PBP5, PBP6 and DacD play different roles in intrinsic \(\beta\)-lactam resistance of *Escherichia coli*

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*Escherichia coli* PBP5, PBP6 and DacD, encoded by *dacA*, *dacC* and *dacD*, respectively, share substantial amino acid identity and together constitute \(\sim 50\%\) of the total penicillin-binding proteins of *E. coli*. PBP5 helps maintain intrinsic \(\beta\)-lactam resistance within the cell. To test if PBP6 and DacD play similar roles, we deleted *dacC* and *dacD* individually, and *dacC* in combination with *dacA*, from *E. coli* 2443 and compared \(\beta\)-lactam sensitivity of the mutants and the parent strain. \(\beta\)-Lactam resistance was complemented by wild-type, but not \(\ddot{d}\)-carboxypeptidase-deficient PBP5, confirming that enzymic activity of PBP5 is essential for \(\beta\)-lactam resistance. Deletion of *dacC* and expression of PBP6 during exponential or stationary phase did not alter \(\beta\)-lactam resistance of a *dacA* mutant. Expression of DacD during mid-exponential phase partially restored \(\beta\)-lactam resistance of the *dacA* mutant. Therefore, PBP5 \(\ddot{d}\)-carboxypeptidase activity is essential for intrinsic \(\beta\)-lactam resistance of *E. coli* and DacD can partially compensate for PBP5 in this capacity, whereas PBP6 cannot.

**INTRODUCTION**

*Escherichia coli* encodes 12 penicillin-binding proteins (PBPs), four of which (PBP4, -5, -6 and DacD) have been reported to have 50-carboxypeptidase (50-CPase) activity (Höltje, 1998; Denome *et al.*, 1999; Ghosh *et al.*, 2008). All these proteins are low molecular mass (LMM) PBPs (Ghuysen, 1991) and are dispensable for survival in *vitro* (Denome *et al.*, 1999). PBP5 and PBP6 are 62% identical at the amino acid level and share 48 and 47% identity with DacD, respectively (Baquero *et al.*, 1996). It has been suggested that these proteins might have similar physiological functions based upon their homology but only PBP5 appears to play a prominent role in maintenance of cell shape (Nelson & Young, 2003; Nelson *et al.*, 2002; Ghosh & Young, 2003). PBP5, PBP6 and DacD are primarily expressed in early exponential, stationary and mid-exponential phases, respectively (Buchanan & Sowell, 1982; Baquero *et al.*, 1996; Santos *et al.*, 2002), which may explain the different roles of these proteins in maintenance of cell shape. The number of PBP5 molecules is also two- to threefold higher than the number of PBP6 molecules in exponentially growing cells (Spratt, 1977; Dougherty *et al.*, 1996).

It has been observed recently that loss of PBP5 makes the cells susceptible to a wide range of \(\beta\)-lactam antibiotics and this can be reversed by expression of PBP5 *in trans* (Sarkar *et al.*, 2010). Similarly, deletion of some PBPs from *E. coli* increases susceptibility to \(\beta\)-lactam antibiotics (Nishimura *et al.*, 1980; Pepper *et al.*, 2006; Georgopapadakou, 1993). However, loss of PBPs may also increase \(\beta\)-lactam resistance indirectly. For example, inactivation of PBP4 triggers overproduction of the AmpC \(\beta\)-lactamase in *Pseudomonas aeruginosa* (Moya *et al.*, 2009).

Based upon the homology of PBP5, PBP6 and DacD we hypothesized that these proteins might play similar roles in \(\beta\)-lactam sensitivity. To test this, we compared the \(\beta\)-lactam sensitivities of PBP6 and DacD mutants individually, and in the presence and absence of PBP5. Deletion of PBP6 and DacD did not change the \(\beta\)-lactam sensitivity of *E. coli*, although expression of DacD *in trans* could partially compensate for the loss of PBP5.

**METHODS**

**Bacterial strains and antibiotics.** Bacterial strains used in this study were derived from *E. coli* 2443 and are listed in Table 1. CS18-2K was a gift from Professor Kevin D. Young, University of Arkansas Medical School, AR, USA. The strains were grown in Luria–Bertani (LB) broth, agar (Hi-Media), Muller-Hinton (MH) broth (Hi-Media) and M9-Glucose minimal medium, supplemented with the required amino acids (arginine, proline, leucine and threonine) and thiamine. Chloramphenicol (20 \(\mu\)g ml\(^{-1}\)), kanamycin (50 \(\mu\)g ml\(^{-1}\)), tetracycline (25 \(\mu\)g ml\(^{-1}\)) and ampicillin (50 \(\mu\)g ml\(^{-1}\)) were added where necessary. Unless otherwise specified, chemicals and reagents were purchased from Sigma.

