A putative low-molecular-mass penicillin-binding protein (PBP) of *Mycobacterium smegmatis* exhibits prominent physiological characteristics of DD-carboxypeptidase and beta-lactamase

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**INTRODUCTION**

Mycobacterial infections cause 1.3 million deaths globally (Murray *et al.*, 2014; World Health Organization, 2013). In general, mycobacteria are resistant to most antibiotics including beta-lactams, limiting treatment options and leading to high mortality rates (Jarlier *et al.*, 1991; Mukhopadhyay & Chakrabarti, 1997). The most commonly used antibiotics in the world since the 1940s are the beta-lactams, but most species of mycobacteria are resistant to beta-lactams due to cell wall impermeability, beta-lactamase production and presence of low-affinity penicillin-binding proteins (PBPs) (Jarlier *et al.*, 1991; Mukhopadhyay & Chakrabarti, 1997; Wang *et al.*, 2006).

Phylogenetically, PBPs and beta-lactamases share common ancestors wherein the former help in the synthesis and remodelling of peptidoglycan and the latter protect the cells by hydrolysing beta-lactam antibiotics. Beta-lactamases are either secretory (in Gram-positive) or periplasmic (in Gram-negative bacteria) whereas PBPs are a set of membrane-bound enzymes that include transglycosylases, transpeptidases, DD-carboxypeptidases (DD-CPases) and endopeptidases. The former two help to cross-link peptidoglycan while the latter two remodel the cell wall (Dzhekieva *et al.*, 2012; Ghosh *et al.*, 2008; Sauvage *et al.*, 2008). PBPs are the target of beta-lactam antibiotics, and alteration in their copy number or affinity towards beta-lactams can confer beta-lactam resistance in a bacterial cell (Hartman & Tomasz, 1984; Henry *et al.*, 2010).

dd-Carboxypeptidases (dd-CPases) are low-molecular-mass (LMM) penicillin-binding proteins (PBPs) that are mainly involved in peptidoglycan remodelling, but little is known about the dd-CPases of mycobacteria. In this study, a putative dd-CPase of *Mycobacterium smegmatis*, MSEM_2433 is characterized. The gene for the membrane-bound form of MSEM_2433 was cloned and expressed in *Escherichia coli* in its active form, as revealed by its ability to bind to the Bocillin-FL (fluorescent penicillin). Interestingly, *in vivo* expression of MSEM_2433 could restore the cell shape oddities of the septuple PBP mutant of *E. coli*, which was a prominent physiological characteristic of dd-CPases. Moreover, expression of MSEM_2433 *in trans* elevated beta-lactam resistance in PBP deletion mutants (ΔdacAdacC) of *E. coli*, strengthening its physiology as a dd-CPase. To confirm the biochemical reason behind such physiological behaviours, a soluble form of MSEM_2433 (sMSEM_2433) was created, expressed and purified. In agreement with the observed physiological phenomena, sMSEM_2433 exhibited dd-CPase activity against artificial and peptidoglycan-mimetic dd-CPase substrates. To our surprise, enzymic analyses of MSEM_2433 revealed efficient deacylation for beta-lactam substrates at physiological pH, which is a unique characteristic of beta-lactamases. In addition to the MSEM_2433 active site that favours dd-CPase activity, *in silico* analyses also predicted the presence of an omega-loop-like region in MSEM_2433, which is an important determinant of its beta-lactamase activity. Based on the *in vitro*, *in vivo* and *in silico* studies, we conclude that MSEM_2433 is a dual enzyme, possessing both dd-CPase and beta-lactamase activities.

**Abbreviations:** dd-CPases, dd-carboxypeptidases; LMM, low-molecular mass; PBP, penicillin-binding protein.

Three supplementary figures, one supplementary table and supplementary methods and references are available with the online Supplementary Material.
In mycobacteria, the beta-lactamas that have been studied include BlaC, BlaS and BlaF of Mycobacterium tuberculosis H37Rv, Mycobacterium smegmatis and Mycobacterium fortuitum, respectively (Flores et al., 2005; Wang et al., 2006). Apart from these, two cephalosporinas have been reported in M. smegmatis: a minor cephalosporinase BlaE, and a group 2e cephalosporinase (Busu et al., 1997; Flores et al., 2005). Similarly, only a few PBPs have been studied in different species of mycobacteria as compared to other organisms such as E. coli (Busu et al., 1992; Bourai et al., 2012; Goffin & Ghyysen, 1998). Work in the arena of mycobacterial PBPs began when DD-CPase activity was reported in the membrane fragments of M. smegmatis (Eun et al., 1978). Thereafter, a few high-molecular-mass (HMM) and low-molecular-mass (LMM) PBPs of mycobacteria were purified and characterized biochemically. HMM-PBPs in mycobacteria include PBP1 and PBP1* (M. leprae); PBP1* (M. tuberculosis); and PnA1 and PnA2 (M. smegmatis) (Busu et al., 1996; Blantia & Basu, 2002; Lepage et al., 1997; Patru & Pavelka, 2010). Most of the HMM-PBPs have high beta-lactam binding affinity, except PBP1 (transglycosylase and transpeptidase) of M. leprae (Busu et al., 1996). Two LMM-PBPs of M. smegmatis have been reported including 49.5 kDa (transpeptidase) and 49 kDa (DD-CPase) PBPs, both of which are inhibited by beta-lactams (Busu et al., 1992; Mukherjee et al., 1996). In M. tuberculosis RV2991, which encodes DacB2, there is also LMM-PBP involved in peptidoglycan synthesis (Bourai et al., 2012). Among all the mycobacterial LMM-PBPs listed so far, none of them possess dual (DD-CPase and beta-lactamase) activity, but in other organisms such as Pseudomonas aeruginosa (PBPP) and Staphylococcus aureus (PPB4), dual activity is reported (Navratna et al., 2010; Smith et al., 2013). In addition to DD-CPases, Ld-transpeptidases (Ldh) such as LdM2 of M. tuberculosis can cross-link peptidoglycan, and their inactivation results in the increased susceptibility to amoxicillin-clavulanate (Gupta et al., 2010).

