A single amino acid substitution in the Ω-like loop of E. coli PBP5 disrupts its ability to maintain cell shape and intrinsic beta-lactam resistance

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Penicillin-binding protein 5 (PBP5), a dd-carboxypeptidase, maintains cell shape and intrinsic beta-lactam resistance in E. coli. A strain lacking PBP5 loses intrinsic beta-lactam resistance and simultaneous lack of two other PBPs results in aberrantly shaped cells. PBP5 expression in trans complements the shape and restores the lost beta-lactam resistance. PBP5 has a ‘Ω-loop’-like region similar to that in class A beta-lactamases. It was previously predicted that Leu182 present in the ‘Ω-like’ loop of PBP5 corresponds to Glu166 in PER-1 beta-lactamase. Here, we studied the physiological and biochemical effects of the Leu182Glu mutation in PBP5. Upon overexpression in septuple PBP mutants, ~75% of cells were abnormally shaped and intrinsic beta-lactam resistance maintenance was partially lost. Biochemically, the purified soluble mutated PBP5 (smPBP5) retained low acylation ability for penicillin. The turnover number of smPBP5 for artificial and peptidoglycan mimetic substrates was ~10-fold less than that of the wild-type. Superimposition of the active-site residues of smPBP5 on PBP5 revealed that perturbation in the orientating key residues may explain the low turnover numbers. Therefore, we establish the involvement of Leu182 in maintaining the physiological and biochemical behaviour of E. coli PBP5.

INTRODUCTION

One of the main bacterial cell-wall components is the peptidoglycan layer, which is indispensable for cellular viability and maintenance of cellular morphology. Penicillin-binding proteins (PBPs) are enzymes required for the cross-linking of the glycan chain (transglycosylase) and peptide chain (transpeptidase). Most of these proteins have three signature motifs, namely SXXK, SXN and KTG, and these motifs are responsible for their respective enzymic activities (Ghuysen, 1991). In Escherichia coli, five PBPs (PBP4, 5, 6, DacD and AmpH) are documented as dd-carboxypeptidase (DD-CPase) (Korat et al., 1991; Baquero et al., 1996; Höljtje, 1998; Nelson & Young 2000, 2001; González-Leiza et al., 2011). These PBPs are thought to prevent the unwanted cross-link formation by removing the terminal d-alanine residue from the pentapeptide side-chain of N-acetyl muramic acid (Höljtje, 1998; Ghosh et al., 2008). PBP5 is the key DD-CPase that helps maintain a uniform cell shape in E. coli (Nelson & Young 2000, 2001; Ghosh et al., 2008). E. coli mutants lacking at least three DD-CPases including PBP5 show aberrant morphology. However, the abnormalities are abolished upon ectopic expression of PBP5 (Nelson & Young, 2000; Ghosh & Young, 2003). The ability of PBP5 to repair the cellular abnormalities depends on a stretch of 20 aa (200–219) around the KTG motif of PBP5, known as the ‘morphology maintenance domain’ (Ghosh & Young, 2003). Apart from maintaining cell shape, PBP5 also plays an important role in maintaining the intrinsic beta-lactam resistance in E. coli (Sarkar et al., 2010). The absence of PBP5 sensitizes the E. coli cell to beta-lactam antibiotics, while in trans expression of the same helps to reverse the lost resistance (Sarkar et al., 2010). The high copy number of PBP5 at the exponential phase of growth may be responsible for trapping the beta-lactam antibiotics, which prevents them from binding to the essential PBPs (Sarkar et al., 2010, 2011).

Several mutation analyses have assessed the activity of PBP5 in vitro. The PBP5 ‘74–90 loop’ deletion mutant, Gly105Asp mutant and the modified Cys115 resulted in the

Abbreviations: AcLAA, N,N’-diacetyl-Lys-d-Ala-d-Ala; AGLAA, L-Ala-γ-D-Glu-L-Lys-d-Ala-d-Ala; CD, circular dichroism; DD-CPase, dd-carboxypeptidase; PBP, penicillin-binding protein; sPBP5, soluble form of wild-type PBP5; smPBP5, soluble mutated PBP5.

