptidoglycan synthesis, wild-type *M. smegmatis* cells containing pMV306-P_{acet}-*gfp-wag31*_{Msmeg} were cultured in 7H9 liquid medium with kanamycin and 0.1 % (w/v) acetamide. After 6 h incubation with shaking (OD₆₀₀=0.5), cells were treated with 1 μ g Van-Alexa568 ml⁻¹, and further incubated for 3.5 h. Cells were then washed with 7H9 liquid medium and examined by microscopy.

To test the effect of Wag31 on chromosome segregation, *wag31* conditional mutant cells were stained with cell-permeant SYTO dye (SYTO Blue Fluorescent Nucleic Acid Stains kit, Molecular Probes). A series of SYTO dyes was tested to determine the optimal dye for DNA staining in *M. smegmatis*. SYTO 45 was chosen since it produced a strong signal with minimal bleaching. To evaluate the effect of Wag31 on chromosome segregation, *wag31* conditional mutant cells were cultured with or without 0.1 % acetamide. After 7 h incubation with shaking, each culture (1 ml) was treated with 5 μ M SYTO 45 for 30 min, washed with fresh 7H9 liquid medium, and examined by microscopy.

Immunoblot analysis. To examine Wag31 in the *wag31* conditional mutant, cells cultured with or without inducer (0.1 % acetamide) were harvested after 48 h of shaken incubation. After washing with 40 mM Tris buffer (pH 7.5), total protein was extracted using TRIzol (Invitrogen), Lysing Matrix B tubes (QBiogene) and the MagNA Lyser device (Roche). A 5 µg sample of total protein from each culture was used for immunoblot analysis using the anti-Wag31 mAb (Hermans *et al.*, 1995) and the Phototope-HRP Western Blot Detection System (Cell Signaling Technology).

To examine Wag31 content in strains expressing three different *wag31* alleles (*wag31_{Mtb}*, *wag31T73A_{Mtb}* and *wag31T73E_{Mtb}*) in the *wag31* conditional mutant, each strain was cultured in 7H9 liquid medium without tetracycline overnight and then inoculated into a fresh 7H9 liquid medium containing 2 ng tetracycline ml⁻¹. Total protein was purified from 26 and 53 h cultures and 5 µg total protein was used for Western blot analysis with anti-Wag31 mAb. Protein from exponentially growing *M. smegmatis* wild-type cells was used as a control.

The levels of the Wag31 protein and its phosphorylation at different growth stages of *M. bovis* BCG were determined by Western blot analysis with the anti-Wag31 mAb and a phospho-Q (Durocher *et al.*, 2000) polyclonal antibody (Cell Signaling Technology). Wild-type *M. bovis* BCG cells were cultured in 7H9 liquid medium, and total protein from mid-exponential, late-exponential, early-stationary and late-stationary phase cells was purified as described above. Western blot analysis with the anti-Wag31 mAb was conducted by running 5 µg protein in a SDS-PAGE gel (10 % Tris/HCl). Because the phosphorylation level of Wag31 has been found previously to be very low in *M. tuberculosis* (Kang *et al.*, 2005), 10-fold more protein (50 µg) was used to detect changes in Wag31 phosphorylation.

RESULTS

Wag31, a member of the *division cell wall (dcw)* cluster, is recruited to new cell poles when cell division is completed or nearly completed

Many Gram-positive and Gram-negative bacteria possess a chromosomal region that contains several genes whose products are involved in cell division and cell wall biosynthesis, and is therefore known as the *dcw* cluster (Massidda *et al.*, 1998; Pucci *et al.*, 1997; Yura *et al.*, 1992). Supplementary Fig. S1 shows that in *M. tuberculosis*, *wag31* is part of the *dcw* gene cluster that contains cell division genes (*ftsZ*, *ftsQ* and *ftsW*) and peptidoglycan biosynthetic genes (*murC*, *murG*, *murD*, *murX*, *murF* and *murE*). The location of *wag31* in this cluster suggests that Wag31 plays a role in cell division and/or cell wall synthesis. The gene encoding Wag31 (Rv2145c) is separated by 268 bp from Rv2146c and by 95 bp from Rv2144c, suggesting that *wag31* is monocistronic. In support of this inference we identified a transcription start site 5' of *wag31* and 3' of the upstream gene Rv2146c (data not shown).

