PBP5, PBP6 and DacD play different roles in intrinsic β -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 ~50 % of the total penicillin-binding proteins of *E. coli*. PBP5 helps maintain intrinsic β -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 β -lactam sensitivity of the mutants and the parent strain. β -Lactam resistance was complemented by wild-type, but not DD-carboxypeptidase-deficient PBP5, confirming that enzymic activity of PBP5 is essential for β -lactam resistance. Deletion of *dacC* and expression of PBP6 during exponential or stationary phase did not alter β -lactam resistance of a *dacA* mutant. Expression of DacD during mid-exponential phase partially restored β -lactam resistance of the *dacA* mutant. Therefore, PBP5 DD-carboxypeptidase activity is essential for intrinsic β -lactam resistance of *E. coli* and DacD can partially compensate for PBP5 in this capacity, whereas PBP6 cannot.

Received 17 October 2010

Revised 12 April 2011

Accepted 24 June 2011

INTRODUCTION

Escherichia coli encodes 12 penicillin-binding proteins (PBPs), four of which (PBP4, -5, -6 and DacD) have been reported to have DD-carboxypeptidase (DD-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, 2001; 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 β -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 β -lactam antibiotics (Nishimura *et al.*, 1980; Pepper *et al.*, 2006; Georgopadakou, 1993). However, loss of PBPs may also increase β -lactam resistance indirectly. For example, inactivation of PBP4 triggers overproduction of the AmpC β -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 β -lactam sensitivity. To test this, we compared the β -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 β -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 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$), tetracycline (25 $\mu\text{g ml}^{-1}$) and ampicillin (50 $\mu\text{g ml}^{-1}$) were added where necessary. Unless otherwise specified, chemicals and reagents were purchased from Sigma.

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Abbreviations: DD-CPase, DD-carboxypeptidase; LMM, low molecular mass; PBP, penicillin-binding protein.

Two supplementary figures are available with the online version of this paper.

Table 1. *E. coli* strains and plasmids used in the study

Strain/plasmid	Genotype/relevant feature	PBP(s) deleted	Source/reference
<i>E. coli</i> strains			
KL743	MG1655 <i>zba-3000::Tn10</i> LAM [−] <i>rph</i> [−]	None	Goldberg (CGSC 6213)
KM32	<i>leuB6 proA2 thr-1 argE3 lacY1 galK2 ara-14 xyl-5 thi-1 rpsL31 mtl-1 tsx-33 supE44 Δ(recC ptr recB recD)::P_{lac}-bet exo (Cam^R)</i>	None	Murphy (1998)
S17-1(λ <i>pir</i>)	<i>recA thi pro hsdR</i> [<i>r_B[−] m_B⁺</i>]	None	Nilsen <i>et al.</i> (2004)
CS18-2K	CS109 (W1485 <i>rpoS rph</i>) Δ <i>dacD::Kan</i>	DacD	Nilsen <i>et al.</i> (2004), K. D. Young, University of Arkansas Medical School
2443	<i>thr-1 leuB6 Δ(gpt-proA)66 argE3 thi-1 rfb_{O8} lacY1 ara-14 galK2 xyl-5 mtl-1 mgl51 rpsL31 kdgK51 supE44 Δ(recC ptr recB recD)::P_{lac}-bet exo (Cam^R)</i>	None	Sandlin <i>et al.</i> (1996)
2443T	2443 <i>zba-3000::Tn10</i> (Tet ^R) (By P1 transduction from KL743)	None	Ghosh & Young (2005)
AG456-1K	2443 Δ <i>dacB ΔpbpG dacA dacC::Kan</i>	4, 5, 7, 6	Nilsen <i>et al.</i> (2004)
AG1O5-1	2443 Δ <i>dacA</i>	5	Sarkar <i>et al.</i> (2010)
SK1O6-3	2443 Δ <i>dacC</i>	6	This work
SK1OD-3	2443 Δ <i>dacD</i>	DacD	This work
SK2O56-3	2443 Δ <i>dacA ΔdacC</i>	5, 6	This work
SK2O5D-3	2443 Δ <i>dacA ΔdacD</i>	5, 6	This work
SK3O6D-3	2443 Δ <i>dacA ΔdacC ΔdacD</i>	5, 6, 6	This work
Plasmids			
pJMSB8	[RP4::2-Tc::Mu-Km::Tn7] λ <i>pir</i> lysogen		Nilsen <i>et al.</i> (2004)
pBAD18-Cam	Expression vector with arabinose-inducible promoter		Guzman <i>et al.</i> (1995)
pPJ5	<i>dacA</i> cloned in pBAD18-Cam		Nelson & Young (2003)
pAG6	<i>dacC</i> cloned in pBAD18-Cam		Ghosh & Young (2003)
pAG544-1	Serine 44 of PBP5 is mutated to alanine		This work
pAGDacD	<i>dacD</i> cloned in pBAD18-Cam		This work

Construction of the PBP deletion mutants. PBP deletion mutants were constructed by P1 transduction from *E. coli* 2443T (Denome *et al.*, 1999). *E. coli* 2443T was previously constructed by inserting a tetracycline marker (Ghosh & Young, 2005). The *dacA* mutant was constructed as described previously (Sarkar *et al.*, 2010). The PBP6 deletion mutant *E. coli* 2443 (SK1O6-3) was constructed by transducing the Δ *dacC::res-npt-res* (Kan^R) allele from AG456-1K (Nilsen *et al.*, 2004), and the *DacD* deletion mutant (SK1OD-3) was produced by transducing Δ *dacD::res-npt-res* (Kan^R) allele from CS18-2K (Nilsen *et al.*, 2004) (Table 1). Double and triple mutants were constructed from parents from which the *res-npt-res* cassette had been resolved (Sarkar *et al.*, 2010) by conjugation with pJMSB8 to transiently express RP4 ParA resolvase (Sarkar *et al.*, 2010; Nilsen *et al.*, 2004; Kristensen *et al.*, 1995). Kanamycin-sensitive isolates were identified by streaking on LB kanamycin agar. Deletions were confirmed by PCR and the absence of corresponding PBPs in the mutants was confirmed by labelling with Bocillin FL (Zhao *et al.*, 1999) (Fig. 1).

