

Supplemental Material for:

Title:

Multiple low-reactivity class B penicillin-binding proteins are required for cephalosporin resistance in enterococci

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Running title — Multiple bPBPs required for cephalosporin resistance

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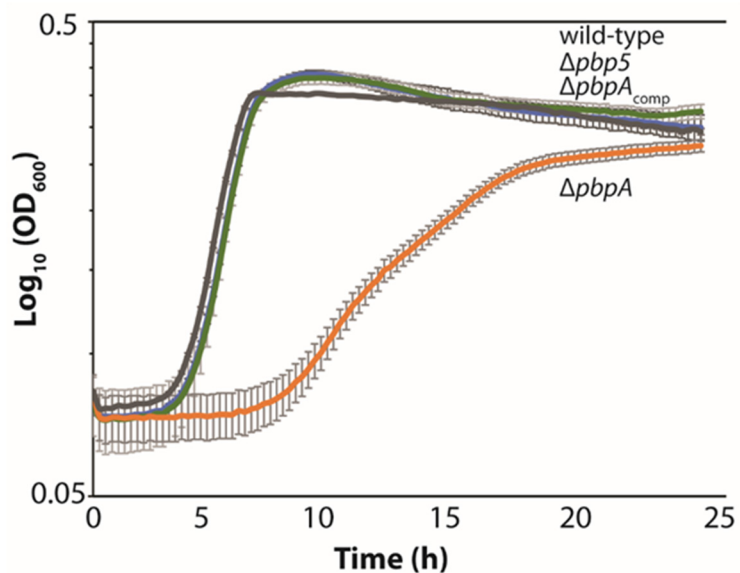


Fig S1. Growth kinetics of *E. faecalis* strains. Stationary-phase cultures were diluted to $\sim 10^5$ cfu/ml in MHB and incubated at 37 C. Culture density was monitored at 15-min intervals using a Bioscreen C plate reader, revealing that the $\Delta pbpA$ mutant exhibited a significant growth defect compared to the other strains tested, which were indistinguishable. Data represent mean \pm standard error from 3 independent experiments. Strains were: wild-type, OG1; $\Delta pbpA$, JL632; $\Delta pbp5$, JL339; $\Delta pbpA_{comp}$, DDJ241.

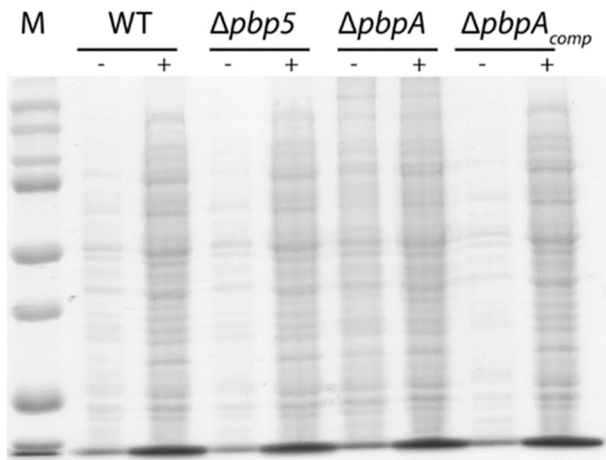


Fig S2. The $\Delta pbpA$ mutant exhibits compromised cell wall integrity. Cells growing exponentially in MHB were collected and treated (or not) with lysozyme, as indicated (- or +), prior to addition of Laemmli sample buffer containing 2% SDS. Samples were subjected to SDS-PAGE and total protein was stained, revealing extensive lysis of the $\Delta pbpA$ mutant in the absence of lysozyme treatment. Strains were: wild-type, OG1; $\Delta pbpA$, JL632; $\Delta pbp5$, JL339; $\Delta pbpA_{comp}$, DDJ241.

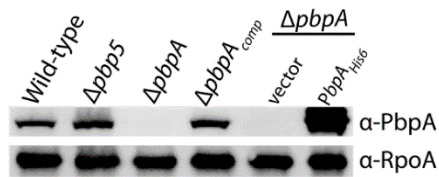


Fig S3. Immunoblot analysis of PbpA(2b) expression. Whole-cell lysates from *E. faecalis* cells growing exponentially in MHB were prepared and subjected to SDS-PAGE with immunoblotting using custom antisera against *E. faecalis* PbpA(2b) or the loading control, RpoA. Strains were: wild-type, OG1; $\Delta pbpA$, JL632; $\Delta pbp5$, JL339; $\Delta pbpA_{comp}$, DDJ241. For the plasmid-carrying $\Delta pbpA$ (JL632) strains, plasmids were: vector, pJRG9; and PbpA_{His6}, pEAW9.