In vegetatively growing *B. subtilis* cells, the Wag31 homologue DivIVA localizes to the cell poles and the septum, where it is bound to the MinCD cell division inhibitor complex so that the FtsZ ring can only form at the mid-cell region (Marston *et al.*, 1998). After assembly of the FtsZ ring, DivIVA is recruited to the mid-cell division site before contraction of the ring begins (Edwards & Errington, 1997). To see if a similar pattern of Wag31

localization occurs in mycobacteria, we constructed and expressed a *gfp-wag31_{Msmeg}* N-terminal fusion driven by an acetamide-inducible promoter in the integrating vector pMV306 (Parish *et al.*, 1997) in *M. smegmatis*, and examined it by fluorescence microscopy.

Growth in the presence of the inducer (0.1 % acetamide) for 7 h showed normal cell morphology and revealed strong, distinct localization of GFP-Wag31_{Msmeg} at the poles but very little at the potential mid-cell position (Fig. 1). The signal was often markedly stronger at one of the poles (the old pole) in cells that were in the process of dividing and in newly formed daughter cells (Fig. 1a, b). Such localized structures were not seen when GFP alone was expressed, indicating that the GFP-Wag31_{Msmeg} localization is not a GFP artefact (Fig. 1c). These findings suggest that the role of Wag31 in mycobacteria is distinct from that of DivIVA in *B. subtilis*, which has been found to localize at cell poles and at the mid-cell position before cell division. To determine whether Wag31 localized in a similar manner in the slow-growing members of the *M. tuberculosis* complex, we constructed an N-terminal *gfp-wag31_{Mtb}* fusion protein from *M. tuberculosis* and expressed it in *M. bovis* BCG. When examined after 16 h of induction with 0.1 % acetamide, GFP-Wag31_{Mtb} showed localization patterns identical to those seen with GFP-Wag31_{Msmeg} (Fig. 1a, inset).

Fig. 1. Localization of GFP-Wag31 in *M. smegmatis*. Images from bright-field DIC and fluorescence microscopy of GFP-Wag31_{Msmeg} were overlapped. The gene for *gfp* or *gfp-wag31* was expressed for 7 h under the control of the inducible acetamidase promoter. (a) *M. smegmatis* strain expressing *gfp-wag31_{Msmeg}*; inset, *M. bovis* BCG expressing *gfp-wag31_{Mtb}*. (b) Magnified image of cells expressing *gfp-wag31_{Msmeg}*. White triangles indicate GFP-Wag31_{Msmeg} signals from the cell poles, and the black triangle indicates a weaker signal where cell division is completed or near completion. The adjacent cell does not have a signal from the mid-cell region. (c) Control *M. smegmatis* strain expressing *gfp* alone.

wag31 is essential in mycobacteria

In a saturation transposon mutagenesis study in *M. tuberculosis*, no *wag31* insertions were isolated, suggesting that *wag31* is essential (Sasseti *et al.*, 2003). To verify that it is essential, and to examine the role of Wag31 in mycobacteria, we constructed a conditional mutant of *wag31* in *M. smegmatis* by disrupting the *wag31* chromosomal locus in the presence of a construct that expresses *wag31*_{Msmeg} from the acetamide-inducible promoter P_{acet} (see Supplementary Fig. S2). The chromosomal *wag31* gene was disrupted in a two-step process. First, the *wag31* inducible expression construct in the integrating vector pMV306 (Parish *et al.*, 1997) was electroporated into wild-type *M. smegmatis*. Transformants were selected and screened by PCR to confirm the presence of the second copy of *wag31* (Supplementary Fig. S2, step A).

To inactivate the native copy of *wag31* in this strain, 212 codons of *wag31* were deleted and replaced with a hygromycin-resistance cassette. This construct was cloned into pRH1351 (Pelicic *et al.*, 1997; Raman *et al.*, 2001) and then electroporated into the strain resulting from step (A) (Supplementary Fig. S2). Sequential selection and counter-selection yielded candidate strains in which the native chromosomal copy of *wag31* was deleted. Disruption of the chromosomal *wag31* was confirmed by PCR (Supplementary Fig. S2b).