In the present study, a putative DD-CPase, MSMEG_2433 of M. smegmatis, was characterized. Physiological and biochemical studies on MSMEG_2433 demonstrated its activity as a DD-CPase both in vitro and in vivo, as well as showing that it exhibited significant beta-lactamase activity. Finally, the structure–function relationship of MSMEG_2433 was assessed through in silico analyses, to explain the behavior behind the observed in vivo and in vitro enzymatic behaviors.

**METHODS**

**Bacterial strains, plasmids, culture media, antibiotics and chemicals.** The bacterial strains and plasmids used in the study are listed in Table 1. Plasmids pBAD18-Cam (Guzman et al., 1995) and pET-28a(+) (Novagen) were used for in vivo expression and in vitro characterization of the protein, respectively. M. smegmatis strain mcC155 (received as a gift from M. Kundu) was grown in Middle Brook 7H9 liquid and 7H11 agar medium (Sigma-Aldrich) supplemented with enrichment media, oleic acid-ADC enrichment (OADC; Difco), 0.35% (w/v) glycerol and 0.05% (w/v) Tween 80. E. coli strains were grown in LB medium (Difco) for expression analysis and purification. Chloramphenicol (20 μg ml⁻¹) and kanamycin (30 μg ml⁻¹) were used to maintain the plasmids, pBAD18-Cam and pET-28a(+), respectively. Unless otherwise indicated, all antibiotics and chemicals were purchased from Sigma-Aldrich and all DNA-modifying enzymes were purchased from New England Biolabs.

**Construction of recombinant plasmids carrying full-length and soluble forms of MSMEG_2433.** To determine the physiological role of MSMEG_2433, the corresponding full-length gene was amplified from the genomic DNA of M. smegmatis mcC155. The amplicon was cloned into a pBAD18-Cam vector at the Nhel and HindIII restriction sites to create the construct pD2433. The construct was sequenced for confirmation (Eurofin MWG Operon, Bangalore, India). Finally, E. coli SK2056-3 (ΔlacAdacC) and AM10C-1 (ΔampC) cells were transformed with pD2433. A soluble MSMEG_2433 construct, lacking 30 aa from the N-terminal and 5 aa from the C-terminal regions, was created by amplifying the truncated gene from the M. smegmatis mcC155 chromosome through PCR. The primers used were as follows: for 5'-CTCTTCATATGACCGCGCAATCCGCG-AGGTCCG-3' and rev 5'-CTCTTCATATGACCGCGCAATCCGCG-AGGTCCG-3' (Chowdhury et al., 2010). The amplicon was cloned into a pET-28a(+) vector at Nhel and HindIII sites, and the construct was named pET2433.

**Site-directed mutagenesis.** The plasmids pD2433 and pET2433 bearing cloned MSMEG_2433 gene were mutated separately by using a site-directed mutagenesis kit (Stratagene) to create plasmids pD33E75A and pET33E75A, respectively. The glutamic acid residue present at the 75th position of the omega-loop-like region of MSMEG_2433 was mutated to alanine (studies related to bioinformatics are detailed later). The forward and reverse primers used for the mutation were 5'-CCGACACCGACGTGGTACGCGTCCG-3' and 5'-CCCCCGACGTGGTACGCGTCCG-3', respectively. Finally, the single amino acid substitutions in the respective clones were confirmed by sequencing (MWG Biotech, Bangalore, India). To study in vivo characteristics, E. coli SK2056-3 (ΔlacAdacC) and AM10C-1 (ΔampC) were transformed with pD33E75A, while for in vitro analysis, BL21(DE3) cells were transformed with pET33E75A.

**Microscopic evaluation of the ectopic expression of MSMEG_2433 in an E. coli septuple PPB mutant.** E. coli septuple PBP mutant strain CS703-1 (Ghosh & Young, 2003) was transformed with the plasmid pD2433, and grown overnight in LB with chloramphenicol (20 μg ml⁻¹) at 37 °C. The overnight culture was diluted to 1% with LB containing chloramphenicol (20 μg ml⁻¹) and further grown to an OD600 of 0.2. The culture was induced with 0.02% (w/v) arabinose for 2 h at 37 °C. Samples (5 μl each) were placed onto the poly-L-lysine coated slides and subjected to phase-contrast microscopy for visualization at ×100 magnification in an Olympus IX71 fluorescence microscope with CCD camera (Olympus). Images captured were processed using CellSens Dimension software (Olympus). CS703-1 cells containing pBAD18-Cam and pPJ5 expressing E. coli PBPP5 were used as negative and positive controls, respectively. Expression of PBPP5 from pPJ5 was induced by using 0.0005% of arabinose (Nelson & Young, 2000).

**Determination of MICs.** The MIC is the concentration of antibiotic where no visible growth is detected, and it is a measure of the effectiveness of an antimicrobial agent. MIC values of the beta-lactam antibiotics, amoxicillin, penicillin, meticillin, cloxacinil, cephalxin, cefoperazone, ceftazidime, aztreonam, imipenem and amoxicillin-clavulanic acid were determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Sarkar et al., 2010; Stubbs et al., 2007). The assays were performed in microtitre plates with an assay volume of 300 μl per well and an inoculum size of 10⁶ cells per well.
The E. coli strains SK2O56-3 and AM1OC-1 with plasmids pD2433 and pD33E75A were used as experimental samples, while plasmid pBAD18-Cam in the respective strains were used as controls. The gene expression was induced by adding 0.2% (w/v) arabinose to the cultures in MH broth and incubating at 37 °C for 18 h. Bacterial growth was assessed at 600 nm by using a Multiskan Spectrum spectrophotometer (Thermo Scientific) at 460 nm. Kinetic data measurements were repeated six times and the most reproducible results are reported.

