Cloning, Expression and Characterization of UDP-N-Acetylglucosamine Enolpyruvyl Transferase (MurA) from Wolbachia Endosymbiont of Human Lymphatic Filarial Parasite *Brugia malayi*

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Abstract

Wolbachia, an endosymbiont of filarial nematode, is considered a promising target for treatment of lymphatic filariasis. Although functional characterization of the Wolbachia peptidoglycan assembly has not been fully explored, the Wolbachia genome provides evidence for coding all of the genes involved in lipid II biosynthesis, a part of peptidoglycan biosynthesis pathway. UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) is one of the lipid II biosynthesis pathway enzymes and it has inevitably been recognized as an antibiotic target. In view of the vital role of MurA in bacterial viability and survival, MurA ortholog from Wolbachia endosymbiont of *Brugia malayi* (wBm-MurA) was cloned, expressed and purified for further molecular characterization. The enzyme kinetics and inhibition studies were undertaken using fosfomycin. wBm-MurA was found to be expressed in all the major life stages of *B. malayi* and was immunolocalized in Wolbachia within the microfilariae and female adults by the confocal microscopy. Sequence analysis suggests that the amino acids crucial for enzymatic activity are conserved. The purified wBm-MurA was shown to possess the EPSP synthase (3-phosphoshikimate 1-carboxyvinyltransferase) like activity at a broad pH range with optimal activity at pH 7.5 and 37°C temperature. The apparent affinity constant (Kᵦ) for the substrate UDP-N-acetylglucosamine was found to be 0.03149 mM and for phosphoenolpyruvate 0.009198 mM. The relative enzymatic activity was inhibited ~2 fold in presence of fosfomycin. Superimposition of the wBm-MurA homology model with the structural model of *Haemophilus influenzae* (Hi-MurA) suggests binding of fosfomycin at the same active site. The findings suggest wBm-MurA to be a putative antifilarial drug target for screening of novel compounds.

Introduction

*Wolbachia* are the maternally inherited intracellular gram negative alphaproteobacteria widely spread among arthropods and filarial nematodes exhibiting a diverse range of associations with their host. In filarial nematodes, they exhibit vertical transmission via oocytes that has promoted evolutionary adaptation and a mutualistic relationship.

Lymphatic filariasis (LF), the cause of long-term disability in tropical and sub-tropical countries is caused by the filarial nematodes, *Wuchereria bancrofti* and *Brugia* species. Over 120 million people are currently infected and one third of these develop major morbidity worldwide [1]. Currently used antifilarial drugs interrupt transmission of infection by principally killing the larval stage called microfilariae (mf) without much effect on the adult parasites. Since the adult filarial worms can survive up to decade in the vertebrate host, repeated annual treatments are recommended for several years to bring the mf density to a very low level that will not transmit infection. *Wolbachia* is obligatory for most species of filarial nematodes as evidenced by the killing of *Wolbachia* following tetracycline and doxycycline treatment that impairs the development and fecundity of worms [2–5]. However, the antibiotics require long course of treatment and are not recommended for use in the young children and pregnant women [6]. In absence of an adulticidal drug together with the threat of drug resistance to mainstay filaricides [7,8], identification and characterization of novel antifilarial drug targets and discovery of novel classes of compounds with different mode of action is urgently required. *Wolbachia* bears extremely low number of predicted genes (~806) as compared to other bacteria [9] which include several unique potential targets [10]. Investigations on a few proteins/enzyme pathways of *Wolbachia* have recently been undertaken such as *Wolbachia* surface protein (WSP), heat shock protein 60 (HSP60), independent phosphoglycerate mutase...
(PcPGM), pyruvate phosphate dikinase (PPDK), enzymes regulating heme, lipid II and lipoprotein biosynthesis [11], NAD-dependent DNA ligase (αBm-LigA) [12] and transcription factor [13]. Specific inhibitors of Wolbachia enzymes have been investigated recently. The benzimidazoles have been shown to inhibit heme biosynthesis pathway [14], acyldepsipeptides inhibit Clp peptidase [15] and heterorotary compounds target rsmD-like rRNA methyltransferase [16]. These inhibitors also exhibited antifilarial activity reassuring Wolbachia as a promising antifilarial drug target.

Peptidoglycan (PG), an essential component of the cell wall provides structural integrity to bacteria against internal osmotic pressure [17]. The enzymes linked to PG synthesis remain conserved among the bacterial species. These have no mammalian counterpart and therefore present an attractive drug target. The annotated genome of Wolbachia reveals the presence of genes required for lipid II precursors for PG biosynthesis including UDP-N-acetylmuramic acid (UDP-NAG) and UDP-N-acetylmuramic acid enolpyruvyl transferase (MurA) [9,18]. However, the role of lipid II in Wolbachia remains unclear since other genes involved in PG synthesis such as those responsible for polymerization of glycans are absent. It is well known that MurA catalyzes the first committed step in the cell wall biosynthesis of bacteria and transfers an enolpyruvyl group from phosphoenolpyruvate (PEP) to UDP-N-acetylmuramyl pentapeptide (UDP-NAG5) to form UDP-N-acetylmuramyl pentapeptide enolpyruvate [19] which is a precursor to UDP-N-acetylmuramylpentapeptide, a requisite building block of bacterial cell wall (Figure 1) [20]. However, it has also been shown that inhibition of lipid II synthesis brings about a detrimental effect on Wolbachia within the insect cell lines [18]. The deletion/ inactivation of MurA gene of Escherichia coli [21], Staphylococcus pneumoniae [22], and Staphylococcus aureus [23] has been shown to cause their death and this has been investigated extensively.

In view of high homology of the protein encoded by UDP-N-acetylmuramic acid enolpyruvyl transferase gene in all the related bacteria, it is quite likely that αBm-MurA may also perform similar function in B. malayi endosymbiont. The structure prediction studies in the current study establish that the key amino acids required for MurA enzymatic activity are conserved in αBm-MurA. In addition, fosfomycin brought about an irreversible inhibition in αBm-MurA activity as also reported earlier in other bacteria [24]. The current investigation deals with the cloning, expression, purification and characterization of B. malayi Wolbachia MurA.