One supplementary table and two supplementary figures are available with the online Supplementary Material.
loss of DD-CPase activity individually due to inefficient deacylation (Nicholas et al., 2003; Nicola et al., 2005a). The particular defects in deacylation reveal the intimate role of the SXN motif, especially Ser110, in the catalytic mechanism (Nicola et al., 2005a). In addition to the active-site residues, Asp175 also serves as a determinant of in vitro DD-CPase activity, but not for penicillin-binding activity (van der Linden et al., 1994).

PBP5 may have beta-lactamase-like activity as it shares a common ancestry with beta-lactamases (Zhang et al., 2007). Nevertheless, PBP5 in its soluble or in membrane-bound form does not show beta-lactamase activity at physiological pH (Sarkar et al., 2010), although PBP5 has an ‘Q-loop’-like region (characteristic for the beta-lactamases) similar to that of TEM-1 beta-lactamases (Zhang et al., 2007; Ghosh et al., 2008). The orientation of His151 in this loop is similar to that of Glu166 in TEM-1, which might help in accessing water molecules during deacylation of beta-lactams by PBP5 (Davies et al., 2001; Ghosh et al., 2008). It has been shown that the soluble form of PBP-A of Thermosynechococcus elongatus possesses a 50-fold increase in penicillin hydrolysis upon L158E mutation at the ‘Q-loop’-like region, but its physiological effect is as yet unknown (Urbach et al., 2008). Recently, based on in silico predictions, it has been hypothesized that the Leu153 of the ‘Q-loop’-like region in E. coli PBP5 could be the best candidate for mimicking the Glu166 residue of class A beta-lactamases (Chakraborty, 2012).

In the present study, we investigate whether the alteration in the ‘Q-loop’ could affect the physiological function of PBP5. Accordingly, the leucine present in the ‘Q-loop’ of both the membrane-bound (at position 182) and soluble (at position 153) constructs of E. coli PBP5 are replaced with a glutamic acid residue. The physiological functions of the mutant in terms of cell shape and intrinsic beta-lactam resistance are assessed. Subsequently, the alterations in functions of the mutant are verified through in vitro biochemical analyses and finally the possible reason for the altered functions are explained by using in silico molecular modelling and superimposition studies.

METHODS

Bacterial strains, reagents and growth conditions. E. coli K-12 strain CS109 and its PBP deletion mutants, CS703-1 (APBP1, 4, 5, 6, 7, AmpC and AmpH) and AM15-1 (APBP5) were used for this study (Nelson et al., 2002; Sarkar et al., 2010) (Table 1). The strains were grown in Luria–Bertani (LB) broth and Mueller–Hinton (MH) broth (HiMedia). Chloramphenicol (20 μg ml⁻¹) or kanamycin (50 μg ml⁻¹) was added wherever necessary. Unless otherwise specified, all the chemical reagents were purchased from Sigma-Aldrich.

Construction of the clones for full-length PBP5L182E and soluble mutated form of PBP5 (smPBP5). The clone for PBP5L182E was created on the membrane-bound form and smPBP5 was created on the soluble form of PBP5 by using a commercially available site-directed mutagenesis kit (Stratagene) to generate the plasmids pP5SL182E and pTS5LE, respectively (Table 1). Plasmid pP5 was used as template to make pP5SL182E, while pT7c-PBP5 was used as template to make pTS5LE. The same set of oligonucleotide primer pairs were used for each reaction: 5′-CCAGACGGTACA-TGGTGGAGATGCTATGTC-GAC₃′ and 5′-CTGACCATCAGCAT- CCTACCATGTACGTGGT₃′ (necessary mutations are indicated in italics). The gene for both the soluble form of wild-type PBP5 (sPBP5) and smPBP5 were cloned from pT7-7K to PET28a(+) at the Ndel and HindIII sites to generate pET5 and pET3LE, respectively (Table 1). Finally, the mutations in the respective clones were confirmed through sequencing by a commercial sequencing service (MWG Biotech).

Assessment of cell shape. The E. coli strain CS703-1 containing pP5SL182E was expressed by inducing with 0.005% arabinose at the early exponential phase, as previously described (Nelson & Young, 2001). Cells were then incubated for 2–4 h and treated with aztreonam at a final concentration of 5 μg ml⁻¹ (Nilsen et al., 2004). Further, cells were washed with 1× PBS (pH 7.4) and cell morphology was checked by fixing the cells onto poly-lysine-coated glass slides; live cells were subsequently visualized under an Olympus IX 71 phase-contrast microscope.