**Construction, expression and purification of sMSMEG_2433.**

The soluble form of MSMEG_2433 (sMSMEG_2433), containing 262 aa, was overexpressed in E. coli BL21(DE3) for 12 h at 4 °C for 18 h. The expressed protein with N-terminal His-Tag was purified by using Ni-NTA affinity chromatography and eluted in the presence of 100 mM and 200 mM of imidazole in 10 mM Tris/HCL, pH 8.0. The expressed protein was dialysed for 12 h at 4 °C with three changes in the same buffer without imidazole and further concentrated by using Amicon Ultra-4 devices (Millipore). The protein was stored at −80 °C with 50% glycerol for further use.

**Fluorescent penicillin labelling.**
The activity of sMSMEG_2433 was checked by labelling it with fluorescent penicillin, Bocillin-FL (Invitrogen) in 10 mM phosphate buffer, pH 7.4 at 35 °C for 30 min (Chowdhury et al., 2010; Zhao et al., 1999). Once denatured by boiling, the protein was analysed through 12% SDS-PAGE and visualized under a Typhoon FLA 7000 (GE Healthcare) as before. The band intensities of the Bocillin-FL bound protein were measured by densitometric scanning (UVP Gel documentation system). The second-order rate constant k₂/K was determined by calculating the pseudo-first-order rate constant kₐ using the equation as follows (Chambers et al., 1994; Chowdhury et al., 2010; Fontana et al., 1985): $k_{a} = -\ln[1 - \frac{[PBP_{a}]}{[PBP_{a}]}]/t$, where ‘PBPₐ’ represents the density (experimentally derived) of the fluorophore associated with sMSMEG_2433-bound Bocillin-FL at time t for a particular Bocillin-FL concentration D, and ‘PBPₐ’ shows the density at which the enzyme was saturated with Bocillin-FL. kₐ was determined by plotting the obtained $-\ln[1 - \frac{[PBP_{a}]}{[PBP_{a}]}]$ values against time and considering the steepest portion at the initial ascending part of the curve. Different kₐ values obtained were plotted against the corresponding Bocillin-FL concentrations to determine k₂/K.

**Kinetic analysis of the interaction of sMSMEG_2433 with peptide substrates.**
The d-D-Case activity of sMSMEG_2433 was determined with the artificial peptide, N²,N⁵-diacyetyl-L-Lys-d-Ala-d-Ala and the peptidoglycan-mimetic pentapeptide substrate, L-Ala-γ-d-Glu-L-Lys-d-Ala-d-Ala in 50 mM Tris/HCl, pH 8.5, with substrate concentrations varying from 0.25 mM to 14 mM in the presence of the purified enzyme (2 μg) as illustrated previously (Chowdhury et al., 2010). d-Alanine was added to the reaction in the measured and compared with a standard d-alanine using a Multiskan Spectrum-1500 Spectrophotometer (Thermo Scientific) at 460 nm. Kinetic data were analysed using the Enzyme Kinetic Module of SIGMAPLOT v12.5 (Systat Software) to determine kinetic parameters including turnover number (kₐ) and substrate concentration at half the maximum velocity (Kₘ). All kinetic experiments were repeated at least four times for consistency to obtain a mean ± SD value.

**Determination of acylation rate constant of sMSMEG_2433 with fluorescent penicillin.**
The efficiency of sMSMEG_2433 binding to penicillin was assessed by the interaction of sMSMEG_2433 with fluorescent penicillin, Bocillin-FL. The interaction was characterized by the rate of acyl–enzyme complex formation which was determined by the second-order rate constant k₂/K at saturating concentrations of the substrate (Carapito et al., 2006; Chowdhury et al., 2010, 2012; Jamin et al., 1993). The acylation rate of sMSMEG_2433 was determined by incubating the enzyme (250 μg) with various concentrations (25, 50 and 100 μM) of Bocillin-FL in 10 mM phosphate buffer, pH 7.4 and incubating for various time intervals ranging from 10 min to 1 h. The reaction was stopped by adding sample buffer containing SDS and beta-mercaptoethanol, and subsequently boiling the sample for 5 min. Bocillin-FL-labelled protein samples were run through 12% SDS-PAGE and visualized under a Typhoon FLA 7000 (GE Healthcare) as before. The band intensities of the Bocillin-FL bound protein were measured by densitometric scanning (UVP Gel documentation system). The second-order rate constant k₂/K was determined by calculating the pseudo-first-order rate constant kₐ using the equation as follows (Chambers et al., 1994; Chowdhury et al., 2010; Fontana et al., 1985): $k_{a} = -\ln[1 - \frac{[PBP_{a}]}{[PBP_{a}]}]/t$, where ‘PBPₐ’ represents the density (experimentally derived) of the fluorophore associated with sMSMEG_2433-bound Bocillin-FL at time t for a particular Bocillin-FL concentration D, and ‘PBPₐ’ shows the density at which the enzyme was saturated with Bocillin-FL. kₐ was determined by plotting the obtained $-\ln[1 - \frac{[PBP_{a}]}{[PBP_{a}]}]$ values against time and considering the steepest portion at the initial ascending part of the curve. Different kₐ values obtained were plotted against the corresponding Bocillin-FL concentrations to determine k₂/K.

**Evaluation of the deacylation rate constant of the Bocillin-FL-sMSMEG_2433 complex.**
The deacylation of the Bocillin-FL-sMSMEG_2433 complex led to the release of free sMSMEG_2433 and the cleaved Bocillin-FL. The rate constant of the deacylation reaction was established by incubating sMSMEG_2433 (50 μg) with Bocillin-FL (50 μM) for 15 min at 37 °C in 10 mM phosphate buffer, pH 7.4. An excess of Penicillin G was added and the amount of Bocillin-FL remaining bound to the protein was determined by removing the...
 aliquots at various time intervals (0, 15, 30, 60 min) as described by Thompson et al. (2002). The intensity of labelled sMSMEG_2433 was measured by densitometric scanning after running it through 12 % SDS-PAGE. The deacylation rate constant (k_d) was determined by measuring the fluorescence of the remaining sMSMEG_2433-bound Bocillin-FL (the acyl–enzyme complex) that was reduced upon time, as described previously (Chowdhury et al., 2010). Similarly, the deacylation rate constant of the bocillin FL-sMSMEG_2433E75A complex was also determined.