**Materials and Methods**

**Animals, Parasites, Infection**

Rodent host Mastomys coucha (GRA ‘Giessen’ strain) were infected with B. malayi (sub-periodic strain) by subcutaneous inoculation of 100 third stage infective larvae (L3) isolated from laboratory bred mosquito vector Aedes aegypti using Baermann technique [25,26]. Jirds (Mastomys natalensis) were infected with 150–200 B. malayi infective larvae by the intraperitoneal route [27]. Adult parasites and microfilariae (mf) were recovered from the peritoneal cavity of jird infected 15–20 weeks earlier. Adult parasites were made free of host tissues under the dissecting microscope and mf were pelleted by passing the peritoneal wash through a 5.0-μm membrane filter in sterile phosphate buffer saline (PBS-pH 7.2). Euthanization of animals was done by injecting overdose of Intraval Sodium (100 mg/kg). Efforts were made to minimize suffering and reduce the number of animals used. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [28].

**Ethics Statement**

The animals used in the study were housed in climatically controlled pathway free rooms at National Animal Laboratory Centre, CSIR - Central Drug Research Institute, Lucknow, India and fed with standard pellet diet with water ad libitum. This study was approved by the Animal Ethics Committee of CSIR - Central Drug Research Institute, Lucknow, India constituted under the rules and guidelines of the Committee for the Purpose of Control and Supervision of Animals (CPCSEA), Government of India (1998). The study bears approval nos. IAEC/2011/120 & IAEC/2011/145.

**Genomic DNA Isolation, Amplification and Cloning of αBm-MurA Gene**

Adult worms were harvested from the euthanized jirds. Worms were washed repeatedly in PBS and genomic DNA was isolated following manufacturer’s protocol (PureLink Genomic DNA mini kit-Invitrogen). The genomic DNA from adult also contains genomic DNA of Wolbachia. 1270 bp αBm-MurA gene (REGG - αBm0740) was amplified from the extracted genomic DNA using forward sense primer - 5’-GATTCCATGCATAAAATATTAG-3’ and reverse antisense primer - 5’-CTCGAGTCAAGGAATAGAGATATCGGCCC-3’ containing

![Figure 1. The first cytoplasmic step of the peptidoglycan biosynthesis.](image-url)
restriction sites BamHI and XhoI (underlined), respectively. The amplification was carried out by mixing 1 mM of each primer, 200 μM of each deoxynucleoside triphosphate (dNTPs), 0.5 unit Taq DNA polymerase, 1x PCR buffer, and 1.5 mM MgCl2 (all from Fermentas) in a thermocycler (Bio-Rad) under conditions at initial denaturation at 94°C/4 min, 29 cycles at 94°C/45 sec, 48°C/45 sec, 72°C/1.30 min and 1 cycle at 72°C/20 min. The amplified PCR product was electrophoresed in agarose gel and eluted by gel extraction kit (PureLink Gel Extraction kit, Invitrogen). Eluted product (~1278 bp) was sub-cloned into pTZ57R/T (T/A) cloning vector (Fermentas) and transformed into competent E. coli DH5α cells. The transformants were screened for the presence of recombinant plasmids with the desired insert by gene specific PCR under similar conditions as mentioned above. Cloning was performed at BamHI and XhoI site in bacterial expression vector pET28a (Novagen) and the plasmid from positive clones was sequenced to confirm the insert.

Wbm-MurA Gene Expression in Various Stages of B. malayi

Different life stages of B. malayi, viz. adult, mf and L3 were recovered as detailed above. RNA was extracted using the TRIzol reagent (Invitrogen) and quantified with a GeneQuant apparatus.
After treatment with DNase I to remove genomic DNA contamination, 3 μg of total RNA from each life stage was used for cDNA synthesis using a first-strand cDNA synthesis kit (Sigma-Aldrich, USA). The target gene was amplified using cDNAs applying conditions as mentioned above. For negative controls, PCR was performed with total RNA in absence of reverse transcriptase, in order to rule out any possibility of DNA contamination in the total RNA samples.

Expression, Purification and Western Blot
The expression of recombinant αBm-MurA was checked in bacterial cells by transforming the recombinant construct in E. coli - Rosetta(DE3)pLysS strain (Novagen). The transformed cells were

Figure 3. Phylogenetic tree showing divergence of MurA. Tree was constructed by NJ method as implemented by the PHYLIP 3.6 package using the input sequences with 1,000 bootstrap replicates. The scale bar represents 0.05 expected amino acid replacement per site as estimated by the program PRODIST of the same package. Number at the nodes represents the age constraint to mean path lengths. Major groups of bacteria are included with the abbreviation given to gram-positive bacteria (GP), Cytophaga-Flexibacter-Bacteroids (CFB) and intracellular Wolbachia (Wolb). Graphical version of the tree was drawn on FigTree program.

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inoculated into 5 ml Luria-Bertani medium and allowed to grow at 37°C in a shaker at 220 rpm. Cultures in logarithmic phase (OD600, 0.5–0.6) were induced for 3 h with different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C. The over-expression of the recombinant (r) wBm-MurA was analyzed by 10% sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) after Coomassie brilliant blue R-250 (Sigma-Aldrich) staining.