Determination of MICs. MIC values were determined by the microtitre plate dilution method as described by Sarkar et al. (2011). Total volume of MH broth in each well was made up to 300 μl by adding overnight-grown culture of the respective E. coli strains at a concentration of ~10⁵ cells per well. The antibiotics were tested by twofold step dilution (Sarkar et al., 2010) and finally the microtitre plates were incubated at 37 °C for 18 h. Bacterial growth was monitored by measuring the optical density at 600 nm using a Multiskan Spectrum spectrophotometer (model 1500; Thermo Scientific). MIC values were tested in triplicate and the experiments were repeated at least six times for accuracy.

Expression and purification of sPBP5 and its mutant (smPBP5). Expression of sPBP5 and smPBP5 was determined in E. coli BL21 Star under control of the T7 promoter. Bulk expression was obtained in the cytoplasmic fraction by growing the cells at 30 °C for 8 h in the presence of 0.05 mM IPTG. The N-terminal His-tag containing sPBP5 and smPBP5 was purified using Ni-NTA affinity chromatography. The proteins were eluted from the affinity column in the elution buffers containing 50 and 100 mM imidazole in a buffer (10 mM Tris/HCl, 300 mM NaCl buffer, pH 8.0) at 4 °C. The eluted fractions were subjected to 12 % SDS-PAGE to determine their molecular masses and their activity was assessed prior to collection. The fractions collected were dialysed for 12 h with three changes of the same buffer without imidazole and were further concentrated by using an Amicon Ultra-4 device (Millipore).

Fluorescent penicillin binding assay. The stability and activity of the PBPs were checked by labelling with Bocillin-FL (Invitrogen), a fluorescent penicillin. The membrane-bound PBPs (~300 μg) and the soluble PBPs (~25 μg) were incubated at 35 °C for 30 min with 50 and 100 μM Bocillin-FL, respectively (Zhao et al., 1999; Chowdhury et al., 2010). Furthermore, the proteins were analysed through 12 % SDS-PAGE and visualized in a Typhoon FLA 7000 scanner (GE Healthcare Bio-Sciences).

Kinetic analysis of smPBP5 with pentapeptide substrate. The DD-CPase activity exerted by both sPBP5 and smPBP5 was checked for the artificial substrate N₀,N₀-diacetyl-Lys-d-Ala-d-Ala (AcLAA) and peptidoglycan mimetic pentapeptide substrate L-Ala-g-γ-d-Glu-Lys-d-Ala-d-Ala (AGLAA) (USV custom peptide synthesis) in 10 mM Tris/HCl buffer (pH 8.0) (Chowdhury et al., 2010). The reactions were carried out in 60 μl for each soluble PBP where 2 mg of each protein was mixed with the respective peptides at a concentration range of 0.25–12 mM. The remaining volume was adjusted with
50 mM Tris/HCl, pH 8.5. The reaction mixture was incubated at 37 °C for 30 min, after which freshly prepared enzyme–coenzyme mixture (140 μl) was added. The enzyme–coenzyme mixture was prepared by mixing 50 mM Tris/HCl (pH 8.5), 0.3 mg FAD ml−1, 10 mg horseradish peroxidase ml−1 and 5 mg d-α-amino acid oxidase ml−1 at a ratio of 20:10:5:1. This resultant mixture was incubated at 37 °C for 5 min. Free β-alanine generated in this reaction was detected using the method described by Frère et al. (1976), and compared with a standard β-alanine solution using a Multiskan Spectrum-1500 spectrophotometer (Thermo Scientific) set at 460 nm. The kinetic parameters for this assay were analysed from the linear regression of the double reciprocal plot (Lineweaver & Burk, 1934).

**Assay of beta-lactamase activity of soluble enzymes.** The beta-lactam hydrolysis of sPBP5 and smPBP5 was determined with different groups of beta-lactam antibiotics in the presence of 10 mM phosphate buffer (pH 7.4). The purified proteins were incubated with the beta-lactam antibiotics at different concentrations and hydrolysis was monitored at their respective wavelengths by using an Eppendorf spectrophotometer. The molar extinction coefficients for turnover of penicillin and nitrocefin were Δε322 = −800 M−1 cm−1 and Δε480 = 15000 M−1 cm−1, respectively (Urbach et al., 2008). The deacylation rate constants (kD) of soluble enzymes were determined by plotting the value of remaining Bocillin-FL versus time (Di Guilmi et al., 2000; Chowdhury et al., 2010). In brief, the enzymes were incubated with Bocillin-FL (50 μM) at 35 °C for 30 min. Penicillin G (3 mM) was added at time 0 and fluorescence intensity was measured by removing the aliquots at certain times and subsequently denaturing the protein.