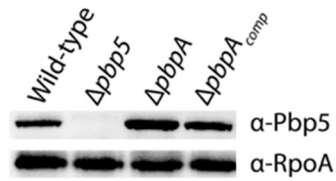


Fig S4. Immunoblot analysis of Pbp5(4) expression. Whole-cell lysates from *E. faecalis* cells growing exponentially in MHB were prepared and subjected to SDS-PAGE with immunoblotting using custom antisera against *E. faecalis* Pbp5(4) or the loading control, RpoA. Strains were: wild-type, OG1; $\Delta pbpA$, JL632; $\Delta pbp5$, JL339; $\Delta pbpA_{comp}$, DDJ241.

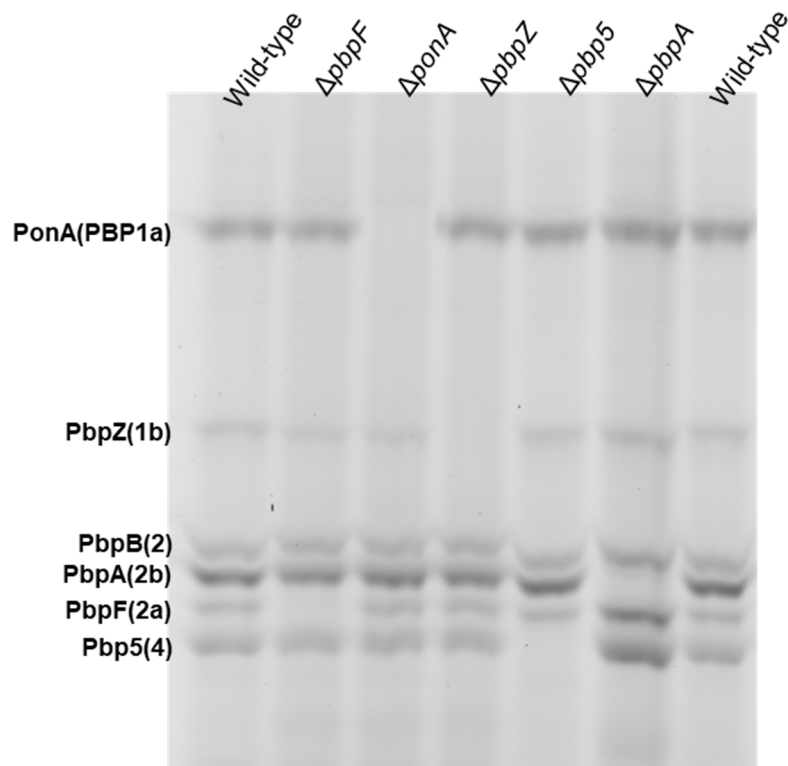


Fig S5. Bocillin FL labeling of a panel of *E. faecalis* PBP deletion mutants enables identification of each of the 6 labeled bands. *E. faecalis* strains growing exponentially in MHB were collected and treated with Bocillin FL to acylate all PBPs. Total cell lysates were subjected to SDS-PAGE and scanned to visualize fluorescently labeled PBPs. Data are representative of more than 3 independent experiments. Strains were: wild-type, OG1; $\Delta pbpA$, JL632; $\Delta pbp5$, JL339; $\Delta pbpZ$, JL629; $\Delta ponA$, JL618; $\Delta pbpF$, JL623. PbpB(2) was identified by elimination because a deletion mutant is not available. We note that using our optimized experimental conditions, we were able to achieve complete separation of all 6 PBPs (unlike the previous study by Arbeloa *et al* (2004) *J. Bacteriol.* **186**:1221), so the pattern of labeled bands and identification of PBPs observed here is not directly comparable to that reported by Arbeloa *et al*.