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**Abbreviations:** 50-CPase, \(\ddot{d}\)-carboxypeptidase; LMM, low molecular mass; PBP, penicillin-binding protein.
PCR product was digested with NheI positive clones were confirmed by DNA sequencing (MWG Biotech). Cam. Transformants were selected on chloramphenicol plates and primer sequence) and ligated into the multiple cloning site of pBAD18-RP4 ParA resolvase (Sarkar et al., 1995). Labeling with Bocillin FL (Zhao et al., 2004), absence of corresponding PBPs in the mutants was confirmed by MICs on LB kanamycin agar. Deletions were confirmed by PCR and the presence of chloramphenicol plates and positive clones were confirmed by DNA sequencing (MWG Biotech).

Site-directed mutagenesis of PBP5. The active-site serine (Ser44) of PBP5 was replaced with alanine (AGC to GCC) through site-directed mutagenesis of the parent plasmid pPJ5, using a QuikChange mutagenesis kit (Stratagene) and oligonucleotide primers 5'-GGGAGGATCCGCGGCGGATCCGGGCGGCGGATCCTGCCGCTGACCAAGAAAATAGTGC-3' and 5'-GAGGAGGATCCGCGGCGGATCCGGGCGGCGGATCCTGCCGCTGACCAAGAAAATAGTGC-3', according to the manufacturer's instructions to produce plasmid pAG544-1 (bold/italic type indicates changed nucleotides).

MIC testing. MICs were determined by a micro-broth dilution method following CLSI guidelines (CLSI, 2007) and as we have described previously (Sarkar et al., 2010; Sarkar & Ghosh, 2008) in 96-well plates in a total volume of 300 μl per well. Wells were inoculated with ~10^5 c.f.u. per well, and antibiotics were tested at concentrations from 1 to 0.006 μg ml⁻¹ by twofold step dilution (Stubbs et al., 2007). The plates were incubated for 18 h at 37 °C and growth of the cultures was monitored by measuring OD₆₀₀ using a Multiskan Spectrum-1500 Spectrophotometer (Thermo Scientific). MIC tests were performed in triplicate and were repeated at least six times.

Complementation experiments. MICs for SK106-3, SK10D-3, SK2056-3, SK205D-3 and the triple mutant SK306D-3 were determined after complementation with plasmids encoding PBP5 (pPJ5), PBP6 (pAG6), DacD (pAGDacD) and mutated PBP5 (pAG544-1), all of which were constructed in the pBAD18-Cam backbone (see Table 1). Prior to MIC assays, cells were grown in the presence of chloramphenicol and 0.2 % glucose to prevent expression from the pBAD promoter. The cells were then washed twice in MH Broth, resuspended in MH broth and treated with a varying amounts of arabinose (0.0005–0.2 % w/v) to induce PBP expression from the plasmids (Fig. 1).

Growth kinetics and time-kill assays. These assays were performed as described previously, with minor modifications (Kumar et al., 1999).
RESULTS

β-Lactams are substrate analogues of the d-alanyl-d-alanine moiety of muropeptides (Park & Strominger, 1957), and kill bacteria by inactivation of high molecular mass PBPs. Inactivation or alteration of the affinities of some HMM PBPs for their substrates can impact sensitivity to β-lactams (Gotoh et al., 1990; Gerrits et al., 2002, 2006; Ghosh et al., 1998). Loss of PBP5 increases E. coli β-lactam sensitivity by four- to eightfold, indicating that LMM PBPs contribute to this phenomenon (Sarkar et al., 2010). PBP6 and DacD share structural similarities with PBP5 so we speculated that these proteins might play similar roles in β-lactam sensitivity.

Loss of PBP6 or DacD alone or in a PBP5 mutant background does not affect β-lactam sensitivity

To test if PBP6 or DacD contribute to intrinsic β-lactam resistance, we compared the β-lactam sensitivities of PBP5, PBP6 and DacD single and multiple mutant strains. PBP5 mutants had four- to eightfold higher sensitivity to β-lactams, consistent with our previous findings (Sarkar et al., 2010). β-Lactam resistance of PBP5 mutants was restored by the expression of wild-type, but not a DD-CPase deficient PBP5 (Ser44 to Ala44), in trans. In contrast, the phenotypes of single PBP6 and DacD mutant strains were indistinguishable from wild-type (Table 2). To test if PBP5 was dominant over and masked contributions of PBP6 and/or DacD, PBP6 and DacD were deleted singly, and in combination, from the PBP5 mutant and the β-lactam resistance phenotypes of these strains were compared. PBP5, PBP5/6 and PBP5/DacD and PBP5/6/DacD mutants yielded almost identical results. These results indicate that DD-CPase activity of PBP5, but not of PBP6 or DacD, is critical to the intrinsic β-lactam resistance of E. coli.