To determine whether *wag31* is essential in *M. smegmatis*, the conditional mutant was plated onto 7H9 solid medium with or without 0.1 % acetamide. Fig. 2(a) shows that the *wag31* conditional mutant is unable to grow in the absence of Wag31 (no inducer), confirming that *wag31* is an essential gene in *M. smegmatis*. To further examine the effect of Wag31 depletion on mycobacterial growth, the *wag31* conditional mutant was cultured in 7H9 liquid medium in the presence or absence of 0.1 % acetamide (Fig. 2b). To observe the effects of conditional depletion of Wag31 would require turnover of existing functional protein. The conditional *wag31* mutant was therefore grown to mid-exponential phase (16 h) in the absence of inducer, prior to inoculation into medium with or without acetamide. While cells with inducer showed normal growth kinetics, the culture lacking the inducer did not display an increase in optical density. Finally, to confirm that this growth phenotype is due to the depletion of Wag31, immunoblot analysis with an anti-Wag31 mAb (Hermans *et al.*, 1995) was performed. Cultures grown with or without inducer (Fig. 2b) were harvested at 48 h, and total protein was purified for subsequent SDS-PAGE and Western blot analysis. Fig. 2(c) shows that while cells cultured with inducer produced Wag31, cells without inducer contained amounts of Wag31 that were nearly undetectable, confirming the linkage between the absence of Wag31 and the growth defect in the conditional mutant.

Fig. 2. *wag31* is an essential gene in mycobacteria. (a) Growth of the *M. smegmatis* *wag31* conditional mutant on 7H9 solid medium. Cells grown on 7H9 agar medium with 0.1 % acetamide (inducer of *wag31* expression) were streaked on fresh 7H9 agar plates with or without 0.1 % acetamide. (b) Growth of the *wag31* conditional mutant in 7H9 liquid medium. Colonies were grown in the presence of inducer on 7H9 agar plates and then cultured overnight in 7H9 liquid medium in the absence of acetamide. Cells were then reinoculated into liquid medium with (■) or without (●) 0.1 % acetamide, and growth was monitored by measurement of OD₆₀₀. (c) Western blot analysis for Wag31 in the *wag31* conditional mutant. A 5 µg sample of total protein from cultures at the 48 h time point in (b) was run in SDS-PAGE, followed by immunoblotting with an anti-Wag31 mAb (F126-2).

Depletion of *wag31* causes a morphological phenotype with asymmetric bulged and round cells

To test whether Wag31 depletion causes any morphological change in *M. smegmatis*, the *wag31* conditional mutant grown on 7H9 agar medium containing 0.1 % acetamide was inoculated into 7H9 liquid medium without acetamide, harvested at serial time points and examined by microscopy (Fig. 3, DIC 1× and DIC 2×). After 7 h, the majority of cells had a bulged tip at one end. Some cells appeared to be in the process of cell division despite the markedly abnormal bulged-tip morphology. At later stages of Wag31 depletion (24 and 48 h), many cells became more rounded and many formed diplococcus-like cells, suggesting incomplete cell division. In addition, we observed that many cells appeared to have lysed, forming large clumps, suggesting that the morphological defect caused by Wag31 depletion produced osmotic instability, consistent with altered cell wall integrity.

In the wild-type background we found that GFP–Wag31_{Msmeg} was localized at the cell pole immediately

before or after the actual separation into two daughter cells (Fig. 1). We therefore examined whether the observed morphological changes of the *wag31* conditional mutant are caused by the absence of Wag31 in the rounded end of the cells by observing the localization of GFP–Wag31_{Msmeg} in the *wag31* conditional mutant. To do this, we subcloned the P_{acet}–gfp–*wag31*_{Msmeg} construct (from pMV306–P_{acet}–gfp–*wag31*_{Msmeg} used in Fig. 1) into a replicating plasmid, pMV261 (Km^r), and introduced this construct into a *wag31* conditional mutant strain in which P_{acet}–*wag31*_{Msmeg} was replaced with P_{tet}–*wag31*_{Msmeg} (Blokpoel *et al.*, 2005; see Methods). Transformants were selected and cultured on 7H9 solid medium with 0.1 % acetamide and 10 ng tetracycline ml^{−1}. Cells were then inoculated into 7H9 liquid medium without either inducer and examined by microscopy as the residual Wag31 was depleted. When examined at about 6 h after inoculation, in almost all the cells that retained the signal, GFP–Wag31_{Msmeg} was exclusively found at the tip of the rod-shaped part of the cell, as shown in Fig. 3b (−inducer). When both inducers were present in the medium, cells had normal morphology and GFP–Wag31_{Msmeg} was localized to both tips of the