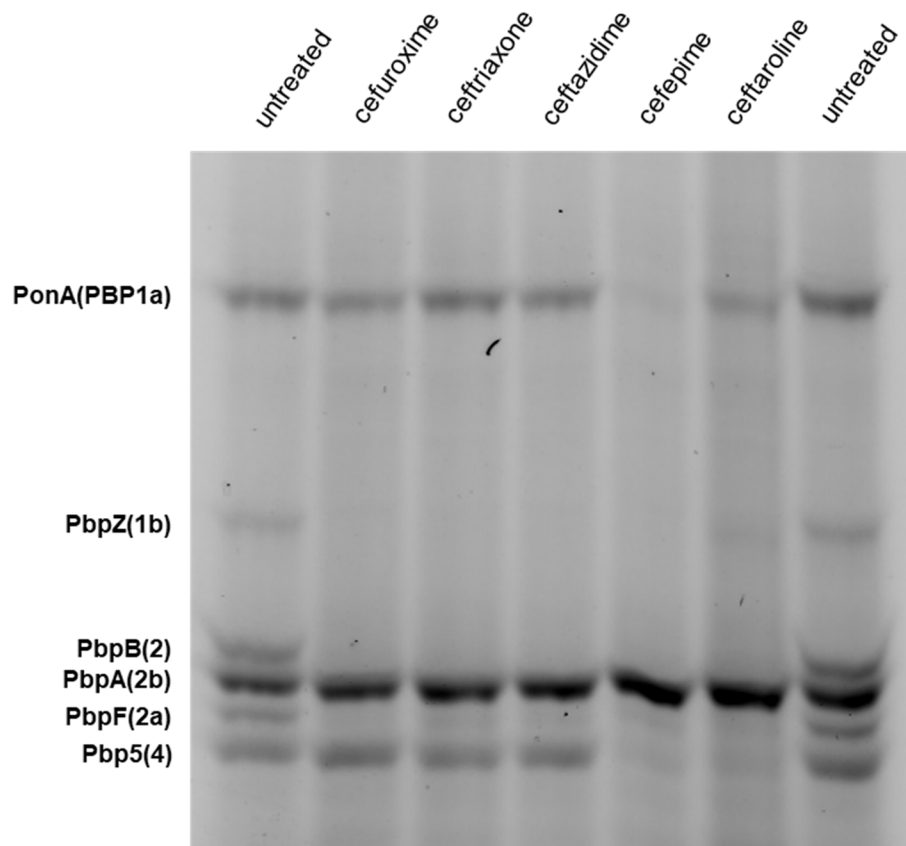


Fig S6. Multiple cephalosporins acylate only a subset of *E. faecalis* PBPs on growing cells. *E. faecalis* OG1 was cultured in MHB to exponential phase and treated (or not) with various cephalosporins (512 $\mu\text{g/ml}$) as indicated. Cells were collected after 20 min and treated with Bocillin FL to acylate any PBPs that had not been previously acylated by cephalosporins. Total cell lysates were subjected to SDS-PAGE and scanned to visualize fluorescently labeled PBPs. Data are representative of more than 3 independent experiments.

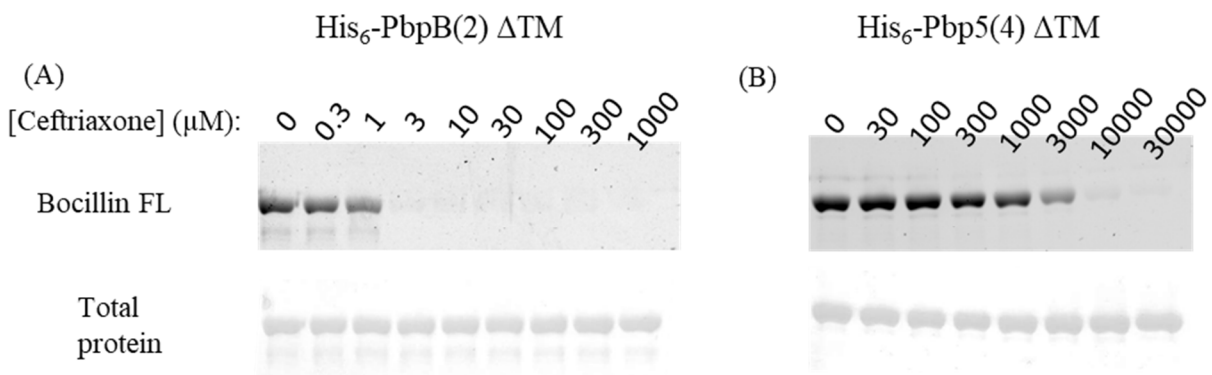


Fig S7. Reactivity of Pbp5(4) and PbpB(2) with ceftriaxone *in vitro*. Purified, recombinant *E. faecalis* His₆-PbpB(2) ΔTM (A) or His₆-Pbp5(4) ΔTM (B) (3 μM) were incubated with the indicated concentrations of ceftriaxone for 20 min, and then treated with excess Bocillin FL. The reactions were subjected to SDS-PAGE and scanned to visualize fluorescently labeled PBPs, followed by Coomassie staining for total protein. Data are representative of 3 independent experiments.