**Determination of kinetic parameters for the interaction between sMSMEG_2433 and other beta-lactams.** The beta-lactamase activity of the purified MSMEG_2433 was determined by measuring the changes in the absorbance at an appropriate wavelength (λ nm) of the substrate using a Kinetic Biospectrometer (Eppendorf). The following wavelengths and molar absorption coefficients (ε) were used for different beta-lactam substrates: benzylpenicillin, 232 nm (ε=560 M⁻¹ cm⁻¹); meticillin, 260 nm (ε=100 M⁻¹ cm⁻¹); oxacillin, 260 nm (ε=258 M⁻¹ cm⁻¹); cephalaxin, 260 nm (ε=7750 M⁻¹ cm⁻¹); cefotaxime, 265 nm (ε=6260 M⁻¹ cm⁻¹); ceferoperazone, 273 nm (ε=5700 M⁻¹ cm⁻¹); cefazidime, 260 nm (ε=8660 M⁻¹ cm⁻¹); nitrocef, 500 nm (ε=15 900 M⁻¹ cm⁻¹); aztreonam, 318 nm (ε=640 M⁻¹ cm⁻¹) and amoxicillin-clavulanic acid, 275 nm (ε=1070 M⁻¹ cm⁻¹). The hydrolysis reactions were conducted at 37 °C in 50 mM sodium phosphate buffer at pH 7.4 with enzyme (0.5 μM) and different beta-lactam substrates (20–500 μM). Initial reaction rates were determined from the linear portion of the hydrolysis curve. Michaelis– Menten constants (K_m and V_max) were derived using the Enzyme Kinetic Module of SIGMAPLOT v12.5 (Systat Software). Turn-over number (k_cat) and catalytic efficiency (k_cat/K_m) of the enzyme were also calculated. Each hydrolysis reaction was repeated in triplicate for accuracy. The inhibitor concentration required to reduce the maximum rate of the reaction by half (K_i value) for clavulanic acid was determined with sMSMEG_2433 using amoxicillin as a substrate. sMSMEG_2433 (0.5 μM) was incubated with different concentrations of the inhibitor clavulamic acid (0.5–50 μM) for 20 min at 30 °C and amoxicillin (20–200 μM) was added to the final assay volume of 100 μl. The K_i value for clavulanic acid was determined using the software GraphPad Prism v6.

**Comparing the penicillin hydrolysis by sMSMEG_2433 and Bacillus cereus penicillinase.** The hydrolysis of penicillin was monitored at 223 nm (ε=560 M⁻¹ cm⁻¹) in the presence of 0.5 μM of sPBP5, sMSMEG_2433 and B. cereus penicillinase (Sigma-Aldrich). The concentration of penicillin used was in the range of 20–500 μM in 50 mM phosphate buffer at pH 7.4. The hydrolysis reactions were monitored for 10 min at 37 °C in a quartz cuvette (path-length 1 cm). Initial velocities for the reactions were determined from the linear portion of the hydrolysis curve. Kinetic parameters (V_max and K_m) were derived using the Enzyme Kinetic Module of SIGMAPLOT v12.5 (Systat Software). Each hydrolysis reaction was performed in triplicate for accuracy.

**3D model building of sMSMEG_2433.** To build a 3D model of sMSMEG_2433, a restraint-based program, MODELLER 9v11, was used (Sali & Blundell, 1993). The MSMEG_2433 sequence (aa) was obtained from NCBI (accession no. ABK71878.1). However, the amino acid sequence of sMSMEG_2433 was used for model building. The crystal structure of a putative PBP homologue of M. tuberculosis, Rv2911 (PDB ID: 2BCF) which has 68% identity with sMSMEG_2433, was used as the template (Fig. S1, available in the online Supplementary Material). Alignment of target protein with the template was performed using the Multrain program that annotates protein sequence alignments with hierarchical clustering (Corpet, 1988). The 3D model was generated by aligning the sequence of sMSMEG_2433 with the corresponding template and final calculations were based on the lowest value of MODELLER objective function (Sali & Blundell, 1993). The model was subjected to energy minimization by using AMBER 03 force field implemented in YASARA. Molecular dynamics (MD) simulation was performed to optimize the intra-molecular interaction and stereochemistry of the structured model (Krieger et al., 2009). The stereo-chemical quality and folding patterns of the model were assessed by using PROCHECK (Laskowski et al., 1993) and Verify 3D (Lüthy et al., 1992). Surface topology analysis (CASTp) was used to measure the active site groove volume of sMSMEG_2433 that determines the substrate binding characteristics of the enzyme and the catalytic mechanism involved (Dundas et al., 2006). Moreover, the secondary structural elements of sMSMEG_2433 were predicted using algorithms PREDICT PROTEIN (Rost et al., 2004), PSIPRED (McCugin et al., 2000) and STRIDE (Heinig & Frishman, 2004).