Fig. 3. Changes in morphology and peptidoglycan synthesis caused by the depletion of Wag31. (a) Morphological changes of the *wag31* conditional mutant during growth without inducer. The *wag31* conditional mutant cells grown on 7H9 solid medium containing 0.1 % acetamide were inoculated into fresh 7H9 medium without inducer. Aliquots of culture were treated with 5 µg Van-Alexa568 ml^{−1} at 5, 22 and 46 h, incubated for another 2 h, washed and examined by microscopy. DIC 1×, DIC images using ×100 objective lens; DIC + Van-Alexa, images for DIC and fluorescent Van-Alexa568 are superimposed; DIC 2×, images produced using a ×100 objective lens magnified twofold by imaging software (Photoshop CS2). White triangles indicate Van-Alexa568 signals found between the two seemingly dividing lollipop-shaped cells. (b) The bulged-tip phenotype is associated with the absence of Wag31. *gfp*–*wag31* was expressed from the acetamide-inducible promoter in a *wag31* conditional mutant in which native *wag31* was expressed from a tetracycline-inducible promoter. Cells were cultured on 7H9 solid medium containing 10 ng tetracycline ml^{−1} and 0.1 % acetamide, and inoculated into fresh 7H9 liquid medium without either inducer. After 7 h, cells were harvested, washed and examined by microscopy. (c) Examination of nascent peptidoglycan synthesis using Van-Alexa568 in wild-type *M. smegmatis*. Exponentially growing *M. smegmatis* cells were treated with 5 µg Van-Alexa568 ml^{−1} for 2 h, washed and examined by fluorescence microscopy.

rod-shaped cell (Fig. 3b, +inducer). A portion of cells (<30%) that had a bulged tip did not show any signal from GFP–Wag31^{Msmeg}, suggesting that these cells may have been lysed and lost GFP–Wag31^{Msmeg}.

The bulged-tip phenotype of the *wag31* conditional mutant is caused by the absence or dispersal of peptidoglycan synthesis

In our previous work, we showed that overexpression of *wag31* produced a large number of short, wide cells compared with control cells containing vector only (Kang *et al.*, 2005). Here we have shown that depletion of *wag31* causes abnormal morphology with a bulged tip at one end of the cell. These phenotypes could result from altered localization of peptidoglycan synthesis and/or cell division proteins. To examine whether Wag31 is present at the site of nascent peptidoglycan synthesis we first identified the site of nascent cell wall synthesis in wild-type *M. smegmatis* cells by using a fluoresceinated vancomycin conjugate (Van-Alexa568, red fluorescence; see Methods). Vancomycin binds the terminal D-Ala-D-Ala of the peptidoglycan precursor, and a vancomycin–fluorescein conjugate (Van-FL) has been used to identify the sites of nascent peptidoglycan synthesis during cell division in Gram-positive bacteria (Daniel & Errington, 2003).

Using conditions similar to those used to localize the sites of nascent peptidoglycan synthesis in *B. subtilis* (Daniel & Errington, 2003), no staining by Van-FL was detected in *M. smegmatis*. Because the lipid-rich mycobacterial cell envelope may be less permeable to this stain, we synthesized another vancomycin conjugate, Van-Alexa568 (Molecular Probes). Using the same concentration of Van-Alexa568 (1 µg ml⁻¹), prominent fluorescent labelling at the cell poles was evident after 7 h incubation, while weaker and dispersed fluorescent labelling was observed in the mid-cell regions (Fig. 3c). The staining pattern of Van-Alexa568 in *M. smegmatis* was similar to that described for Van-FL staining of *Corynebacterium glutamicum*, a closely related actinomycete (Daniel & Errington, 2003). While it is possible that differences in the lipid envelope between the cell pole and other parts of the cell result in this pattern of localized staining, these results strongly suggest that nascent peptidoglycan synthesis in mycobacteria occurs at the cell poles and mid-cell but not along the entire length of the rod.