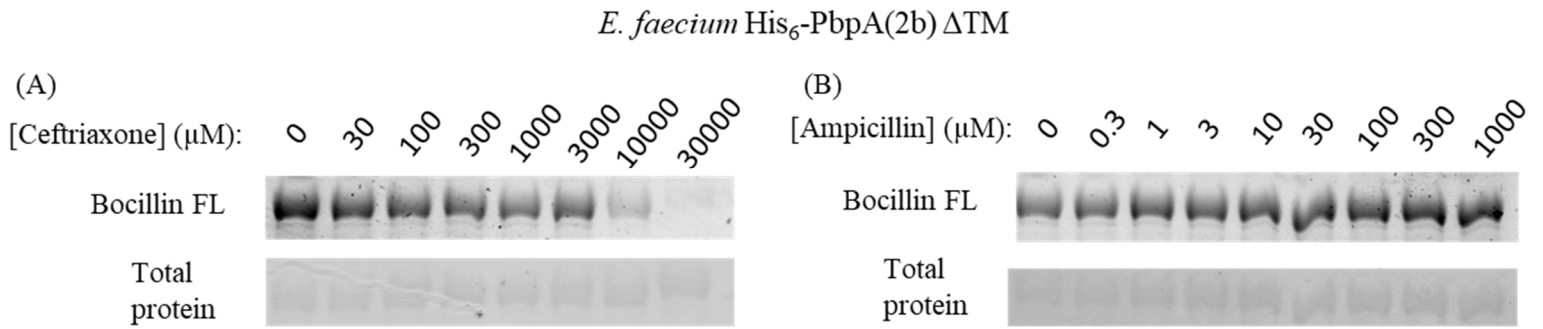


Fig S8. PbpA(2b) of *E. faecium* exhibits intrinsically low reactivity for ceftriaxone *in vitro*. Purified, recombinant *E. faecium* His₆-PbpA(2b) ΔTM (3 μM) was incubated with the indicated concentrations of ceftriaxone (A) or ampicillin (B) for 20 min, and then treated with excess Bocillin FL. The reactions were subjected to SDS-PAGE and scanned to visualize fluorescently labeled PbpA(2b), followed by Coomassie staining for total protein. Data are representative of 3 independent experiments.