For purification, 300 ml Luria-Bertani medium containing 100 μg/ml-chloramphenicol and 50-μg/ml kanamycin were inoculated with freshly transformed pET28a recombinant construct and grown at 37°C/220 rpm to an OD of ~0.6. For expressing the recombinant protein in soluble form, the culture was further grown at 24°C for 20 min., induced by the addition of 0.2 mM IPTG and further grown for 22 h at 24°C/130 rpm. The recombinant wBm-MurA was purified by affinity chromatography using Ni2+ chelating resin which binds to (His)-6-tag fusion peptide derived from the pET28a vector. Harvested cell pellet was resuspended in 15 ml of chilled lysis buffer (20 mM Tris-pH 7.4, 300 mM NaCl, 1 mM EDTA, 10% Glycerol, 1% Triton X-100, 0.5 mM phenyl-methanesulfonyl fluoride [PMSF], 5 mM β-mercaptoethanol) and incubated for 45 min on ice with 1mg/ml of lysozyme (Sigma-Aldrich). The suspension was disrupted by sonication (20 cycles; 10 sec pulse at 20% amplitude with 30 sec interval after each pulse) on ice, and pelleted at 12,500 rpm for 30 min. The supernatant was incubated at 4°C for 1 h with 3 ml Ni-NTA resin (Qiagen,) in a column pre-equilibrated with lysis buffer. The column was subsequently washed with lysis buffer only and then with wash buffer (20 mM Tris-pH 7.4, 300 mM NaCl, 1 mM EDTA, 150 mM PMSF) containing different concentrations of imidazole (20, 40 and 60 mM). The purified recombinant protein was eluted with wash buffer containing 300 mM imidazole. All the washing, elution and dialysis step were performed at 4°C. For purity check, 100 μl from each eluted fraction was mixed with an equal volume of 2x sample buffer (10 mg/ml Bromophenol Blue, 4.4% SDS, 0.5 M Tris-Cl,

Figure 4. Confirmation of cloned construct, expression, purification and Western blotting of recombinant wBm-MurA. A: The cloned gene within the expression vector pET28a was checked by restriction-digestion. Lane 1, molecular size marker (GeneRuler 1 kb Plus DNA Ladder, Thermo Scientific); lane 1–2, Restricted plasmid (insert-1278 bp); lane 3, un-restricted construct (pET28a containing the insert). B: Coomassie-stained SDS-polyacrylamide gel of recombinant wBm-MurA over-expressed in Rosetta(DE3)pLysS E. coli strain with a His tag fusion protein. Lane 1, molecular mass markers (Puregene 4 Color Prestain protein ladder, Genetix); lane 2, uninduced E. coli lysate; lane 3; E. coli lysate after 22 h induction with 0.2 mM IPTG at 24°C; lane 4, flowthrough after passing the supernatant through an Ni-NTA column; lane 5, 10 column volumes eluted with wash buffer containing 60 mM imidazole; lane 6, purified wBm-MurA recombinant fusion protein eluted with wash buffer containing 300 mM imidazole. C: Western blot developed with diaminobenzidine using mouse anti-His monoclonal antibody as primary antibody and HRP-conjugated anti-mouse IgG (lane 2) as secondary antibody; lane 1, molecular mass markers (Puregene 4 Color Prestain protein ladder, Genetix).

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Figure 5. Stage specific expression of wBm-MurA gene and the enzyme. A: Expression of wBm-MurA gene. The full-length DNA (1278 bp, wBm-MurA gene) was amplified from the cDNA of three life- stages of B. malayi using gene specific primers. Lane 1, molecular size markers (GeneRuler 1 kb DNA Ladder, Thermo Scientific); lane 2, infective larvae; lane 3, adults (both sexes); lane 4, microfilariae. Lane 5, 6 and 7 are controls containing PCR products from infective larvae, adults and microfilariae respectively in absence of reverse transcriptase. B: Endogenous protein (wBm-MurA) expression. Western blot was performed with anti-wBm-MurA antibody to confirm the presence of wBm-MurA. Lane 1, molecular mass markers (Puregene 4 Color Prestain protein ladder, Genetix); lane 2, microfilariae; lane 3, infective larvae; lane 4, adult worms (both sexes); and lane 5, purified wBm-MurA protein (positive control).

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Characterization of MurA from Wolbachia of Brugia malayi

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300 mM β-mercaptoethanol) and analyzed on 10% SDS-PAGE along with un-induced sample fraction. The protein was dialyzed against Buffer A (20 mM Tris-Cl-pH 7.4, 250 mM NaCl, 50 mM imidazole) and subsequently against Buffer B (20 mM Tris-Cl-pH 7.4, 250 mM NaCl). Concentration of the eluted fractions was estimated by the Bradford method using Bovine serum albumin (BSA) as standard [29]. The resolved purified recombinant protein was transferred to a nitrocellulose membrane in a mini-blot transfer assembly (Bio-Rad). The membrane was blocked in 3% skimmed milk for 2 h at room temperature (RT). After blocking, the membrane was incubated at RT with mouse anti-His antibody (Novagen, USA) at 1:2000 dilution. The membrane was washed thrice with PBS containing 0.5% Tween 20 and then incubated with goat anti-mouse IgG-HRP conjugate (Sigma, USA) at a dilution of 1:10,000 for 2 h at RT. The blot was developed with 3,3′-diaminobenzidine tetra hydrochloride (DAB) and H2O2 (Sigma-Aldrich).

Raising Polyclonal Antibodies Against wBm-MurA

For the generation of polyclonal antibodies to wBm-MurA, 20 µg of wBm-MurA recombinant protein mixed with Freund’s complete adjuvant (FCA, Sigma, USA) in 100 µl volume was administered subcutaneously in six eight-week old BALB/c mice. Further two booster doses of same amount of protein were given with Freund’s incomplete adjuvant (FIA, Sigma, USA) on the day 15 and 21 post first immunization. Anti-wBm-MurA serum was collected from the blood collected on day 30 post first immunization.

Stage Specific Endogenous Presence of wBm-MurA Enzyme

To observe the presence of wBm-MurA protein in adults, L3 and mf of B. malayi, the crude extracts from each life stage were resolved on 10% SDS-PAGE prior to the Western blot. The target protein was recognized with polyclonal antibody to wBm-MurA. Crude extract were prepared by homogenization and sonication of 20 adult worms (female and male), ~4000 L3 and ~5000 mf in 400 µl PBS, each containing protease inhibitor cocktail (Sigma, USA) followed by the centrifugation at 12,000 ×g for 30 min. Samples for SDS-PAGE were prepared by mixing the supernatants with an equal volume of 2x sample buffer and heated for 5 min at 100°C. The separated protein fractions from SDS-PAGE

Figure 6. Immunolocalization of wBm-MurA in female B. malayi adult worm by confocal microscopy. A–A‴ & B–B‴: Adult female worm was incubated with anti-wBm-MurA polyclonal antibody followed by re-incubation with secondary FITC-IgG antibody and counterstaining with DAPI (control). A, B and C images are in Phase contrast; A′ and B′ demonstrate green fluorescence signal (dots) generated by FITC confirming the presence of wBm-MurA; C′ has no green fluorescence signal in absence of specific antibody; A″, B″, C″ show blue signals produced by DAPI indicating the presence of nuclear DNA; A‴, B‴, C‴ are the merged images of phase contrast and the fluorescence. All the images were captured at 63X oil objective, u, uteri and c, lateral chord.
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were then transferred to the nitrocellulose membrane and the remaining steps were same as appended above for His-fused αBm-MurA Western blotting except that the anti-αBm-MurA mouse serum was used as a primary antibody (1:5000 dilutions). The purified recombinant αBm-MurA served as a positive control.