**Kinetic study of the interaction of soluble enzymes with penicillin.** The acylation rate k2/K (i.e. the rate of formation of acyl–enzyme complex with subsaturating concentration of the substrate) was obtained from a time-course study of enzyme–substrate complex formation with Bocillin-FL (Chambers et al., 1994; Chowdhury et al., 2010). sPBP5 and smPBP5 were incubated with different concentrations of Bocillin-FL (substrate) for 30, 60, 90 and 120 s. To stop the reaction, denaturing buffer was added and samples were analysed by 12% SDS-PAGE. The labelled proteins were quantified using a Typhoon FLA 7000 scanner. The deacylation rate constants (kD) of soluble enzymes were determined by plotting the value of remaining Bocillin-FL versus time (Di Guilmi et al., 2000; Chowdhury et al., 2010). In brief, the enzymes were incubated with Bocillin-FL (50 μM) at 35 °C for 30 min. Penicillin G (3 mM) was added at time 0 and fluorescence intensity was measured by removing the aliquots at certain times and subsequently denaturing the protein.

**RESULTS**

**Site-directed mutagenesis at the ‘Ω-type loop’ affects the cell shape-maintaining ability of PBP5.** Strain CS703-1, derived from *E. coli* (CS109), lacks seven non-essential PBPs, and is morphologically deformed (Nelson & Young, 2001; Ghosh et al., 2006). *In trans* expression of PBP5 in this deletion mutant restores cell shape to near normal, i.e. rod-shaped (Nelson & Young, 2000; Ghosh & Young, 2003). To check whether the alteration in the ‘Ω-type loop’ of PBP5, i.e. the Leu182Glu mutation, could affect its ability to revert the abnormal cell shape of CS703-1, the deformities of CS703-1 cells were checked upon ectopic expression of both PBP5 and PBP5L182E. Surprisingly, after complementing with PBP5 and its L182E mutant individually in CS703-1, levels of abnormalities in cell shape were

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**Table 1. List of *E. coli* strains and plasmids**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/features</th>
<th>Source or reference</th>
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<tbody>
<tr>
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<td>W1485 rpoS rph</td>
<td>C. Schnaitman</td>
</tr>
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<td>AM15-1</td>
<td>CS109APBP5</td>
<td>Sarkar et al. (2010)</td>
</tr>
<tr>
<td>CS703-1</td>
<td>CS109APBP1pBAD18</td>
<td>Nelson et al. (2002)</td>
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**Plasmid**

<table>
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<th>pP5</th>
<th><em>E. coli</em> PBP5 cloned in pBAD18 Cam</th>
<th>Kevin D. Young</th>
</tr>
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<tr>
<td>pT7-cPBP5</td>
<td><em>E. coli</em> sPBP5 cloned in pT7-7k</td>
<td>Robert A. Nicholas</td>
</tr>
<tr>
<td>pET5</td>
<td><em>E. coli</em> sPBP5 cloned in pET28a(+)</td>
<td>This study</td>
</tr>
<tr>
<td>pPS1L182E</td>
<td><em>E. coli</em> PBP5L182E cloned in pBAD18 Cam</td>
<td>This study</td>
</tr>
<tr>
<td>pTSSLE</td>
<td><em>E. coli</em> sPBP5L153E (smPBP5) cloned in pET28a(+)</td>
<td>This study</td>
</tr>
<tr>
<td>pET5LE</td>
<td><em>E. coli</em> sPBP5L153E (smPBP5) cloned in pET28a(+)</td>
<td>This study</td>
</tr>
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</table>
reduced to about 16 and 75 %, respectively, as compared with the strain without any complementation (Fig. 1, Table S1, available in the online Supplementary Material). As the genes encoding PBP5 and PBP5L182E were cloned in pBAD18, strain CS703-1 with pBAD18 was taken as a control.