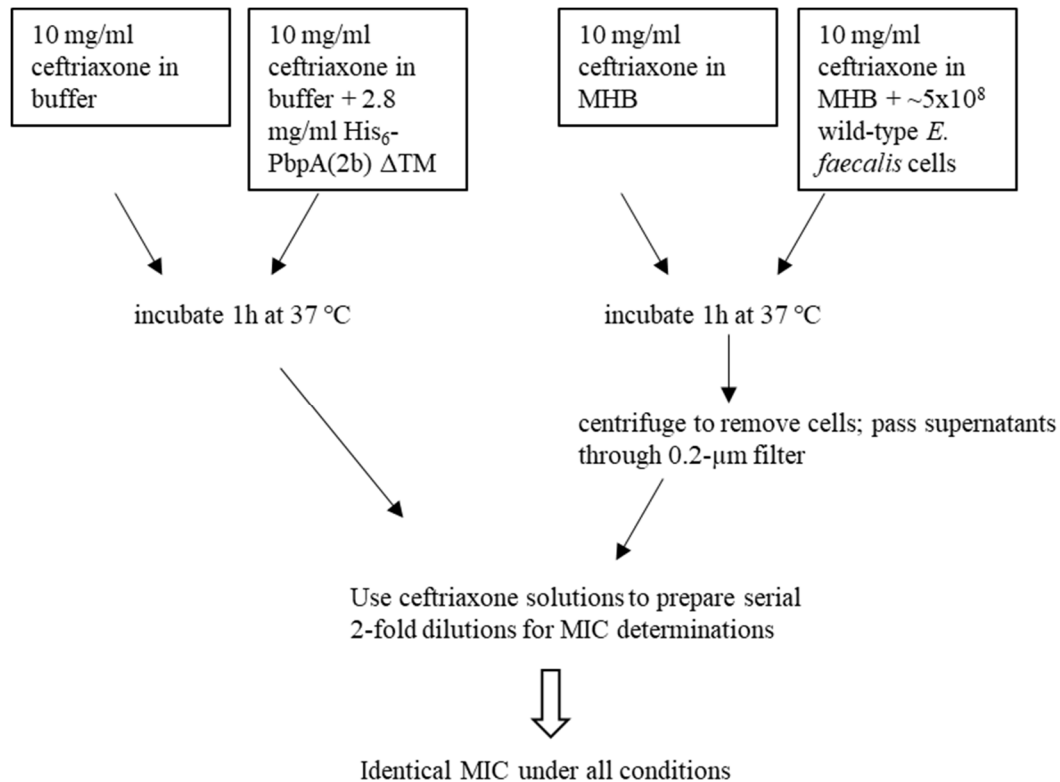


Fig S9. PbpA(2b) does not hydrolyze ceftriaxone. To test the possibility that PbpA(2b) was being initially acylated by ceftriaxone but subsequently hydrolyzed (thereby enabling subsequent acylation by Bocillin FL, and reducing the concentration of intact, active ceftriaxone in solution), we incubated ceftriaxone with either purified, recombinant *E. faecalis* PbpA(2b) ΔTM protein or with live *E. faecalis* cells for 1 h at 37 °C according to the scheme depicted above. We then used a bioassay (MIC assay) to compare the concentration of active ceftriaxone remaining in solution after incubation. The resulting MIC values were identical in all cases, indicating that neither PbpA(2b) ΔTM protein nor enterococcal cells were able to hydrolyze ceftriaxone.

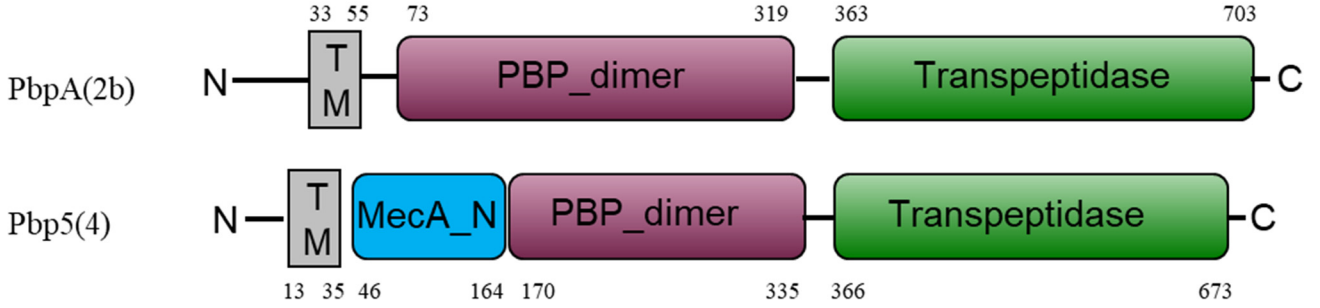


Fig S10. Domain architecture of PbpA(2b) and Pbp5(4). The domain architecture of PbpA(2b) and Pbp5(4) from *E. faecalis* OG1 was analyzed using SMART (Simple Modular Architecture Research Tool; <http://smart.embl-heidelberg.de/>) and is depicted. Numbers above and below the diagrams indicate residue numbers of the domain boundaries in the respective full-length proteins. The transmembrane domains (TM) are depicted with gray rectangles.