Overexpression of DacD, but not PBP6, partially restores β-lactam sensitivity of PBP5 mutants

DacD and PBP6 are expressed at lower copy numbers than PBP5. This suggests that these proteins might not have been expressed at sufficient levels to affect β-lactam resistance. To test this, we overexpressed PBP5, DacD and PBP6 from inducible multi-copy plasmids in PBP5 mutant strains and assessed β-lactam resistance. PBP5 restored β-lactam resistance of PBP5/6 and PBP5/DacD mutants, whereas overexpression of PBP6 had no effect. DacD partially (25–50 %) restored β-lactam resistance of PBP5/6 and PBP5/DacD/DacD mutants. These results suggest PBP5 and DacD might have related roles in β-lactam resistance.

Expression of PBP6 during stationary phase does not restore β-lactam resistance of PBP5 mutants

PBP5, PBP6 and DacD expression are maximal at different points in E. coli development (Ghosh et al., 2008). For example, PBP6 expression increases in stationary phase (Santos et al., 2002) whereas PBP5 levels peak during exponential growth. Thus, we tested if expressing these PBPs in different growth phases affected β-lactam resistance (Fig. 2). PBP expression from plasmids was induced by adding arabinose to the cultures in early exponential, mid-exponential or stationary phase. Subinhibitory levels of ampicillin (0.25 μg ml⁻¹) or cefadroxil (2 μg ml⁻¹) were used to assess growth inhibition. Expression of PBP5 in trans in early exponential phase completely restored β-lactam resistance of PBP5 mutants. DacD partially restored β-lactam resistance when it was expressed in mid-exponential phase. Expression of PBP6 in any growth phase failed to affect β-lactam resistance. These results suggest that PBP5 and DacD mediate β-lactam resistance of E. coli in different growth phases.

Different β-lactam resistance phenotypes of PBP mutants are not related to growth rate

Growth rate is an important determinant of β-lactam sensitivity because these compounds primarily affect cells during exponential phase (Anwar et al., 1990). We suspected that minor variations in growth rate of different LMM PBP mutants might be reflected by increased
sensitivity to subinhibitory concentrations of $\beta$-lactams. To test this, we compared the growth rates of LMM PBP mutant strains in the presence of subinhibitory concentrations (half, quarter and eighth of the MIC) of ampicillin and cefadroxil. Growth inhibition of all of the LMM PBP mutants was proportional to the concentration of antibiotics and was not affected by genotype (Supplementary Fig. S1, available with the online version of this paper). Similar growth rates in all the mutants indicated that differences in intrinsic $\beta$-lactam resistance of PBP5, PBP6 and DacD mutants are not related to growth rate.

**Analysis of killing kinetics indicate that PBP5, PBP6 and DacD have different roles in intrinsic $\beta$-lactam resistance**

MIC assays can obscure differences in the speed of killing by antibiotics, so we performed killing kinetics assays using a range of antibiotic concentrations near the MIC of the parent strain to test whether LMM PBP mutants had different phenotypes. Ampicillin and cefadroxil were chosen as representatives for penicillins and cephalosporins in these assays (see Methods and Kumar *et al.*, 2008 and MacLeod *et al.*, 2009 for details). The general patterns observed in the experiments above were duplicated in these assays. Briefly, expression of PBP5 fully and DacD partially complemented $\beta$-lactam resistance in PBP5 mutants whereas PBP6 had no effect (Supplementary Fig. S2, available with the online version of this paper).

**DISCUSSION**

We showed that loss of PBP6 and DacD from wild-type *E. coli* does not affect the $\beta$-lactam sensitivity of PBP5 mutants and that expression of PBP5 in trans fully restores wild-type $\beta$-lactam resistance to a PBP5/6/DacD triple

**Table 2. $\beta$-Lactam sensitivities (MIC) of *E. coli* 2443 and the PBP mutants**

All concentrations are in $\mu$g ml$^{-1}$.