**RESULTS**

**Ectopic expression of MSMEG_2433 complements morphological defects in an E. coli septuple PBP mutant**

In order to establish the DD-CPase activity of MSMEG_2433, it was essential to find out whether the enzyme is functional in vivo. To check this, we expressed MSMEG_2433 from pD2433 in the E. coli septuple PBP mutant CS703-1, which exhibits morphological deformities as reported by Denome et al. (1999) (Fig. 1a). Like E. coli PBP5, ectopic expression of MSMEG_2433 restored the uniform morphological

![Fig. 1. Complementation of E. coli septuple PBP mutant CS703-1 by ectopic expression of MSMEG_2433. All photographs are displayed at x100 magnification. (a) CS703-1; (b) MSMEG_2433 expressed in E. coli CS703-1 from pD2433; (c) PBP 5 expressed in E. coli CS703-1 from pJP5.](image-url)
Biochemical function of MSMEG_2433

phenotype in the aberrant E. coli septuple PBP mutant (Fig. 1b). Similar morphology was observed upon the regulated expression of PBP5 in CS703-1 (Fig. 1c) (Denome et al., 1999). This result confirmed that MSMEG_2433 possesses a cell-shape-maintaining property, which is likely for an enzyme with Dd-CPase activity.

Ectopic expression of MSMEG_2433 restores beta-lactam resistance of Dd-CPase mutants

E. coli SK2056-3 is devoid of Dd-CPases including PBP5 and 6, rendering it sensitive to beta-lactam agents as the Dd-CPases provide intrinsic beta-lactam resistance (Sarkar et al., 2010, 2011). To check whether expression of MSMEG_2433 in trans was able to restore the lost beta-lactam resistance in SK2056-3, we monitored the change in beta-lactam sensitivity by evaluating the MIC values of various beta-lactams (Table 2). Alteration of MICs for beta-lactams was evident in SK2056-3, except for imipenem. Among the penicillin group of antibiotics, the MIC values were higher (eightfold change) for meticillin and cloxacillin. For the cephalosporin group, increases in MIC values by eightfold for cephalaxin and two- to fourfold for cefoperazone, ceftazidime and aztreonam were detected. Overall, ectopically expressed MSMEG_2433 in SK2056-3 made the cells two- to eightfold more resistant to the beta-lactams tested than the mutant itself, which led us to assume that there was an extended spectrum beta-lactamase (ESBL)-like behaviour present in MSMEG_2433. Interestingly, in the presence of clavulanic acid (1.25 mg l⁻¹), an ESBL inhibitor, the MIC value of amoxicillin in SK2056-3/pD2433 remained unchanged as compared with the parent strain, which was likely due to complete inhibition of MSMEG_2433 by clavulanic acid. MIC values obtained exceeded the MIC values of the parent E. coli strain, which was unlikely for an enzyme with only Dd-CPase activity. Therefore, the result led us to believe that in addition to in vivo Dd-CPase activity, MSMEG_2433 might possess beta-lactamase activity. Its beta-lactamase activity was further proved by ectopic expression of MSMEG_2433 in E. coli deletion mutant of AmpC beta-lactamase, the AM10C-1 strain, which resulted in a higher level of antibiotic resistance as compared with AM10C-1 which was used as a control (Table S1).

To check whether there was any correlation with the beta-lactamase activity of MSMEG_2433 and the increased beta-lactam resistance in the host, we replaced the glutamic acid (Glu 75) present in the omega-loop like region of MSMEG_2433 with alanine. It is noteworthy to mention that a Glu residue present in the omega-loop like region is often responsible for exerting beta-lactamase activity (Adachi et al., 1991; Nemmara et al., 2011). Upon in trans expression of MSMEG_2433E75A in SK2056-3 and AM10C-1, beta-lactam resistance for the penicillin group of antibiotics was reduced by approximately 10-fold and 16-fold, respectively (data not shown), as compared with MSMEG_2433, indicating the involvement of Glu75 in maintaining intrinsic penicillin-resistance. All the above results with regards to antibiotic resistance led us to investigate the molecular nature of MSMEG_2433.

sMSMEG_2433 exhibits strong Dd-CPase activity with peptide substrates

After purification of soluble MSMEG_2433 through affinity chromatography, approximately 7–9 mg of purified protein was obtained from 1 l of culture. The molecular mass of the sMSMEG_2433 was approximately 33 kDa, as determined by 12 % SDS-PAGE (Fig. 2). However, when it was determined accurately through MALDI-TOF analysis, the molecular mass obtained was 33.3 kDa. Circular dichroism (CD) data showed that the protein was in the native conformation and the percentages of α-helix, β-sheet and random coil were 31.3 %, 19.2 % and 49.6 %, respectively (see Supplementary Methods for CD/MALDI-TOF methodology). As the enzyme was initially annotated as Dd-CPase in the NCBI database, the Dd-CPase activity of the sMSMEG_2433 was confirmed through a Dd-CPase assay as described previously (Chowdhury et al., 2010). Furthermore, detailed kinetic analyses were performed with different concentrations of the peptide substrates.

The Dd-CPase activity of sMSMEG_2433 was determined by using a peptidoglycan mimetic pentapeptide substrate, L-Ala-γ-D-Glu- L-Lys-D-Ala-D-Ala and an artificial substrate, N⁶,N⁵-diacetyl-L-Lys-D-Ala-D-Ala (Chowdhury et al., 2010, 2012). The hyperbolic plots obtained are shown in Table S1.

<table>
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<th>Antibiotic</th>
<th>SK2056-3/pBAD (mg l⁻¹)</th>
<th>SK2056-3/pBAD-MSMEG_2433 (mg l⁻¹)</th>
<th>Fold change</th>
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</table>

Table 2. Beta-lactam sensitivities (MIC) of SK2056-3/pBAD and ectopically expressed MSMEG_2433 in SK2056-3/pBAD
Fig. 3(a) and Fig 3(b), respectively. Although in both mycobacteria and E. coli the native substrate has meso-diaminopimelic acid (DAP) at the third position of the peptide, instead of L-lysine, we used an artificial substrate, N\textsubscript{a},N\textsubscript{e}-diacetyl-L-Lys-D-Ala-D-Ala, and a peptidoglycan-mimetic pentapeptide substrate, L-Ala-\textsubscript{c}D-Glu-L-Lys-D-Ala-D-Ala as the native substrate, based on their usage reported earlier (Basu \textit{et al.}, 1992; Kumar \textit{et al.}, 2012). It is noteworthy to mention that L-Lys is present at the respective position in Gram-positive peptidoglycan. Therefore, use of such substrates was justified scientifically. The alpha carbon of L-lysine or meso-DAP are both in the L-configuration in the native peptidoglycan precursors, and the overall backbone of both of the peptides has the same configuration, regardless of the side chains. Moreover, it does not change the stereochemical configuration of the cleavage site of carboxypeptidases, i.e. terminal D-Ala-D-Ala, which is true for its overall substrate binding and enzyme activity. The tripeptide, Ac\textsubscript{2}L-Lys-D-Ala-D-Ala, which is analogous to the carboxy-terminal tripeptide component of the peptidoglycan precursor, has been used as conventional substrate for DD-CPase assay for the last few decades.