We have shown in Fig. 1 that Wag31 is localized at the cell poles and is recruited to the mid-cell position when cell division is completed or nearly complete. This localization of Wag31 overlaps with the polar locus of nascent peptidoglycan synthesis, as shown in Fig. 3(c). We thus tested whether the bulged end of the *wag31* conditional mutant was associated with a defect in peptidoglycan synthesis. To address this possibility, cells grown in 7H9 liquid medium without inducer to allow depletion of Wag31 (Fig. 3a, DIC 1×) were treated with 5 µg Van-Alexa568 ml⁻¹ for 2 h, washed and then examined by

fluorescence microscopy. After 7 h of Wag31 depletion, a strong Van-Alexa568 signal (nascent peptidoglycan synthesis) could be detected from the normal rod-shaped tip. In contrast, the bulged area which lacks GFP–Wag31, as shown in Fig. 3(b), showed an absent or very dispersed Van-Alexa568 signal (Fig. 3a, DIC + Van-Alexa). Taken together, these data suggest that the absence of Wag31 causes a defect in polar peptidoglycan synthesis, which in turn results in the bulged-tip morphology. In many cases, Van-Alexa568 signals (white triangles) were found between the two seemingly dividing lollipop-shaped cells, indicating active cell wall synthesis at the septal locus. Consistent with this interpretation, as cells became more rounded, as shown after 24 and 48 h of Wag31 depletion, Van-Alexa568 did not show localized staining, suggesting absent or highly dispersed cell wall synthesis. At these later time points, many cells were in the form of fused diplococci, indicating that these cells were defective in completing cell division.

Location and intensity of Wag31 staining correspond to those of nascent peptidoglycan synthesis at cell poles in wild-type *M. smegmatis*

Data from the *wag31* conditional mutant stained with Van-Alexa568 strongly suggested that Wag31 plays a critical role in regulating nascent peptidoglycan synthesis at cell poles. If this were the case, we hypothesized that there would be temporal and spatial correlation between Wag31 and nascent peptidoglycan synthesis in wild-type *M. smegmatis* cells. To address this hypothesis, wild-type *M. smegmatis* cells containing P_{acet}-gfp-*wag31*^{Msmeg} were cultured to exponential phase (OD₆₀₀=0.5) in 7H9 liquid medium with 0.1% acetamide and stained with Van-Alexa568 (1 µg ml⁻¹) in order to examine the localization of both GFP–Wag31 and nascent peptidoglycan synthesis. Fig. 4 shows that the location and intensity of Van-Alexa568 staining correspond very closely to those of GFP–Wag31. This result strongly indicates that Wag31 plays a key role in localized peptidoglycan synthesis at cell poles in mycobacteria.

Wag31 is not required for chromosome segregation

In sporulating *B. subtilis*, DivIVA has been shown to be important in chromosome segregation through its interaction with the *oriC* complex (Thomaides *et al.*, 2001). Since Wag31 is a homologue of DivIVA, we investigated whether Wag31 is also involved in chromosome segregation in mycobacteria, by staining DNA in the conditional *wag31* mutant. To investigate the effect of Wag31 on chromosome segregation in mycobacteria, the *wag31* conditional mutant cells were cultured in the presence or absence of the inducer (0.1% acetamide). After 7 h incubation, 1 ml of each culture was treated with 5 µM of SYTO 45 (Molecular Probes) for 30 min, washed with fresh 7H9 liquid medium, and examined by fluorescence microscopy. Cells grown in the presence of inducer showed

Fig. 4. Co-localization of GFP-Wag31 and nascent peptidoglycan synthesis. Wild-type *M. smegmatis* cells containing $P_{\text{acet}}\text{-gfp-wag31}$ were cultured to exponential phase in 7H9 liquid medium with 0.1 % acetamide and stained with $1 \mu\text{g Van-Alexa568 ml}^{-1}$ for 3.5 h. Cells were then washed and examined by fluorescence microscopy. Images from Van-Alexa568 staining are shown in red (upper row) and GFP-Wag31 signals are shown in green (middle row). The two fluorescent images were then overlapped with the bright-field DIC image (bottom row).

normal DNA segregation, with two distinct chromosomes separated by the septum of the rod-shaped cell (Fig. 5, +inducer). Cells without the inducer produced the bulged tip, and the bulged-tip area and the rounded cells were both stained with SYTO 45, suggesting that DNA segregation is not dependent on the presence of Wag31 in mycobacteria (Fig. 5, –inducer). While DNA is clearly present in both the rod-shaped and the round end of the cells, these data do not exclude a role for Wag31 in accurate chromosome segregation.