To compare the cellular expression level and stability of PBP5L182E with that of wild-type PBP5, both PBP5 and PBP5L182E were expressed under control of the arabinose promoter and isolated from the membrane of CS703-1 after 12 h of incubation at 37 °C. Note that the expression level and stability of the proteins could have been checked by an immune-blotting technique, but this was not employed here because the antibodies developed against PBP5 may also cross-react with DacD, which was present in the CS703-1 cells, and it shares considerable identity with PBP5 (Potluri et al., 2010). Moreover, for assessing the stability of PBP5 and its mutant, immune-blotting might not be ideal because the PBP5 antibodies, in addition to binding to the active protein, can also bind to its inactive variant. Therefore, it was more meaningful to assess the in vivo expression for a considerable period of time and the stability of these proteins by labelling them subsequently with a substrate such as Bocillin-FL. Upon labelling equal amounts of each protein with Bocillin-FL (Fig. 2a), similar intensities of the bound Bocillin-FL were found associated with PBP5 and PBP5L182E, indicating that both the PBPs were active to the same extent during their expression in CS703-1. The overall results indicate that the mutation L182E in PBP5 almost aborted its ability to restore the normal rod shape in E. coli strain CS703-1.

**PBP5L182E can partially maintain intrinsic beta-lactam resistance in E. coli**

Deletion of PBP5 is known to sensitize E. coli cells to beta-lactam antibiotics. Ectopic complementation of PBP5 restores the lost beta-lactam resistance of AM15-1 (PBP5 deletion mutant of CS109, an E. coli K-12 strain), as PBP5 possibly traps beta-lactams (Sarkar et al., 2010). To check whether the Leu182Glu mutation can alter the intrinsic beta-lactam resistance property of PBP5, the beta-lactam sensitivity of AM15-1 was tested by expressing PBP5L182E ectopically, using 0.0005 % arabinose. Unlike the wild-type, PBP5L182E was not able to restore the lost beta-lactam resistance of AM15-1 completely (Table 2). This indicates that PBP5L182E partially lost its ability to maintain intrinsic beta-lactam resistance in E. coli. PBP5 might thus have lost its ability to exert efficient DD-CPase activity in vivo, which is required for maintaining cell shape. It also retains its ability to bind beta-lactams, although the binding ability to beta-lactams may have been reduced.

**smPBP5 exhibits poor DD-CPase activity**

To determine whether PBP5L182E had retained the ability to exert DD-CPase activity it was necessary to analyse the biochemical nature of PBP5L182E in vitro and to compare the same with the wild-type PBP5. To verify the biochemical function of PBP5L182E, its soluble form (smPBP5) was generated. Although PBP5 is a membrane-bound protein, for ease of purification, both the genes encoding sPBP5 and smPBP5 were cloned in pET28a(+) and finally the proteins were purified by Ni-NTA affinity chromatography. The concentrations of the proteins produced were ~1 mg ml⁻¹. The molecular mass of the purified proteins was ~44.0 kDa and both enzymes were active as confirmed by labelling them with Bocillin-FL and subsequent visualization by 12 % SDS-PAGE (Fig. 2b). sPBP5 and smPBP5 were checked for their DD-CPase activity. Both AcLAA and AGLAA were used (Chowdhury et al., 2012) as substrates for the assay. The turnover numbers for AcLAA and AGLAA, i.e. $k_{cat}$ value, of smPBP5 indicated ~10-fold lower product formation by smPBP5 as compared with sPBP5 (Table 3a). Therefore, the result indicates that smPBP5 has indeed lost the ability to exert in vitro DD-CPase activity for both

![Fig. 1.](image-url) Cell shape of CS703-1 was monitored at 1000 × magnification with expression of PBP5L182E. Row 1, without aztreonam; row 2, with aztreonam (5 µg ml⁻¹). From left to right: column 1, CS703-1 with pBAD18 Cam; column 2, CS703-1 with pPJ5; column 3, CS703-1 with pPJ5L182E.
AcLAA and AGLAA substrates as compared with sPBP5, consistent with the result obtained from our in vivo study. Hence, we can infer that the L182E mutation not only affects in vivo physiology of PBP5, but also affects the physiological functions in vitro.