Immunolocalization of αBm-MurA in B. malayi by Confocal Microscopy

For observing the αBm-MurA distribution in the parasites, confocal microscopy was undertaken. The adult female worm and mf were fixed overnight in 4% paraformaldehyde in M9 buffer (22 mM KH2PO4, 42 mM Na2HPO4, 86 mM NaCl, and 1 mM MgSO4. 7H2O; pH 7.2) at 4°C and further processed, as described earlier [30]. Anti-αBm-MurA polyclonal antibody was used as primary antibody (1:500) while FITC (Fluorescein isothiocyanate; Sigma) conjugated IgG (1:200) was used as the secondary antibody, both diluted in the M9 buffer with 0.5% BSA respectively. After every step washing was done four times with the M9 buffer containing 0.05% Tween-20. The worms and mf were incubated with 4',6'-diamidino-2-phenylindole (DAPI, 100 ng/ml; Sigma) for 5 min for the DNA staining and parasites were mounted on glass slide in 90% glycerol and 10% p-

Figure 7. Immunolocalization of αBm-MurA in microfilariae of B. malayi by confocal microscopy. A–A’**: Microfilariae were incubated with anti-αBm-MurA polyclonal antibody followed by re-incubation with secondary FITC-IgG antibody and counterstaining with DAPI. B–B’**: Microfilariae were incubated with pre-immune serum followed by incubation with secondary FITC-IgG antibody and counterstaining with DAPI (control). A, B images are in Phase contrast; A’ demonstrate green fluorescence signal (dots) generated by FITC confirming the presence of αBm-MurA; B’ has no green fluorescence signal in absence of specific antibody; A”, B” show blue signals produced by DAPI indicating the presence of nuclear DNA; A’’, B’’ are the merged images of phase contrast and the fluorescence. All the images were captured at 63X oil objective.

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Figure 8. Effect of temperature, pH and ions on αBm-MurA enzymatic activity. A: Temperature dependent enzyme activity profile assayed at various temperatures (20–60°C). B: pH dependent enzymatic activity at various pH (4.5–10). C: Effect of different ions (10 mM) on αBm-MurA activity as compared to the control with no ions. Each Data point represents average of the three independent measurements. Error bars represent corresponding SEM.

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phenylenediamine (Sigma, USA) in PBS. Slides were analyzed under a Carl Zeiss LSM 510 META (Zeiss, Jena, Germany) confocal laser scanning microscope equipped with 405nm diode, Argon multiline (458, 477, 488, 514nm), 561 nm DPSS and HeNe 633 nm lasers. Plan-apochromat 63X/1.4 NA oil DIC objective and Plan-apochromat 40X/0.95 NA DIC objective along with appropriate excitation and emission filter sets were used for imaging. 488 nm and 405 nm laser lines were used for excitation of FITC and DAPI respectively. As a negative control, the same procedure was executed after treating parasites with BALB/c preimmune serum.

**Figure 9. Kinetics profile of wBm-MurA and inhibition of enzymatic activity by fosfomycin.** A: The effect of UDPAG concentration on wBm-MurA activity ($K_m$: 0.03149 mM). B: The effect of PEP concentration on wBm-MurA activity ($K_m$: 0.009198 mM). $K_m$ values were calculated by using the Michaelis-Menton plot. C: Relative effect of the fosfomycin on wBm-MurA activity. D: The effect of PEP concentration on the inhibitory activity of fosfomycin, A value on X-axis is without fosfomycin. Each Data point represents an average of the three independent measurements. Error bars represent corresponding SEM.

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**Sequence Analysis and Phylogeny**

BLASTP [http://blast.ncbi.nlm.nih.gov/Blast.cgi] search was made with the wBm-MurA as the query sequence for identifying similar domain sequences. Domain analysis was done on SMART server [31,32]. To identify the conserved regions, sequence alignments of MurA were generated with ClustalW2 [33]. Phylogenetic tree was constructed by neighbor-joining methods using the programs NEIGHBOR and PROTDIST of the PHYLIP package v3.6 [34]. The programs SEQBOOT and CONSENSE from the same package were used to estimate the confidence limits of branching points from 1000 bootstrap replication. For this, twenty four homologous protein sequences...
of MurA were retrieved from NCBI databases using BLASTP and aligned using ClustalW software and these are; *Deinococcus radiodurans* (NP_294847.1), *Thermotoga maritima* (NP_227924.1), *Streptococcus pneumoniae* (YP_006700754.1), *Enterococcus faecalis* (WP_002367154.1), *Bacillus subtilis* (NP_391357.1), *Chlamydia trachomatis* (YP_000443514.1), *Porphyromonas gingivalis*.

**Figure 10. The homology modeling.**

A (cyan): Shows the simple unliganded structural model of MurA of *H. influenzae* (Hi-MurA, PDB: 2RL1).

B: Technical view of the fosfomycin interactions during inhibition of the Hi-MurA.

C (magenta): 3D refined homology structure model of wBm-MurA.

D: Superimposition of the wBm-MurA homology model with the structure model of Hi-MurA. Green stretch (Cys124) in the loop of wBm-MurA model fits with a similar green stretch (Cys115) of Hi-MurA which is the active site for fosfomycin.