Phosphorylation of Wag31 is important for optimal growth of *M. smegmatis*

In our previous work, we showed that moderate over-expression of the wild-type *wag31_{Mtb}* or phosphomimetic *wag31T73E_{Mtb}* in *M. smegmatis* produced cells that are more rounded than the control cells containing the non-phosphorylatable *wag31T73A_{Mtb}* allele or the vector alone (Kang *et al.*, 2005). The different morphological effects of these three *wag31_{Mtb}* alleles strongly suggested that phosphorylation of Wag31 plays an important role in its regulation of cell division/morphology in mycobacterial cells. To further study the function of Wag31 phosphorylation, we determined the effect of three *wag31_{Mtb}* alleles on growth of *M. smegmatis* by introducing and independently expressing the wild-type *wag31_{Mtb}*, *wag31T73A_{Mtb}* or *wag31T73E_{Mtb}* alleles in the conditional *wag31* mutant of

Fig. 5. Chromosome segregation in the *wag31* conditional mutant. *wag31* conditional mutant cells grown on 7H9 agar medium containing inducer (0.1 % acetamide) were inoculated into fresh 7H9 liquid medium in the presence or absence of the inducer. After 7 h of incubation with shaking, each culture (1 ml) was treated with $5 \mu\text{M SYTO 45}$ for 30 min, washed and examined by fluorescence microscopy. The upper row shows the fluorescent images of induced and uninduced cultures, and the lower row shows the superimposed images from DIC and fluorescence microscopy.

M. smegmatis. To do this, we constructed new conditional mutant strains of *wag31* in which the acetamide-inducible $P_{\text{acet}}\text{-wag31}_{\text{Msmeg}}$ construct at the *attB* locus was replaced with tetracycline-inducible $P_{\text{tet}}\text{-wag31}_{\text{Mtb}}$, $P_{\text{tet}}\text{-wag31T73A}_{\text{Mtb}}$ or $P_{\text{tet}}\text{-wag31T73E}_{\text{Mtb}}$ (Pashley & Parish, 2003). In the absence of tetracycline, none of the transformants could grow on solid 7H9 medium containing the appropriate antibiotics, confirming the low basal expression from the P_{tet} promoter. However, when plated on solid 7H9 medium containing $5 \text{ ng tetracycline ml}^{-1}$, all three transformants were able to grow. This result suggests that while Wag31 itself is essential for the survival of mycobacterial cells, the phosphorylation of Wag31 is not essential to the function of Wag31, but rather may be important for the fine-tuning of Wag31 function.

To examine in more detail the effect of phosphorylation of Wag31 on growth, each transformant was cultured overnight in 7H9 liquid medium in the absence of tetracycline and then inoculated into fresh 7H9 liquid medium containing $2 \text{ ng tetracycline ml}^{-1}$ (Fig. 6a). The choice of this concentration of tetracycline was based on preliminary growth experiments with different concentrations of tetracycline ($1\text{--}20 \text{ ng ml}^{-1}$) to identify the concentration that produced growth and Wag31 protein levels comparable to those in wild-type *M. smegmatis* (data not shown). During exponential phase, the *wag31* conditional mutant containing $P_{\text{tet}}\text{-wag31T73E}_{\text{Mtb}}$ (mean doubling time, $g=4.30 \text{ h}$) showed the highest growth rate, cells harbouring $P_{\text{tet}}\text{-wag31}_{\text{Mtb}}$ ($g=4.95 \text{ h}$) showed an intermediate growth rate, and cells with $P_{\text{tet}}\text{-wag31T73A}_{\text{Mtb}}$ ($g=5.75 \text{ h}$) showed the lowest growth rate.