**Penicillin acylates smPBP5 at a lower rate compared with sPBP5**

To determine the penicillin-binding efficacies of sPBP5 and smPBP5, kinetic parameters for acylation and deacylation were determined with fluorescent penicillin, Bocillin-FL, by calculating acylation and deacylation rate constants. The resultant rate of acylation (k$_2$/K) of smPBP5 for Bocillin was ~40% lower than that of sPBP5 (Table 3b), indicating that the L153E mutation affected the penicillin-binding efficiency of smPBP5. Furthermore, the rate of dissociation of penicillin from these enzymes, as determined by calculating the deacylation rate constant (k$_3$), showed an ~10% lower rate in smPBP5 than in sPBP5 (Table 3b). The L153E mutation has thus reduced the rate of penicillin binding to sPBP5 by 40% without significantly affecting its dissociation.

**The L153E mutation does not alter the beta-lactamase activity of sPBP5 in vitro**

The glutamic acid at the $\Omega$-loop of class A beta-lactamase is believed to provide water molecules for the hydrolysis of beta-lactam antibiotics (Banerjee et al., 1998; Sun et al., 2003). To determine whether the replacement of Glu at Leu153 in the purified soluble construct of PBP5 could change in beta-lactamase activity, the hydrolysis of both penicillin and cephalosporin group antibiotics were studied. However, there was no substantial difference in the hydrolysis pattern of penicillin and nitrocefin by smPBP5 as compared with sPBP5 (data not shown).

**Replacement of Leu153 to Glu disrupts the association of the key residues in the sPBP5 active site**

To understand the reason behind the lower DD-CPase activity of smPBP5, both biophysical and in silico studies were conducted. The structural model of smPBP5 was built to compare with the crystal structure of wild-type PBP5. The similarities in the secondary structure analyses of the soluble proteins by both CD and in silico prediction (Fig. S1) revealed that there was no significant change in overall folding of smPBP5 (Table 4). In addition, both the proteins (sPBP5 and smPBP5) were apparently stable within a physiological temperature range of 28–40 °C, as revealed by the thermal stability assessment through CD (Fig. S2).
However, superimposition study of the residues present in the catalytic sites revealed a drastic deviation in γ-OH of both Ser44 and Ser110 and in ε-NH2 of Lys47 (Fig. 3). In smPBP5, both γ-OH of Ser44 and ε-NH2 of Lys47 are moved away from each other by 1.90 and 2.18 Å (0.190 and 0.218 nm), respectively. Thereby, the distance between Ser44 and Lys47 has been increased (Fig. 3), preventing H-bond formation. In addition, the γ-OH of Ser110 has also deviated by 2.14 Å creating an inter-atomic distance of 4.2 Å between Ser110 and Lys213 in smPBP5 whereas it is 2.7 Å in sPBP5 (Fig. 3). As a result, the greater distance between Ser110 and Lys213 might affect H-bond formation in smPBP5. This may interfere in the decacylation of peptidoglycan mimetic substrates while exerting DD-CPase activity (Sauvage et al., 2008), although the decacylation of Bocillin is not greatly affected (Table 3b). Due to the adverse changes in the organization of the key residues in smPBP5, the groove volume of the active site has been decreased by almost 200 Å3, which is the most likely reason for its lower ability to catalyse peptidoglycan mimetic substrates (Fig. 4).

DISCUSSION

Functional limitation of the L182E mutant of PBP5

The expression of PBP5 offers sufficient DD-CPase activity to preserve the consistency in the cell morphology (Nelson & Young, 2001) and to maintain the intrinsic beta-lactam resistance property in E. coli (Sarkar et al., 2011). The partial inability of PBP5L182E to reverse the cell shape abnormalities of CS703-1 to normal suggests impairment in its DD-CPase activity (Fig. 1). To verify the physiological reason for the particular mutation, the biochemical activity of smPBP5 was examined, revealing an ~10-fold lower DD-CPase activity with the peptidoglycan mimetic pentapeptide substrate than sPBP5 (Table 3a). Therefore, it seems clear that the L182E mutation of PBP5 drastically affects the DD-CPase activity, both in vivo and in vitro.