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Wolbachia endosymbiont strain TRS of Wolbachia determined and eventually Neisseria meningitidis (WP_002245281.1), Wolbachia endosymbiont of Drosophila melanogaster (NP_966909.1), Wolbachia endosymbiont strain TRS of Brugia malayi (NP_198570.1), Wolbachia endosymbiont of Culex quinquefasciatus (WP_007302175.1), Rickettsia prowazekii (NP_220950.1) and Aquapafus aennis (NP_213879.1). Viewing and re-annotation were done on FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree).

wBm-MurA Activity Assay

In E. coli, MurA catalyzed the enzymatic reaction with the release of free inorganic phosphate (Pi) and this method was used for the enzymatic assays. Reaction was performed in a 96 well plate at 37°C for 10 min in a final volume of 50 μl reaction mixture containing 50 mM Tris-HCl-pH 7.5, 10 mM KCl, 1 mM dithiothreitol (DTT), 10% (v/v) glycerol, 0.6 mM UDPAG (Sigma), and 3 μg of pre-dialyzed wBm-MurA as described earlier [24,35]. 1 mM PEP (Sigma) was added to initiate the reaction. The amount of Pi released due to PEP cleavage was quantified by malachite green assay kit following manufacturer’s protocol (Cayman Chemical Company, USA). Absorbance was read at 620 nm and compared with the standard phosphate solutions. Standard curve was plotted using phosphate standards and regression analysis was performed to estimate the liberated Pi.

Inhibition of wBm-MurA Activity by Fosfomycin

Fosfomycin inhibits MurA by making a covalent adduct with the active residue. To determine its inhibitory effects on enzymatic activity, different concentrations (1 to 50 mM) of fosfomycin were pre-incubated with the assay mixture for 15 min and the enzymatic reactions were initiated by addition of 5 mM of PEP. Fosfomycin acts as a PEP analogue, therefore to examine the competitive profile with the PEP, another assay was performed in which the reaction was initiated with different concentrations (1–50 mM) of PEP and a fixed concentration (30 mM) of fosfomycin. The activity was analyzed for both the assays mentioned above except for the longer incubation time of 1 h due to expected low release of inorganic phosphate. Concentrations of all the other substrates were same as in the activity assay reaction mixture.

Homology Modeling

For examining the perseverance of the active sites and the probable interactions with fosfomycin, a homology model of the wBm-MurA was generated using Phyre2 server - Imperial College, London [37], which has different component suite for efficient modeling (multi-template modeling by Poing 1.0, template detection by HHpred 1.51 and disorder prediction by Psi-pred 2.5). The generated model was further refined through Mod-Refiner server [38]. For evaluation and validation of the model, Ramachandran Plot was generated from PROCHECK [39]. Knowledge-based energy curve for calculating z-score was done by using ProSA-web [40]. Secondary structure analysis was carried out on PDBsum server [41]. All the visualizations were performed on PyMOL (The Pyn Mol Molecular Graphics System, Schrodinger, LLC).

Statistical Analysis

All the measurements were performed in triplicate and repeated thrice to correct the trial errors. Data were analyzed with the help of statistical software GraphPad Prism (version 6.01).

Results

Sequence and Phylogenetic Analysis

Database search revealed that MurA gene exists in nearly all the bacterial species. wBm-MurA shares high degree of similarity with MurA homologues of other Wolbachia species (Figure 2). It exhibits 88.94 and 80.71% homology with MurA of Wolbachia of Drosophila melanogaster (NP_966909) and Culex molestus (CDH8571) respectively. Varying degree of similarity was observed with others bacterial species such as, 41.05% with Escherichia coli (WP_023568349), 42.24% with Vibrio cholerae (WP_001887759), 40.57% with Haemophilus influenzae (YP_005827986), 41.23% with Bordetella pertussis (WP_014906110), 43.68% with Rickettsia rickettsii (WP_012151046), 43.06% with Streptococcus pneumoniae (WP_023396463) and 35.53% with Chlamydia pecurum (YP_008583337). In addition, the residues involved in ligand interactions in MurA of E. coli (Cys115, Asp305, Lys22, Arg120, Asp369 and Leu370) [22,42] are conserved among wBm-MurA as described earlier [24,35]. All the measurements were performed in triplicate and repeated thrice to correct the trial errors. Data were analyzed with the help of statistical software GraphPad Prism (version 6.01).

Determination of Kinetic Constants

To determine the kinetic constants at optimum pH and temperature, the above enzymatic assay was performed in presence of various concentration of one substrate with a fixed concentration of another one. Varying concentration of UDPAG (0.0 to 6.4 mM) in presence of 1 mM PEP or varying concentration of PEP (0.0 to 6.4 mM) in presence of 5 mM UDPAG was used. Samples were analyzed at 620 nm for the Pi activity over a period of 10 minutes. The average activity (nmol Pi/min) was determined and eventually K_m and V_max values were estimated by fitting the curve through non-linear regression by plotting Michaelis-Menton graph [36].
**wBm-MurA was Cloned, Expressed and Purified**

The αBm-MurA gene of *Wolbachia* of *B. malayi* was successfully amplified and cloned in T/A vector. It was further sub-cloned in bacterial expression vector pET28a which was transformed into *E. coli* Rosetta strain. The recombinant construct was confirmed by restriction digestion with respective enzymes (Figure 4A) and also sequenced and it did not show any alteration in the amplified product. The soluble form of recombinant αBm-MurA was expressed with fused (His)-tag at 24°C after inducing with 0.2 mM IPTG for 22 h at 24°C (Figure 4B). Extraction and purification yielded 0.3 mg of αBm-MurA per liter of culture. The recombinant protein was localized with anti-His antibody through Western blot at ~51 kDa (Figure 4C).

**wBm-MurA is Present in the Various Life-stages of *B. malayi***

As shown in Figure 5A, the αBm-MurA gene was found to be expressed in all the major life-stages of *B. malayi* which can be related to the presence of *Wolbachia* in all these stages. The polyclonal antibodies raised against the recombinant αBm-MurA protein reacted in the blot with αBm-MurA present in the crude extracts prepared from adults, L3 and mf. A characteristic band was visualized at ~47 kDa while the purified αBm-MurA taken as a positive control protein revealed ~51 kDa band (Figure 5B). This marginal shifting in recombinant αBm-MurA protein band could be either due to presence of His-tag, or some processing of MurA in vivo, or the presence of unintentional extra residues at N and C terminus of the recombinant protein during cloning.