During the DD-CPase assay the peptide (both tripeptide and pentapeptide) was hydrolysed and the concomitant release of terminal D-Ala was measured spectrophotometrically. The high \( k_{\text{cat}} \) and low \( K_m \) values indicated a higher \( k_{\text{cat}}/K_m \) ratio and strong affinity of the enzyme for both the artificial and pentapeptide substrates (Table 3) (Chowdhury \& Ghosh, 2011; Ghuysen, 1991; Leyh-Bouille \textit{et al.}, 1981; Nguyen-Distèche \textit{et al.}, 1982). The high \( k_{\text{cat}}/K_m \) ratio of MSMEG\textsubscript{2433} with both peptide substrates made its DD-CPase activity comparable to most PBPs that are recognized as conventional DD-CPases (Chowdhury \textit{et al.}, 2012). Therefore, the results obtained by using the tripeptide and pentapeptide substrates reflected the \textit{in vitro} DD-CPase activity of MSMEG\textsubscript{2433}.

\textbf{sMSMEG\textsubscript{2433} shows a low acylation rate but a significant deacylation rate towards penicillin}

The acylation rate constant of sMSMEG\textsubscript{2433} is \( 108.36 \pm 5.56 \text{ M}^{-1}\text{s}^{-1} \), which indicated a slower rate of acyl-enzyme complex formation as compared to other DD-CPases, i.e. PBP5 for which it has been measured as \( 800 \pm 50.3 \text{ M}^{-1}\text{s}^{-1} \) (Chowdhury \textit{et al.}, 2010, 2012). The deacylation reaction, in which hydrolysed beta-lactam is released from the covalent acyl-enzyme complex, was described as first-order rate constant \( k_{+3} \). The obtained deacylation rate, \( (k_{+3}) \), of labelled sMSMEG\textsubscript{2433} indicated a significantly high deacylation efficiency of sMSMEG\textsubscript{2433}, which is not usually observed for DD-CPase PBPs (Chowdhury \textit{et al.}, 2010, 2012). On the other hand, the deacylation rate constant of labelled sMSMEG\textsubscript{2433E75A} was \( 20 \pm 1.8 \times 10^{-5} \text{ s}^{-1} \), which was approximately 30\% lower than that of sMSMEG\textsubscript{2433}, indicating the import-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{sMSMEG_2433_analysis.png}
\caption{SDS-PAGE analysis of purified sMSMEG\textsubscript{2433}. Lane 1: protein molecular mass marker. Lane 2: uninduced pellet. Lane 3: uninduced supernatant. Lane 4: induced pellet. Lane 5: induced supernatant. Lane 6: purified sMSMEG\textsubscript{2433}. Lane 7: purified sMSMEG\textsubscript{2433} labelled with Bocillin-FL.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{DD-CPase_kinetics.png}
\caption{Hyperbolic plot of DD-CPase kinetics with (a) pentapeptide substrate; (b) artificial substrate. Data represent mean $\pm$ SD of four replicates.}
\end{figure}
ance of glutamic acid present in the omega-loop-like region during deacylation.

sMSMEG_2433 exhibits extended spectrum Class A beta-lactamase-like behaviour

As sMSMEG_2433 showed a high deacylation rate towards fluorescent penicillin, we determined the rate of hydrolysis and specificity of various beta-lactams with sMSMEG_2433. The kinetic parameters of sMSMEG_2433 with different beta-lactams are listed in Table 4. sMSMEG_2433 hydrolysed many oxyimino-cephalosporins, aztreonam and penicillins but not imipenems. According to the $V_{\text{max}}$ values obtained, the enzyme could hydrolyse the penicillin group of antibiotics at a faster rate than the cephalosporin group. Based on the $K_m$ values, the substrate specificity of sMSMEG_2433 appeared highest for penicillin and lowest for nitrocefin. Among different beta-lactams tested, the turnover number ($k_{\text{cat}}$) was higher for meticillin and oxacillin. According to the $k_{\text{cat}}/K_m$ values, the enzyme was most effective against oxacillin and least effective against nitrocefin. Substrate hydrolysis by sMSMEG_2433 was not inhibited in the presence of EDTA, indicating that it was not a metallo-beta-lactamase. Interestingly, in the presence of clavulanic acid and amoxicillin, the beta-lactamase activity of sMSMEG_2433 was competitively inhibited and the $K_i$ for clavulanic acid was 0.859 M. Therefore, based on the results obtained, sMSMEG_2433 may be termed as a class A serine beta-lactamase, in which the active site serine was involved in the hydrolytic process.

sMSMEG_2433 shows lower beta-lactamase activity than B. cereus penicillinase

Penicillin was hydrolysed by both B. cereus penicillinase and sMSMEG_2433, but not by sPBP5 (a DD-CPase). Substrate saturation curves for the above reactions were plotted using Sigmaplot v12.5 (Fig. S2). The $V_{\text{max}}$ and $K_m$ values of B. cereus penicillinase with penicillin were 2.71 μM s$^{-1}$ and 78.95 μM, respectively. The $k_{\text{cat}}$ and catalytic efficiency for the above reaction were 5.4 s$^{-1}$ and 0.0683 μM$^{-1}$ s$^{-1}$, respectively. Comparing the kinetic parameters of B. cereus penicillinase with sMSMEG_2433 (Table 4), it can be said that the rate of penicillin hydrolysis was higher for B. cereus penicillinase than sMSMEG_2433. The $K_m$ value for B. cereus penicillinase was lower compared with sMSMEG_2433, suggesting that B. cereus penicillinase had a higher affinity for penicillins compared with sMSMEG_2433 and the catalytic efficiency of penicillinase was comparatively higher for penicillin than sMSMEG_2433. Overall, the results indicated that although sMSMEG_2433 had the ability to hydrolyse penicillin efficiently, it was not as good as the B. cereus penicillinase.