Additionally, we find that PBP5 with the L182E mutation lacks the ability to reverse the lost intrinsic beta-lactam resistance in ΔPBP5 mutant of E. coli (Table 2). Sarkar et al. (2010) suggested that the high copy number PBP5 traps beta-lactam antibiotics to shield over other essential PBPs. Thus, if the rate of acylation of PBP5 were lowered towards beta-lactam substrates, the ability of PBP5 to act as a trap

<table>
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<th>Substrate</th>
<th>sPBP5</th>
<th>smPBP5</th>
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<tr>
<td></td>
<td>$K_m$ (mM)</td>
<td>$k_{cat}$ (s⁻¹)</td>
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<tr>
<td>AGLAA</td>
<td>0.434 ± 0.06</td>
<td>1.97 ± 0.2</td>
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<tr>
<td>AcLAA</td>
<td>0.538 ± 0.03</td>
<td>2.45 ± 0.4</td>
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Table 3. Kinetic parameters (means ± sd) of the enzymes with pentapeptides (a) and penicillin (b)

(a)

(b)

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<th>Substrate</th>
<th>sPBP5</th>
<th>smPBP5</th>
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<tr>
<td></td>
<td>$k_2/K$ (M⁻¹ s⁻¹)</td>
<td>$k_3$ (s⁻¹)</td>
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<tr>
<td>Penicillin (Bocillin-FL)</td>
<td>730 ± 17.9</td>
<td>14.9 ± 1.7</td>
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Table 4. Percentage distribution of secondary structures obtained by using different techniques

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<tr>
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<tr>
<td>sPBP5</td>
<td>22.60 28.71 48.69</td>
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<tr>
<td>smPBP5</td>
<td>21.41 27.39 51.2</td>
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Fig. 3. Superimposition of the active-site residues of sPBP5 (1NZO) over smPBP5 (the root-mean-square deviation is 0.91 Å). The residues of sPBP5 and smPBP5 are coloured in green and magenta, respectively. In smPBP5, the γ-OH of Ser110, γ-OH of Ser44 and ε-NH2 of Lys47 are twisted from their original position by 100.28°, 85.34° and 125.80°, respectively.

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<tr>
<td>smPBP5</td>
<td>21.41 27.39 51.2</td>
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</table>
would diminish. Indeed, the rate of acylation for smPBP is ~40% lower for the penicillin substrate than sPBP5 (Table 3b). Therefore, such reduction in the acylation rate might cause the partial inability of PBP5L182E to reverse intrinsic beta-lactam resistance.

The mechanism behind the inefficiency of smPBP5 to exert low DD-CPase activity, especially towards peptidoglycan mimetic substrates, lies in the packing of the active site. The distance between γ-OH of Ser44 and ε-NH₂ of Lys47 is increased in smPBP5, which may result in an unsuccessful interaction and possibly weakens the nucleophilicity of the active-site Ser44 (Davies et al., 2001; Stefanova et al., 2002). Thus, greater distance may also hinder the acylation rate of the peptidoglycan mimetic substrates (Table 3a). Moreover, Ser110, which helps in hydrolysing peptide substrate(s) by polarizing water molecules with the help of Lys213 (Nicola et al., 2005b), also undergoes deviation in smPBP5, thereby raising the distance between γ-OH of Ser110 and ε-NH₂ of Lys213. Such alteration in distance might hamper the stability of the acyl–enzyme complex during deacylation (Malhotra & Nicholas, 1992). Furthermore, as there is a reduction in the active-site groove volume by ~25% in smPBP5 compared with sPBP5, the space at the binding pocket is possibly inadequate for proper fitting of the pentapeptide substrate (Fig. 4). Hence, the release of terminal d-alanine may be less during reaction with DD-CPase, lowering the enzymic efficiency of smPBP5. However, the interaction of smPBP5 with the relatively smaller substrate Bocillin-FL is barely affected as it can fit comfortably into the groove (Table 3b). A similar effect has been reported for E. coli DacD (Chowdhury et al., 2012). Therefore, the present observation is in line with our earlier speculation that although there is no remarkable difference in overall folding of the enzyme, the activity of PBP5 towards the substrate depends not only on the orientation of the active-site residues but also on the active-site groove volume. The discrete organization of the active-site residues in smPBP5 may thus be attributed to two large deflections, one between Ser44 and Lys47 and another between Ser110 and Lys213, which adversely affect their orientation and the active-site groove volume. This in turn impedes enzymic activity both in vivo and in vitro. We conclude that Leu182 plays a pivotal role in maintaining the physiology of E. coli PBP5.

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Crystal structure of wild-type penicillin-binding protein 5

Penicillin binding protein 5

dacB

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