**Confocal Immune Localization Revealed the Endogenous Presence of wBm-MurA in Adult *B. malayi* and mf**

The polyclonal antibody raised against αBm-MurA in BALB/c mice reacted with αBm-MurA protein within the adult female worm (Figure 6). Green fluorescence signal generated by binding of fluorescein isothiocyanate (FITC) tagged secondary antibody indicated the presence of αBm-MurA expressed in *Wolbachia* within the uteri of the adult female worm and hypodermal lateral chords. The adult treated with the pre-immune BALB/c serum did not reveal such signal. *Wolbachia* and *B. malayi* DNA showed blue staining with DAPI (4',6'-diamidino-2-phenylindole). αBm-MurA was also localized as intense green signals in the isolated mf while those incubated with the pre-immune serum did not show any fluorescence (Figure 7).

**Kinetics of wBm-MurA**

The optimal pH and temperature were found to be 7.5 and 37°C respectively for enzymatic reaction (Figure 8A & B). The reaction mixture containing αBm-MurA exhibited the release of inorganic phosphate unlike controls where the recombinant enzyme was either absent or was heat inactivated (Figure S1). The enzyme activity was inhibited in presence of manganese, copper, cobalt, ferrous and zinc ions (Figure 8C). Other ions have negligible effect on the activity as compared to potassium which was found to promote activity just like sodium. Based on initial velocity and the optimal conditions, the steady state kinetics was directly calculated. The *Km* values for substrates were: UDPAG, 0.03149 mM; PEP, 0.009198 mM and the *Vmax* value for UDPAG and PEP were calculated as 1.397 mM/min/mg and 0.5378 mM/min/mg respectively (Figure 9A & B).

**Inhibitory Effect of Fosfomycin**

Fosfomycin inactivates αBm-MurA enzyme activity in a concentration dependent manner (Figure 9C). The enzymatic activity was found to be decreased by almost 2 fold at 50 mM concentration of fosfomycin at a fixed concentration of PEP. As the concentration of PEP increased, the enzymatic activity also increased (Figure 9D). This suggests that fosfomycin is a competitive inhibitor for αBm-MurA as its inhibitory effect can be overcome by increasing the concentration of PEP.

**Homology Modeling**

The three dimensional model (Figure 10C) was 100% simulated at >90% confidence by using three different bacterial MurA as structure templates viz., *E. coli* (PDB: 1UAE) [44], *H. influenzae* (PDB: 2RL1) [45] and *Enterobacter cloacae* (PDB: 1EJD) [46] selected by sequence similarity with αBm-MurA. The Ramachandran plot for a refined αBm-MurA homology model revealed that 91.9% residues were in the most favored region, 5.3% in the additional allowed region, 2.1% in the generously allowed region and 0.5% in the disallowed region (Figure S2A), thus making the model geometrically acceptable. The z-score of αBm-MurA homology modelled well within the range of experimentally determined similar X-ray solved protein structures (Figure S2B). The secondary structure analysis provides details that can be used to determine their topological features alongside the existing MurA solved structures. The final model consisted of 18 helices, 6 sheets, 24 strands, 5 beta hairpins, 1 beta bulge, 4 beta alpha beta motifs, 37 helix-helix interactions, 22 beta turns and 2 gamma turns which were consistent with the known MurA structures (Figure S2C). The model also has a surface loop containing the active site Cys124 at the specific region which was predicted to interact with the fosfomycin during enzyme inactivation. An overlay of our αBm-MurA modeled complex with the MurA of *H. influenzae* template liganded with fosfomycin (Figure 10B) reveal proper superimposition of the active site in both the structures (Figure 10D). It is therefore suggested that the inhibitor (fosfomycin) would interact with the Cys124 of αBm-MurA as in case of MurA of *H. influenzae*.