Fig. 6. Effect of Wag31 phosphorylation on growth of *M. smegmatis*. *wag31* conditional mutants were constructed in which one of three *wag31* *Mtb* alleles, P_{tet} -*wag31*_{*Mtb*}, P_{tet} -*wag31*T73A_{*Mtb*} or P_{tet} -*wag31*T73E_{*Mtb*}, was expressed under the control of a tetracycline-inducible promoter. (a) Differential growth phenotypes among the three strains. Each strain was cultured in 7H9 liquid medium with 2 ng tetracycline ml⁻¹ and growth was examined by measuring OD₆₀₀. These data were collected from three independent cultures of each strain containing P_{tet} -*wag31*_{*Mtb*} (●), P_{tet} -*wag31*T73A_{*Mtb*} (▲) or P_{tet} -*wag31*T73E_{*Mtb*} (■). Error bars represent 1 SD. (b) Western blot analysis to examine Wag31 protein content in the strains expressing the three different *wag31* alleles. Total protein was purified from exponential- (26 h) and stationary-phase cells (53 h), and 5 µg of total protein was used for Western blot analysis with anti-Wag31 mAb. Protein from exponentially growing *M. smegmatis* wild-type cells is also shown, and demonstrates a Wag31 protein content similar to that in the inducible expression strains. (c) Levels of Wag31 and its phosphorylation (indicated by the double-headed arrow) at different growth stages of *M. bovis* BCG. For Western blot analysis with anti-Wag31 mAb, 5 µg of total protein was loaded in each lane; for Western blot analysis with anti-^PTQ antibody, 50 µg per lane was used. Lanes: 1, mid-exponential phase; 2, late exponential phase; 3, early stationary phase; 4, late stationary phase. In addition to Wag31, additional bands were detected by the anti-^PTQ antibody, consistent with our previous immunoblot data (Kang *et al.*, 2005).

Western blot analysis with anti-Wag31 mAb confirmed that this growth phenotype is not due to differences in expression levels of each construct (Fig. 6b). When examined by microscopy, no clear difference in morphology was found among the three strains harvested at serial time points (data not shown).

In a Western blot analysis of cell lysates from different growth phases with anti-Wag31 mAb, we found that the *wag31* gene was constitutively expressed from exponential through to stationary phase. Probing with an anti-phospho-(S/T)Q antibody that recognizes the phosphorylation site in Wag31, however, we found that Wag31 is mainly phosphorylated during exponential phase in *M. bovis* BCG (Fig. 6c). This result is consistent with our previous finding that the *pknA/B* operon is maximally transcribed during exponential phase and that its transcription drops dramatically during stationary phase. In the context of these observations, the differential growth phenotypes of cells expressing *wag31* alleles that encode substitutions at the phosphoacceptor site (Fig. 6a) suggest that precise regulation of Wag31 phosphorylation at different growth phases is important for optimal growth of mycobacteria.

DISCUSSION

In this work we determined that *wag31* is an essential gene whose product localizes to cell poles, and that depletion of

Wag31 results in a dramatic morphological change in which one end of the cell becomes rounded. Together with data showing co-localization of GFP-Wag31 and nascent cell wall synthesis at the cell poles in both the *wag31* conditional mutant and wild-type strains of *M. smegmatis*, these results indicate that Wag31 is important for maintaining cell shape and cell wall integrity by localizing peptidoglycan synthesis to the cell poles. This role in regulating cell wall synthesis is consistent with the chromosomal locus of *wag31* being in the *dcw* cluster, which includes six *mur* genes encoding enzymes that function in the biosynthesis of peptidoglycan precursor molecules.

While our data show that polar localization of Wag31 is critical for maintaining the rod shape of mycobacteria, Wag31 does not appear to play a major role in formation or function of the mid-cell division complex, in contrast to the function of DivIVA in some other species. In vegetatively growing *B. subtilis* cells, for example, the Wag31 homologue DivIVA localizes at the cell poles and the septum. At the cell poles, DivIVA-GFP is bound to the MinCD cell division inhibitor complex so that the FtsZ ring can only form at the mid-cell region (Marston *et al.*, 1998). After assembly of the FtsZ ring, DivIVA then is recruited to the mid-cell division site before contraction of the ring begins (Edwards & Errington, 1997). Our experiments in mycobacteria, however, which lack MinC or MinD homologues, revealed intense localization of

GFP–Wag31_{Msmeg} at the poles but not at the septum, suggesting that in mycobacteria Wag31 does not play a major role in septum formation. This idea is consistent with the hypothesis that peptidoglycan synthesis required for septation is independent from that required for cell elongation (Donachie & Robinson, 1987).