The omega-loop-like region and three conserved motifs of PBPs constitute the active site of the modelled protein

The active site containing the signature motifs (STIK, SGN and KTG) lies in the cleft between the five-stranded anti-parallel $\beta$ sheet, at the adjunction site of an $\alpha$-helical cluster

### Table 3. Kinetic parameters with peptide substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{\text{max}}$ (nmol min$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$k_{\text{cat}}/K_m$ (mM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artificial substrate ($N^\alpha,N^\varepsilon$-diacetyl-$\gamma$-L-Lys-D-Ala-D-Ala)</td>
<td>1.9900 ± 0.1300</td>
<td>4.270 ± 0.4536</td>
<td>0.4773 ± 0.0300</td>
<td>0.1095 ± 0.0200</td>
</tr>
<tr>
<td>Pentapeptide (L-Ala-$\gamma$-D-Glu-L-Lys-D-Ala-D-Ala)</td>
<td>1.5500 ± 0.1600</td>
<td>4.6400</td>
<td>0.4639 ± 0.0337</td>
<td>0.3710 ± 0.0200</td>
</tr>
</tbody>
</table>

*mean ± SD.

### Table 4. Kinetic parameters with beta-lactam group antibiotics

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>$V_{\text{max}}$ (μM s$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$k_{\text{cat}}/K_m$ (μM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>2.2570</td>
<td>125.44</td>
<td>4.6400</td>
<td>0.036000</td>
</tr>
<tr>
<td>Meticillin</td>
<td>3.2700</td>
<td>152.53</td>
<td>6.7280</td>
<td>0.044100</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>2.6960</td>
<td>142.80</td>
<td>5.5400</td>
<td>0.038700</td>
</tr>
<tr>
<td>Cephalaxin</td>
<td>0.1077</td>
<td>138.13</td>
<td>0.2216</td>
<td>0.001604</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.4949</td>
<td>210.40</td>
<td>1.0180</td>
<td>0.004830</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>0.4597</td>
<td>250.73</td>
<td>0.9458</td>
<td>0.003770</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>0.2101</td>
<td>152.67</td>
<td>0.4323</td>
<td>0.002830</td>
</tr>
<tr>
<td>Nitrocefin</td>
<td>0.2037</td>
<td>578.70</td>
<td>0.4191</td>
<td>0.000724</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>1.3554</td>
<td>395.80</td>
<td>2.7880</td>
<td>0.007043</td>
</tr>
</tbody>
</table>
and the omega-loop-like region (Fig. 4a). The stereochemical arrangement of the catalytic residues in the conserved motifs forms an intermolecular network at the active site, as shown in Fig. S3. Surface topology analysis (CASTp) predicted that in the modelled protein, STIK, SGN and KTG were involved with the active site cleft formation and the active site cleft volume determined was 1461.1 Å³ (Fig. 4b). The model generated contains two ordered arrays of amino acids organized to form an omega-loop-like region. As revealed by STRIDE analysis, two long stretches of omega-loop-like region span between Asp68 to Gly84 and Gly130 to Thr152, forming an active-site pocket of the enzyme. In the model obtained, the omega-loop-like structure acts as a structural scaffold for Glu75 and projects it towards the active site cleft of the enzyme. It has been reported earlier that Glu166, a conserved residue of the omega-loop in class A beta-lactamases, plays a key role in beta-lactam hydrolysis by promoting water molecules to the active site cleft of the enzyme (Banerjee et al., 1998; Massova & Mobashery, 1998). In the present model, Glu75 present in the omega-loop-like region is predicted to play a similar role in beta-lactam hydrolysis. This prediction is supported by the results obtained from the analysis of the E75A mutant of sMSMEG_2433, wherein the deacylation efficiency had decreased by approximately 30% as compared with sMSMEG-2433.

**DISCUSSION**

**sMSMEG_2433 exhibits higher DD-CPase activity with artificial and pentapeptide substrates**

In all the PBPs possessing DD-CPase activity, the formation of an acyl enzyme intermediate is a crucial step where the lysine of an SXXK tetrad acts as a proton acceptor for nucleophilic attack by serine (Chowdhury & Ghosh, 2011; Zhang et al., 2007). A large displacement of the SXN motif from the active site impairs the DD-CPase activity in a Lys213Arg mutant of *E. coli* PBP5 (Malhotra & Nicholas, 1992). A distance of 3.02 Å between Lys206 (KTG) and Ser100 (SXN) in the sMSMEG_2433 model might favour the formation of hydrogen bonds, facilitating the DD-CPase activity of the soluble enzyme. The distance is suitable for the formation of a hydrogen bond bridge that includes a hydrolytic water molecules, peptide substrate and active site signature motifs (Chowdhury & Ghosh, 2011). Therefore, polarization of a hydrolytic water molecules onto the active site of the enzyme may lead to hydrolysis of peptide substrates with an increase in DD-CPase activity. In addition, the large active site groove volume (1461.1 Å³) of the sMSMEG_2433 model, estimated through surface topography (CASTp) analyses, can create a high degree of substrate accessibility at the catalytic site of the enzyme. These factors may be helpful in explaining the high DD-CPase activity of sMSMEG_2433 similar to other LMM-PBPs, e.g. PBP5 (Chowdhury et al., 2010).