**Discussion**

*Wolbachia* was first identified in 1924 [47] however, it created enormous interest among scientific community in recent years. Up to 76% of the insect species along with other invertebrates including nematodes are reported to be infected with *Wolbachia* [48,49]. In filarial nematodes, *Wolbachia* exerts control over the host viability and fecundity [50,51]. Moreover, their role in the development of filarial pathology as a result of immune responses generated against the endosymbiont and/or their released products has also been established [52,53]. Fully annotated genome of *Wolbachia* of *B. malayi* and other known *Wolbachia* provides opportunities for delineating functional pathways involved in host-parasite interaction. Peptidoglycan (PG) biosynthesis is one of the pathways within the bacteria which are essential for both cell division and cell wall elongation processes. Till date, only two reports are available on the functional attributes of the enzymes involved in PG synthesis in *Wolbachia* [18,54]. In the current investigation, characterization of one of such enzyme i.e. UDP-N-acetylglucosamine enolpyruvyl transferase of *B. malayi* by *Wolbachia* (αBm-MurA) has been undertaken which is evolutionary conserved throughout the bacterial species. The gene sequence of αBm-MurA was successfully cloned, over-expressed and purified. We faced initial difficulty in purifying this His-tagged recombinant enzyme in soluble form since majority of the expressed protein was present in the inclusion bodies while soluble part contained very small amount of the protein. The protein present in the soluble part did not bind to Ni-NTA beads. Several attempts were made...
to bring the protein in soluble fraction such as low temperature, low IPTG concentration, purification buffers and variable pH which resulted in to marginal increase in its solubility. The recombinant enzyme was functionally active and appeared as a monodispers, low ionic strength solution. The gene expression and the protein immuno-blots, western blots, SDS-PAGE gels and the recombinant enzyme verification demonstrated the recombinant enzyme expressed in E. coli. Localization of αBm-MurA by confocal microscopy using anti-αBm-MurA antibody further demonstrated its presence in the Wolbachia within the hypodermal chord and inside the uteri of female parasite i.e. in the developing embryos and mf. All this indicates that the enzyme is abundant during various stages of development of B. malayi. αBm-MurA contains a single active EPSynthase (pfam) domain that catalyses the chemical reaction with the release of inorganic phosphate. The optimal activity assay conditions such as pH and temperature defined the catalytic profile of the enzyme and the optimal enzymatic reaction occurred at pH 7.5. Interestingly, the cytoplasmic pH in E. coli is also maintained at pH 7.5 which may possibly reflect the activity of this enzyme at similar pH [55]. αBm-MurA was active at various temperatures with optimal activity at 37°C. Several ions did not exhibit any significant effect on the enzyme activity except potassium which markedly enhanced it. The K_{m} value of αBm-MurA for the substrate UDPAG (0.03149 mM) was comparatively lower than that of other bacterial MurA (E. cloacae, 0.080 mM; S. pneumoniae, 0.244 mM; S. mutans, 0.12 mM and S. aureus, 0.168 mM) with the only exception being E. coli (0.015 mM) [56,57]. For PEP (0.009198 mM) the value was higher than that of E. coli (0.004 mM) and E. cloacae (0.0083 mM), while lower than that of S. mutans (0.086 mM) and S. pneumoniae (0.037 mM) [56,57]. On the basis of these K_{m} values, αBm-MurA appears to have lower affinity for both the substrates (UDPAG & PEP) as compared to E. coli MurA. It is difficult to explain the inferior substrate affinity in the current investigation may be attributed to the intracellular adaptation of symbiotic Wolbachia in B. malayi. Fosfomycin, a broad spectrum antibiotic irreversibly inhibits MurA. It acts as a PEP analogue and binds to MurA [21] leading to bacterial lysis and death [58]. αBm-MurA enzymatic activity was demonstrated to be reduced in the presence of fosfomycin, however, PEP at increasing concentrations limits this inhibitory effect suggesting that fosfomycin competes with the PEP for its activity. Sequence and alignment analysis of αBm-MurA with other bacterial MurA shows conservation of five important amino acid residues viz. Lys22, which participates in the formation of covalent adducts with PEP and fosfomycin [59], Cys115 and Asp369 and Leu370 which facilitate the deprotonation step [60], Asp369 and Leu370 which facilitate the deprotonation step [60], Asp369 and Leu370 which facilitate the deprotonation step [60].

**Supporting Information**

**Figure S1 Malachite Green Assay of recombinant αBm-MurA at 37°C.** The enzyme reaction mixture with boiled αBm-MurA (A), without αBm-MurA (B) and (C) with purified recombinant αBm-MurA. Each Data point represents average of the five independent measurements. Error bars represent corresponding SEM. (TIF)

**Figure S2 Quality and assessment of the αBm-MurA homology model.** A: Ramachandran plot from the PROCHECK server revealed the acceptable geometry of the αBm-MurA homology model. B: The z-score of αBm-MurA homology model. The score (−10.67) generated through ProSA-web server is within the range of experimentally similar X-ray solved MurA protein structures. C: Diagrammatic representation of the secondary structural elements of αBm-MurA. (TIF)

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**Author Contributions**

Conceived and designed the experiments: MS SMB. Performed the experiments: MS MV KM SMB. Analyzed the data: MS MV KM SMB. Contributed reagents/materials/analysis tools: SMB KM. Wrote the paper: MS SMB.
References

1. WHO (2012) Global Programme to Eliminate Lymphatic Filariasis: progress report in 2011. Weekly Epidemiological Record 37: 346–356.

2. Taylor MJ, Hoerauf A (1999) Wolbachia bacteria of filarial nematodes. Parasitol Today 15: 437–442.

3. Bandi C, McCall JW, Genchi C, Corona S, Venco L, et al. (1999) Effects of tetracycline on the filarial worms Brugia pahangi and Dirofilaria immitis and their bacterial endosymbionts Wolbachia. Int J Parasitol 29: 357–364.

4. Langworthy NG, Renz A, Mackenstedt U, Henkle-Duhrsen K, de Bronsvoort MB, et al. (2000) Macroflarial activity of tetracycline against the filarial nematode Onchocerca ochengi: elimination of Wolbachia precedes worm death and suggests a dependent relationship. Proc Biol Sci 267: 1063–1069.

5. Voronin D, Cook DA, Steven A, Taylor MJ (2012) Autophagy regulates Wolbachia populations across diverse symbiotic associations. Proc Natl Acad Sci USA 109: E1631–E1646.

6. Gheulin E, Haimanantara T, DePasse JV, Zhang X, Oskar Y, et al. (2009) Brugia nagasakiae MurA and MurZ: evolution within a human-pathogenic nematode. PLoS Biol 7: e121.

7. James CE, Hudson AL, Davey MW (2009) Drug resistance mechanisms in helmints: is it survival of the fittest? Trends Parasitol 25: 328–333.

8. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

9. Shahab M, Misra-Bhattacharya S (2012) Combating Mosquito-Borne Lym-
defilariasis. Adv Parasitol 60: 245–284.

10. Pfarr K, Hoerauf A (2005) The annotated genome of Brugia malayi: implications for neglected tropical diseases. PLoS Negl Trop Dis 3: e525.

11. McCall JW, Malone JB, Hyong-Sun A, Thompson PE (1973) Mongolian jirds as a rich source of developing larvae, adult filariae, and microfilariae. J Parasitol 59: 140–149.

12. Shrivastava N, Nag JK, Misra-Bhattacharya S (2012) Molecular characterization of NAD+−dependent DNA ligase from Wolbachia endosymbiont of lymphatic filarial parasite Brugia malayi. PLoS One 7: e41113.

13. Li Z, Carlow CK (2012) Characterization of transcription factors that regulate the type IV secretion system and riboflavin biosynthesis in Brugia malayi. PLoS One 7: e51597.

14. Foster J, Ganatra M, Kamal I, Ware J, Makarova K, et al. (2005) The mitochondrial genome of Bacteroides fragilis: insights into the genome organization of Bacteroidetes. J Bacteriol 177: 177–187.