What is the mechanism by which first one and then both ends of the cell lose their rod shape and become rounded when Wag31 is depleted? A previously described model for the generation of rod-shaped cells by actinomycetes such as mycobacteria proposes that *de novo* peptidoglycan synthesis limited to the cell poles gives rise to this shape, with progressive elongation of the rod until cell division occurs (Daniel & Errington, 2003). Our data are strongly supportive of this model and indicate a critical role of Wag31 in the polar localization of peptidoglycan synthesis during cell growth. Our data also demonstrate an asymmetry in this process, with accumulation of Wag31 beginning at the new pole only at the final stages, or after completion, of cell division. Thus, the old pole shows a strong GFP–Wag31 signal and strong staining of nascent peptidoglycan by Van-Alexa, whereas the division site shows minimal GFP–Wag31 signal until after cytokinesis. In the wild-type, following cell division, Wag31 accumulates at the new pole, and the rod-shape is maintained by polar peptidoglycan synthesis at both poles. In the *wag31* conditional mutant strain without ongoing *wag31* expression, however, the new pole is unable to accumulate Wag31 and thus is unable to direct localized peptidoglycan synthesis to the new pole. In this model, the resulting absent or diffuse cell wall synthesis at the new pole, possibly caused by an imbalance between cell wall synthesis and cell wall hydrolysis, results in a round end of the cell while the other end (the old pole with residual Wag31) maintains its rod shape. Over time, residual Wag31 is depleted from the old pole, and the cells become completely round and more susceptible to lysis.

This model suggested by our data raises several questions for further investigation. What is the mechanism by which Wag31 localizes to the poles? How does it direct peptidoglycan synthesis? Does Wag31 recruit the cell wall synthesis machinery to the poles, or does it play some other regulatory role? Protein–protein interactions are likely to be critical to Wag31 localization and function, and this protein contains at least two predicted protein interaction domains (Flardh, 2003).

Another subject for investigation is the role of phosphorylation of Wag31 in modulating its function. The higher growth rate during exponential phase of the strains expressing the *wag31T73E_{Mtb}* allele relative to those harbouring the *wag31_{Mtb}* or *wag31T73A_{Mtb}* alleles suggests that regulation of the activity of Wag31 via phosphorylation is important during active cell growth in mycobacteria. This result is consistent with our previous and current data indicating that the expression levels of the kinase genes responsible for phosphorylating Wag31 (*pknA*

and *pknB*) are highest during exponential-phase growth, as is the extent of phosphorylation of Wag31 *in vivo* (Kang *et al.*, 2005).

Our results thus identify and provide insights into a novel mechanism for the regulation of localized cell wall synthesis in mycobacteria via polar localization of Wag31. This function may then be further modulated by reversible phosphorylation in response to environmental conditions, including those encountered during infection. Wag31 and DivIVA homologous proteins have two predicted coiled-coil regions that are interrupted by a highly variable sequence (Flardh, 2003). Interestingly, this highly variable sequence contains the unique phosphorylation site of Wag31 (T73). None of the DivIVA homologues are known to be phosphorylated, nor is the phosphorylation site in Wag31_{Mtb} conserved in other bacterial genera.

Our data thus suggest that the signal transduction pathway involving Wag31 and PknA/B plays a unique role in regulating peptidoglycan synthesis at cell poles in mycobacteria. This Wag31-regulated process is essential for growth and normal rod-shaped cell morphology in mycobacteria, and thus identifies a novel target for the development of new anti-tuberculars. Further elucidation of proteins with which Wag31 interacts and the role of Wag31 phosphorylation in these interactions will provide insights into the mechanisms by which Wag31 and peptidoglycan synthesis are targeted to the cell poles. These insights will be important in understanding the regulation of mycobacterial morphology and cell division, and in understanding aspects of *M. tuberculosis* growth regulation that may play a role in the pathogenesis of tuberculosis.

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