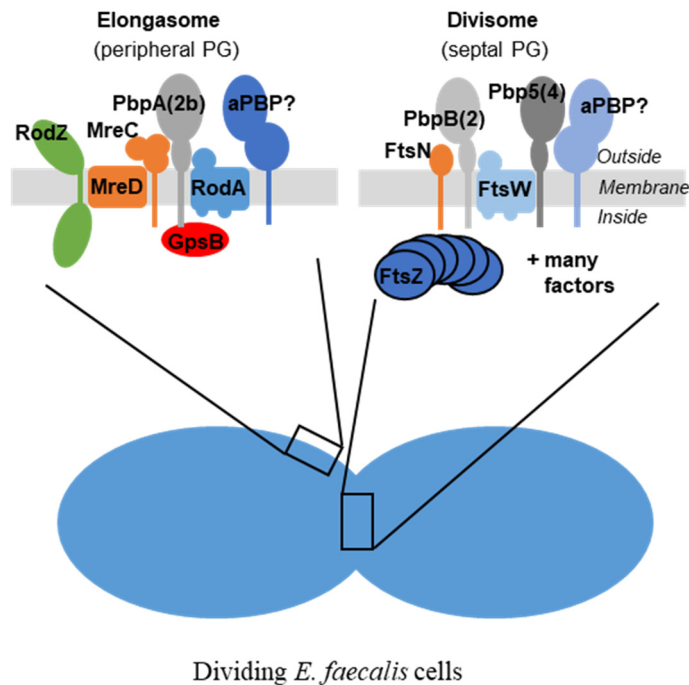


Fig S11. Model for functionally and spatially distinct enterococcal peptidoglycan synthases. We speculate that PbpA(2b) and Pbp5(4) belong to distinct peptidoglycan synthesis machines that are responsible for synthesizing peptidoglycan at different locations in the cell (*bottom*). One PG synthase (the divisome; *right*) builds PG at the division septum, which we speculate contains PbpB(2), Pbp5(4), and possibly one or more aPBPs in complex with FtsW (SEDS family glycosyltransferase) and other factors involved in cell division (e.g. FtsZ, FtsN, and others not shown for simplicity). A second PG synthase (the elongasome, *left*) builds peripheral PG, which we speculate contains PbpA(2b) and possibly one or more aPBPs in complex with RodA (SEDS family glycosyltransferase), MreC, MreD, RodZ, and the cytoplasmic protein GpsB. The activity of both PG synthases is required for growth and division; hence, we propose each synthase must contain 1 of the low-reactivity bPBPs encoded in enterococci to enable growth in the presence of cephalosporins.

Table S1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	routine cloning host	lab stock
BL21(DE3)	<i>E. coli</i> protein expression host	lab stock
<i>E. faecalis</i>		
OG1	Wild-type reference strain (MLST 1)	(1)
V583	<i>vanB</i> -containing clinical isolate (MLST 6)	(2)
CK221	V583 Δ <i>ermB</i>	(3)
JL339	OG1 Δ <i>pbp5</i> (Δ S11-A677)	(4)
JL618	OG1 Δ <i>ponA</i> (Δ G7-K771)	This work
JL623	OG1 Δ <i>pbpF</i> (Δ F18-D709)	This work
JL629	OG1 Δ <i>pbpZ</i> (Δ N16-N797)	This work
JL632	OG1 Δ <i>pbpA</i> (Δ F6-E707)	This work
DDJ241	JL632 (<i>OG1RF_10894-10895</i>):: <i>pbpA</i>	This work
JL639	CK221 Δ <i>pbpA</i> (Δ F6-E707)	This work
JL640	CK221 Δ <i>pbp5</i> (Δ S11-A677)	This work
<i>E. faecium</i>		
1141733	Wild-type reference strain, clinical isolate (MLST 327)	(5)
Plasmids		
pJH086	<i>E. faecalis</i> allelic exchange vector (Cm ^R); <i>pheS</i> * counterselection	(6)
pDV75-2	<i>E. faecalis</i> allelic exchange vector enabling ectopic integration of genes at the <i>OG1RF_10894-10895</i> intergenic locus (Cm ^R); <i>pheS</i> * counterselection	(7)
pDDJ227	pDV75-2 carrying <i>pbpA</i> plus 400 bp upstream of its start codon (from <i>E. faecalis</i> OG1)	This work
pBK2	cCF10-inducible expression vector (Cm ^R), carries <i>lacZ</i> (Cm ^R)	(8)
pJLL105	pBK2 with <i>lacZ</i> removed (Cm ^R)	(6)
pJLL264	pJLL105 carrying antisense <i>E. faecium pbpA</i>	This work
pJRG9	enterococcal expression vector carrying constitutive P23s promoter (Cm ^R)	(9)
pEAW9	pJRG9 carrying <i>pbpA-His₆</i> from <i>E. faecalis</i> OG1	This work
pJLL222	pET28b:: <i>pbp5</i> Δ TM (Δ 1-35) from <i>E. faecalis</i> OG1	This work
pJLL262	pET28b:: <i>pbpB</i> Δ TM (Δ 1-50) from <i>E. faecalis</i> OG1	This work
pJLL257	pET28b:: <i>pbpA</i> Δ TM (Δ 1-55) from <i>E. faecalis</i> OG1	This work
pJLL265	pET28b:: <i>pbpA</i> Δ TM (Δ 1-58) from <i>E. faecium</i> 1141733	This work

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