15. Rana AK, Chandra S, Siddiqi MI, Mura-Bhattacharya S (2012) Molecular characterization of an rsmD-like rRNA methyltransferase from the endosymbiont of Brugia malayi: what it means for progress in antifilarial medicine. PLoS Med 2: e110.

16. Shrivastava N, Nag JK, Mura-Bhattacharya S (2012) Molecular characterization of NAD+-dependent DNA ligase from Wolbachia endosymbiont of lymphatic filarial parasite Brugia malayi. PLoS One 7: e41113.

17. Li Z, Carlow CK (2012) Characterization of transcription factors that regulate the type IV secretion system and riboflavin biosynthesis in Brugia malayi. PLoS One 7: e51597.

18. Lentz CS, Halls V, Hannam JS, Niebel B, Struthung U, et al. (2013) A selective inhibitor of heme biosynthesis in endosymbiotic bacteria elicits antifilarial activity in vitro. Chem Biol 20: 177–187.

19. Schierf J, Vollmer J, Lammer C, Specht S, Lentz C, et al. (2015) The ClpP peptidase of Wolbachia endobacteria is a novel target for drug development against filarial infections. J Antimicrob Chemother 68: 1790–1799.

20. Rana AK, Chandra S, Siddiqi MI, Mura-Bhattacharya S (2012) Molecular characterization of an rsmD-like rRNA methyltransferase from the endosymbiont of Brugia malayi: antifilarial activity of specific inhibitors of the enzyme. Antimicrob Agents Chemother 57: 3014–3016.

21. Vollmer W, Blanot D, de Pedro MA (2008) Peptidoglycan structure and architecture. FEMS Microbiol Rev 32: 149–167.

22. Schierf J, Vollmer J, Schneider T, Julicher K, Johnston KL, et al. (2013) Requirement of lipid II biosynthesis for cell division in cell wall-less Wolbachia endobacteria of arthropods and filarial nematodes. Int J Med Microbiol 303: 140–149.

23. Bugg TD, Walsh CT (1992) Intracellular steps of bacterial cell wall peptidoglycan biosynthesis: enzymology, antibiotics, and antibiotic resistance. Nat Rev Microbiol 2: 215–221.

24. Barrau H, Kovac A, Boniface A, Sowa M, Gober S, et al. (2008) Cytoplasmic steps of peptidoglycan biosynthesis. FEMS Microbiol Rev 32: 168–207.

25. Brown ED, Vivas EI, Walsh CT, Kopher R (1995) MurA (MurZ), the enzyme that catalyzes the first committed step in peptidoglycan biosynthesis, is essential in Escherichia coli. J Bacteriol 177: 4194–4197.

26. Du W, Brown JR, Sylvester DR, Huang J, Chalker AF, et al. (2000) Two active forms of UDP-N-acetylglucosamine:emoproyl transferase in gram-positive bacteria defined. J Biol Chem 275: 41435–41451.

27. Blake KL, Onnij AJ, Mening-Lecureux D, Henderson PJ, Bestock JM, et al. (2009) The nature of Staphylococcus aureus MurA and MurZ and approaches for detection of peptidoglycan biosynthesis inhibitors. Mol Microbiol 72: 335–343.

28. Hoerauf A, Brown JR, Lane WS, Haley TM, Ichikawa Y, et al. (1994) Kinetics, stoichiometry, and identification of the reactive thiolate in the inactivation of UDP-GlcNAc:enolpyruvyl transferase by the antibiotic fosfomycin. Biochemistry 33: 10646–10651.

29. Suzuki T, Sasaki JG (1979) A mass dissocation technique for determining infective rate of filarial vectors. Jpn J Exp Med 49: 117–121.

30. Sanger J, Landerer M, Kimmig J (1981) Filarial infections of Musca domestica and their relevance for experimental chemotherapy. Acta Trop 30: 277–281.

31. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

32. Gheulin E, Haimanantara T, DePasse JV, Zhang X, Oskar Y, et al. (2009) Brugia nagasakiae MurA and MurZ: evolution within a human-pathogenic nematode. PLoS Biol 7: e121.

33. Felsenstein J (2005) PHYLIP (Phylogeny Inference Package) version 3.6. Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948.

34. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, et al. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948.

35. Foster J, Ganatra M, Kamal I, Ware J, Makarova K, et al. (2005) The mitochondrial genome of Bacteroides fragilis: insights into the genome organization of Bacteroidetes. J Bacteriol 177: 4194–4197.

36. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

37. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

38. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

39. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

40. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

41. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

42. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

43. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

44. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

45. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

46. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

47. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

48. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

49. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

50. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

51. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.

52. Wolbachia and the physiology of filarial nematodes. PLoS Med 2: e110.
ase from Cariogenic Streptococcus mutans. Journal of Hard Tissue Biology 21: 17–24.

58. Kahan FM, Kahan JS, Cassidy PJ, Kropp H (1974) The mechanism of action of fosfomycin (phosphonomycin). Ann N Y Acad Sci 235: 364–386.

59. Samland AK, Amrhein N, Macheroux P (1999) Lysine 22 in UDP-N-acetylglycosamine enolpyruvyl transferase from Enterobacter cloacae is crucial for enzymatic activity and the formation of covalent adducts with the substrate phosphoenolpyruvate and the antibiotic fosfomycin. Biochemistry 38: 13162–13169.

60. Eschenburg S, Priestman M, Schonbrunn E (2005) Evidence that the fosfomycin target Cys113 in UDP-N-acetylglycosamine enolpyruvyl transferase (MurA) is essential for product release. J Biol Chem 280: 3757–3763.

61. De Smet KA, Kempwell KE, Gallagher A, Duncan K, Young DB (1999) Alteration of a single amino acid residue reverses fosfomycin resistance of recombinant MurA from Mycobacterium tuberculosis. Microbiology 145: 3177–3184.

62. Baum EZ, Montenegro DA, Licata I, Turchi I, Webb GC, et al. (2001) Identification and characterization of new inhibitors of the Escherichia coli MurA enzyme. Antimicrob Agents Chemother 45: 3182–3188.

63. Silver LL (2006) Does the cell wall of bacteria remain a viable source of targets for novel antibiotics? Biochem Pharmacol 71: 996–1005.