Cloning. The *dacD* gene was amplified by PCR from *E. coli* 2443 genomic DNA using oligonucleotide primers 5'-CTCTCTGCTAGC-AGGAGGAATTCACCATGAAACGCCGTCTTATTATTGC-3' and 5'-CTCTCTAAGCTTTCAGGCCTTATGGTGGAATAATCACT-CAGGC-3'. A Shine–Dalgarno sequence (in bold) was incorporated in the forward primer. PCR conditions were 5 min at 95 °C, followed by 30 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. The PCR product was digested with *NheI* and *HindIII* (underlined in the primer sequence) and ligated into the multiple cloning site of pBAD18-Cam. Transformants were selected on 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'-CGCCGCGATCCTGCCGCCCTGACCAAAATGATG-3' and 5'-CATCATTTTGGTCAGGGCGGCAGGATCGCGGCG-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 μ l per well. Wells were inoculated with $\sim 10^5$ c.f.u. per well, and antibiotics were tested at concentrations from 1 to 0.006 μ g 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 SK1O6-3, SK1OD-3, SK2O56-3, SK2O5D-3 and the triple mutant SK3O6D-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.*,

Fig. 1. Labelling of PBP5 with fluorescent penicillin. Isolated membranes (protein content $\sim 300 \mu\text{g}$) were labelled with Bocillin FL ($50 \mu\text{M}$) at 30°C for 30 min, solubilized with 1 % Sarkosyl and analysed by using 12 % SDS-PAGE ($100 \mu\text{g}$ protein per lane). Lanes: 1, *E. coli* 2443; 2, SK2O56-3; 3, SK2O56-3 expressing PBP5 from the arabinose promoter in the presence of an optimal concentration of arabinose (0.0005%) at exponential phase; 4, SK2O56-3 expressing PBP6 from the arabinose promoter with arabinose (0.2%) at stationary phase; 5, SK2O56-3 expressing DacD from the arabinose promoter with arabinose (0.001%) at mid-exponential phase; 6, SK2O56-3 complemented with pAG544-1 with arabinose (0.2%).

2008; MacLeod *et al.*, 2009). Briefly, cells (10^5 c.f.u. ml^{-1}) were inoculated into MH broth containing ampicillin or cefadroxil (representative antibiotics from penicillin and cephalosporin groups, respectively). The cultures were incubated at 37°C and viable cells were enumerated by plating on LB and MH agar.

RESULTS

β -Lactams are substrate analogues of the D-alanyl-D-alanine moiety of mucopeptides (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 PBP5/6/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 ($0.25 \mu\text{g ml}^{-1}$) or cefadroxil ($2 \mu\text{g ml}^{-1}$) 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

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

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

<i>E. coli</i> strain	Amoxicillin	Piperacillin	Ampicillin	Penicillin G	Cefadroxil	Cefalexin	Cefalothin	Cefaclor
2443	4	1	2	125	16	8	16	4
AG1O5-1	1	0.25	0.5	32	4	2	2	0.5
AG1O5-1 + PBP5	4	1	2	125	16	8	16	4
SK1O6-3	4	1	2	125	16	8	16	4
SK1OD-3	4	1	2	125	16	8	16	4
SK1O6-3 + PBP6	4	1	2	125	16	8	16	4
SK2O56-3	1	0.25	0.5	32	4	2	2	0.5
SK2O56-3 + PBP5	4	1	2	125	16	8	16	4
SK2O56-3 + PBP6	1	0.25	0.5	32	4	2	2	0.5
SK2O56-3/pAG544-1	1	0.25	0.5	32	4	2	2	0.5
SK2O56-3 + DacD	2	0.5	1	64	8	4	4	2
SK2O5D-3	1	0.25	0.5	32	4	2	2	0.5
SK2O5D-3 + DacD	2	0.5	1	64	8	4	4	2
SK3O6D-3	1	0.25	0.5	32	4	2	2	0.5
SK3O6D-3 + DacD	2	0.5	1	64	8	4	4	2

sensitivity to subinhibitory concentrations of β -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 β -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 β -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 β -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 β -lactam sensitivity of PBP5 mutants and that expression of PBP5 *in trans* fully restores wild-type β -lactam resistance to a PBP5/6/DacD triple

Fig. 2. Phase expression studies in the presence of (a) ampicillin ($0.25 \mu\text{g ml}^{-1}$) and (b) cefadroxil ($2 \mu\text{g ml}^{-1}$). The strains are *E. coli* 2443 (in the absence of antibiotic; $\circ$), *E. coli* 2443 ($\blacklozenge$), SK2O56-3 ($\circ$), SK2O56-3 expressing PBP5 ($\blacksquare$), SK2O56-3 expressing PBP6 ($\blacktriangle$) and SK2O56-3 expressing DacD ($\blackstar$).

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.

ACKNOWLEDGEMENTS

The authors are deeply indebted to David E. Nelson for editing of the manuscript. This work has been supported in parts by research grants from the Indian Council for Medical Research (ICMR) Department of Biotechnology (DBT), Government of India, to A. S. G. M. D. was supported by a grant from the Council of Scientific and Industrial Research (CSIR) to A. S. G.

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Edited by: U. Dobrindt