<table>
<thead>
<tr>
<th><em>E. coli</em> strain</th>
<th>Amoxicillin</th>
<th>Piperacillin</th>
<th>Ampicillin</th>
<th>Penicillin G</th>
<th>Cefadroxil</th>
<th>Cefalexin</th>
<th>Cefalothin</th>
<th>Cefaclor</th>
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<tr>
<td>2443</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>125</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>AG1O5-1</td>
<td>1</td>
<td>0.25</td>
<td>0.5</td>
<td>32</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>AG1O5-1 + PBP5</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>125</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>SK1O6-3</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>125</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>SK1OD-3</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>125</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>4</td>
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<tr>
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<td>4</td>
</tr>
<tr>
<td>SK2O56-3</td>
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<td>2</td>
<td>2</td>
<td>0.5</td>
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<tr>
<td>SK2O56-3 + PBP5</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>125</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>4</td>
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<tr>
<td>SK2O56-3 + PBP6</td>
<td>1</td>
<td>0.25</td>
<td>0.5</td>
<td>32</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>SK2O56-3 + DacD</td>
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<td>1</td>
<td>64</td>
<td>8</td>
<td>4</td>
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<tr>
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<td>0.25</td>
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<td>32</td>
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<td>2</td>
<td>2</td>
<td>0.5</td>
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<tr>
<td>SK2O5D-3 + DacD</td>
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<td>0.5</td>
<td>1</td>
<td>64</td>
<td>8</td>
<td>4</td>
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<td>2</td>
</tr>
<tr>
<td>SK3O6D-3</td>
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<td>2</td>
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<td>0.5</td>
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<tr>
<td>SK3O6D-3 + DacD</td>
<td>2</td>
<td>0.5</td>
<td>1</td>
<td>64</td>
<td>8</td>
<td>4</td>
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<td>2</td>
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**Fig. 2.** Phase expression studies in the presence of (a) ampicillin (0.25 $\mu$g ml$^{-1}$) and (b) cefadroxil (2 $\mu$g ml$^{-1}$). The strains are *E. coli* 2443 (in the absence of antibiotic; –), *E. coli* 2443 (●), SK2O56-3 (○), SK2O56-3 expressing PBP5 (■), SK2O56-3 expressing PBP6 (▲) and SK2O56-3 expressing DacD (★).
mutant strain. β-Lactam sensitivity of PBP5 mutants is related to PBP5 DD-CPase activity because catalytically inactive PBP5 did not complement this mutation. Although expression of PBP6 in any growth phase failed to restore resistance to a PBP5 mutant, expression of DacD during mid-exponential phase partially restored β-lactam resistance. Collectively, these results indicate that PBP5 mediates intrinsic β-lactam resistance in wild-type E. coli whereas PBP6 and DacD do not. Separately, the results imply that the cellular functions of DacD and PBP5 may be more closely related to one another than they are to PBP6.

A few hypotheses could explain the different roles of PBP5 and PBP6. One possibility is that PBP5 and PBP6 have similar enzymic functions but that loss of PBP6 fails to effect β-lactam resistance because the protein is not present when β-lactam antibiotics act against the cells. Consistent with this, PBP6 accumulates in stationary phase cells whereas expression of PBP5 is maximal in exponential phase cells. However, overexpression of PBP6 in exponential phase cells did not affect β-lactam resistance. Thus, if PBP5 maintains resistance by ‘trapping’ β-lactam antibiotics, as we have previously proposed (Sarkar et al., 2010), PBP6 does not seem to be able to perform this function, regardless of when it is expressed. A caveat is that if other co-factors that modulate PBP6 activity are also expressed in stationary phase, PBP6 expressed during exponential phase might not be fully active. Biochemical and in silico analyses suggest other explanations for the different roles of PBP5 and PBP6. PBP5 and PBP6 both bind β-lactams efficiently. The acylation rate ($k_2/K$) of PBP6 for β-lactams and some small DD-CPase substrates, such as Ac2-L-Lys-D-Ala-D-Ala, is actually slightly higher than that of PBP5. However, PBP6 cannot bind the peptidoglycan mimetic pentapeptide substrate (Chowdhury et al., 2011). This has been linked to differences in the microarchitecture of the active sites of these proteins (Chowdhury & Ghosh, 2011). Also, PBP5 deacylates β-lactam (Bocillin FL) more rapidly than does PBP6 (Chowdhury et al., 2010). Thus, the effective pool of PBP5 available to bind β-lactams could be larger than that of PBP6 even if their protein copy numbers in cells are similar.

DacD partially rescued β-lactam resistance of PBP5 mutants, suggesting that these proteins have related functions in vivo. Baquero et al. (1996) previously proposed that DacD compensates for other DD-CPases in their absence and our observations support this interpretation. DacD can utilize pentapeptide muropeptides and maintained 5% of the residual DD-CPase activity of an E. coli strain from which all of the other DD-CPases had been deleted (Baquero et al., 1996). That 5% might be sufficient to impart normal physiological function (Waxman & Strominger, 1983). Unfortunately, little is known about the structure, substrate specificity and/or kinetics of this enzyme at present (Nelson et al. 2002; Ghosh et al., 2008). We hope to address these questions in the future because DacD homologues are present in a wide variety of pathogenic Gram-negative bacteria and our data suggest that these proteins could play unappreciated roles in β-lactam resistance.

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