**sMSMEG_2433 shows a slow acylation rate but significant deacylation rate towards penicillin**

Generally, the beta-lactam hydrolysis mechanism is mediated by two principal processes, acylation and deacylation (Stec et al., 2005). In acylation, the serine residue of SXXK is deprotonated to execute a nucleophilic attack at the carbon atom in the carbonyl group of the beta-lactam ring, and active site residues form a conducive environment for hydrogen bond formation around their respective amide bonds (Massova & Mobashery, 1998; Vakulenko et al., 1999). In the sMSMEG_2433 model generated, an oxyanion hole is formed at the active site comprising Ser45, which can be occupied by the beta-lactam carbonyl as suggested by Adachi et al. (1991) and Sorci et al. (2014). Hydrolytic water molecules at the active site may contribute to forming a proton bridge between the Glu75/Lys48 couple and the nucleophilic hydroxyl residue of Ser45. A high degree of beta-lactam tenancy of the oxyanion hole positions Ser45 for nucleophilic addition to the beta-lactam carbonyl group (Fisher & Mobashery, 2009; Sorci et al., 2014). The oxyanion hole enables the reaction intermediates to move towards the transition state for acylation, as reported by Wilkinson et al. (1999, 2003). However, an inter-atomic distance of 5.44 Å between Ser45 and Lys48 of STIK might be unfavourable for hydrogen bond formation, lowering the nucleophilicity of the active site Ser45 and hence decreasing the acylation rate convincingly.

It is well known that in class A beta-lactamases, the conserved glutamic acid of the omega-loop-like region promotes water molecules during the deacylation step of the beta-lactamase reaction (Adachi et al., 1991; Nemmara et al., 2011). The presence of the omega-loop-like structure in the sMSMEG_2433 model might drive the side chain of Glu75 into the active site, thereby bringing water molecules between the Glu75 and Lys48. It is important to mention...
that the decacylation of acyl enzyme intermediate involves the formation of hydrogen bridges bound with an activated water molecule to Glu75, releasing Ser45 from the beta-lactam ring in its protonated form (Stec et al., 2005). Structural analysis of the sMSMEG_2433 model predicts a high decacylation rate owing to the presence of the omega loop, which is in agreement with the high value of the decacylation rate constant of the enzyme obtained kinetically. It is worth mentioning that water access promoted by the omega-loop-like region might not be the only reason for the efficient hydrolysis of acyl-enzyme intermediates. Efficient beta-lactam hydrolysis might occur due to participation of other side chains during the decacylation step of the beta-lactam hydrolysis reaction. Hence, further research is required to find the exact mechanism behind the beta-lactamase behaviour of sMSMEG_2433. Nevertheless, the reason for the slower acylation rate but faster decacylation rate in sMSMEG_2433 can be explained from the large volume of the active site cleft. The huge active groove might reduce the chances of binding of small Bocillin-FL to the active site, but at the same time creates a more accessible area for a hydrolytic water molecule.

**Involvement of MSMEG_2433 in cell shape maintenance**

Ectopic expression of MSMEG_2433 maintains the uniform cell shape of the deformed *E. coli* septule PBP mutant, and sMSMEG_2433 exhibits DD-CPase activity with peptide substrates. Therefore, it seems likely that MSMEG_2433 might be involved in determining cell shape in *M. smegmatis* mc^2^155. However, the exact molecular basis of the DD-CPase activity of MSMEG_2433 in maintaining cell shape in the *E. coli* septule deletion mutant remains unclear. It has been previously suggested that in Gram-negative organisms, the time of peptidoglycan recycling is often associated with induction of class C beta-lactamases (Jacobs et al., 1997; Normark, 1995; Olsson et al., 1983). DD-CPases are associated with peptidoglycan remodelling events as they prevent unwanted cross-link formation, by removing the terminal D-Ala from penta-peptides (Hölter, 1998; Nicholas et al., 2003). Hence, it can be presumed that the bi-functional enzyme MSMEG_2433 is possibly expressed in mycobacteria (Gram-positive organisms) when both the DD-CPase and beta-lactamase activities are required at the same time, though little is known about the role of beta-lactamases in cell wall physiology and the exact time of their expression in the mycobacterial cell-cycle.

The detailed *in vitro*, *in silico* and *in vivo* analyses together suggest the dual nature of MSMEG_2433 in possessing both DD-CPase and beta-lactamase activity, which is the first such finding among the identified mycobacterial PBPs. The above support is suggested by the evidence from ectopic expression of MSMEG_2433 that restores the cell shape in the *E. coli* septule PBP mutant, and introduces several fold increases in beta-lactam resistance in the surrogate strain, high decacylation rate constants and beta-lactamase-like kinetic parameters. Moreover, the dual nature of MSMEG_2433 is supported by *in silico* analyses of the enzyme. Possibly, the beta-lactamase-like character of sMSMEG_2433 has evolved upon inclusion of an omega-loop-like structure, which may be correlated with the lower decacylation efficiency of the mutant MSMEG_2433 protein and the reduction in MIC values upon its expression as compared with WT protein. In addition, we can speculate that the beta-lactamase-like nature of this dual-functional PBP might partially contribute to the development of beta-lactam resistance in *M. smegmatis*.

It is noteworthy to mention that within the *M. smegmatis* genome, another gene named MSMEG_2432 is situated at a distance of 70 nt upstream to MSMEG_2433. There is a fair chance of single promoter-based regulation of both the genes, i.e. these two genes are possibly the components of an operon. However, no experimental evidence is available to establish their operon-like nature, although ‘ProOpDB’ (Taboada et al., 2012) predictions go against such a concept. As both these genes are predicted to be putative DD-CPase, it will be important to know the function(s) of MSMEG_2432, in the context of MSMEG_2433, as in the future this may further enlighten us about the physiology of DD-CPases in mycobacteria.

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