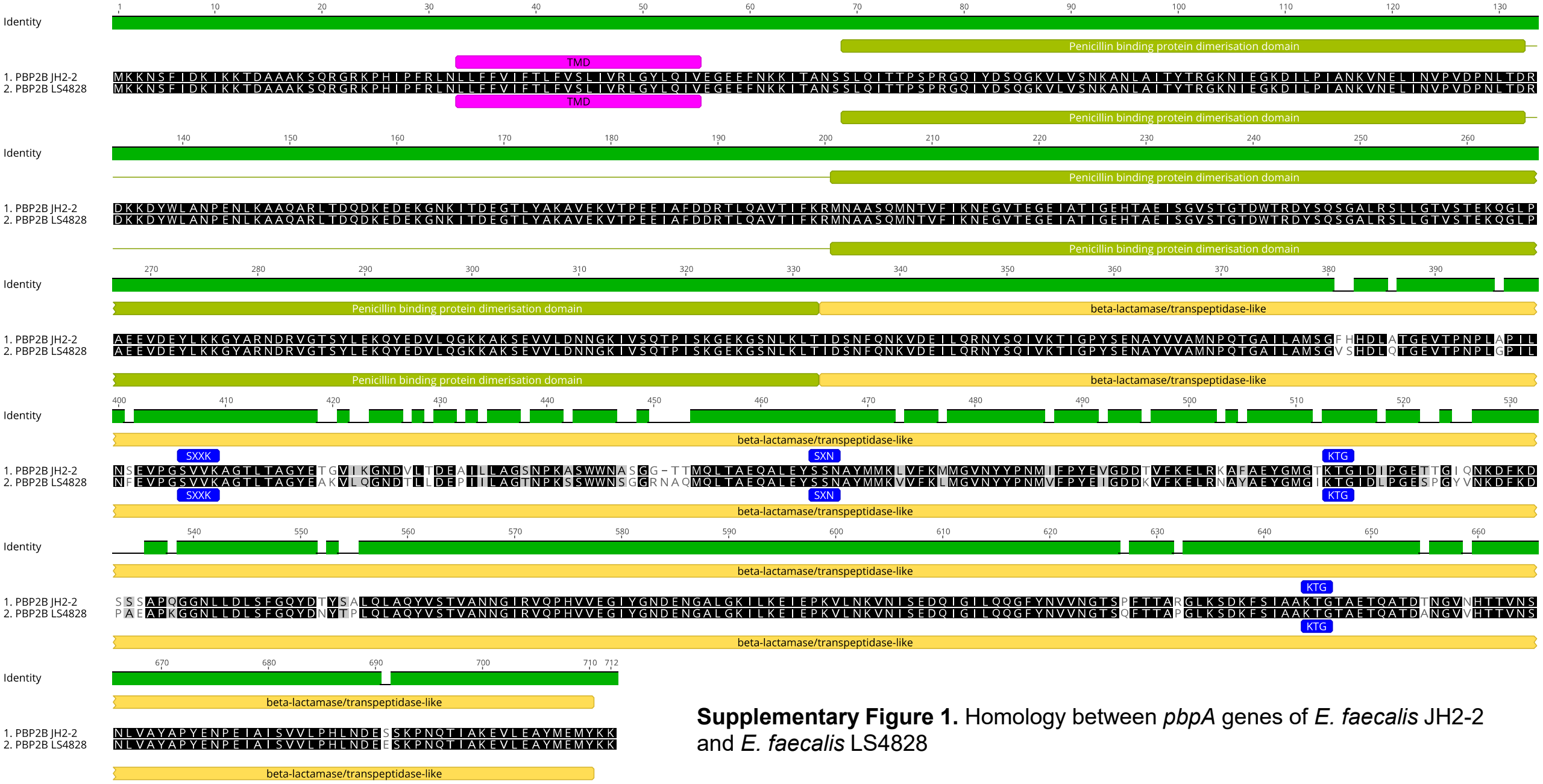
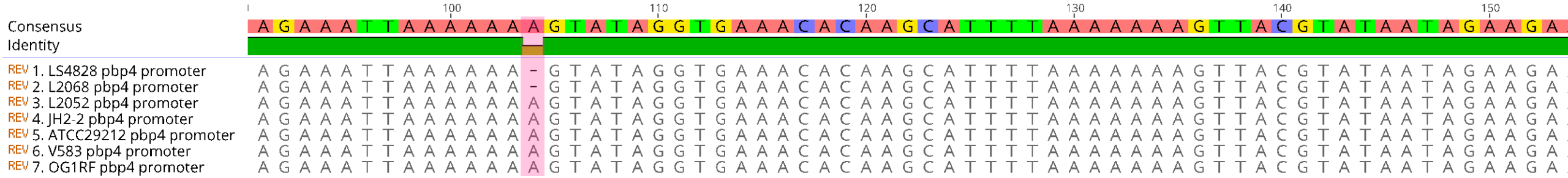
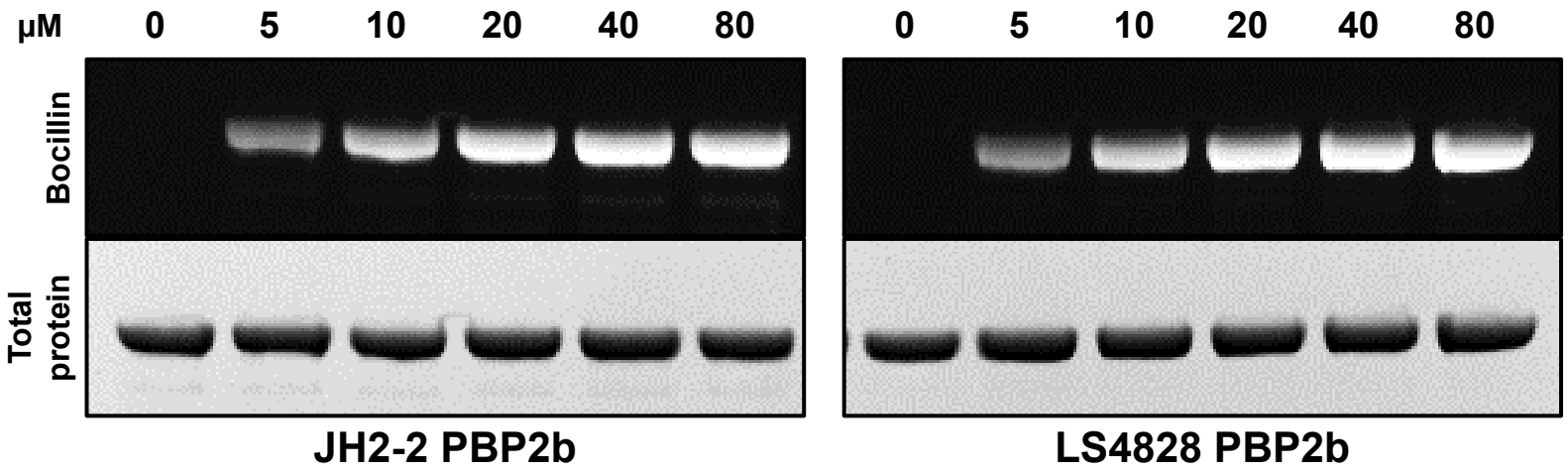


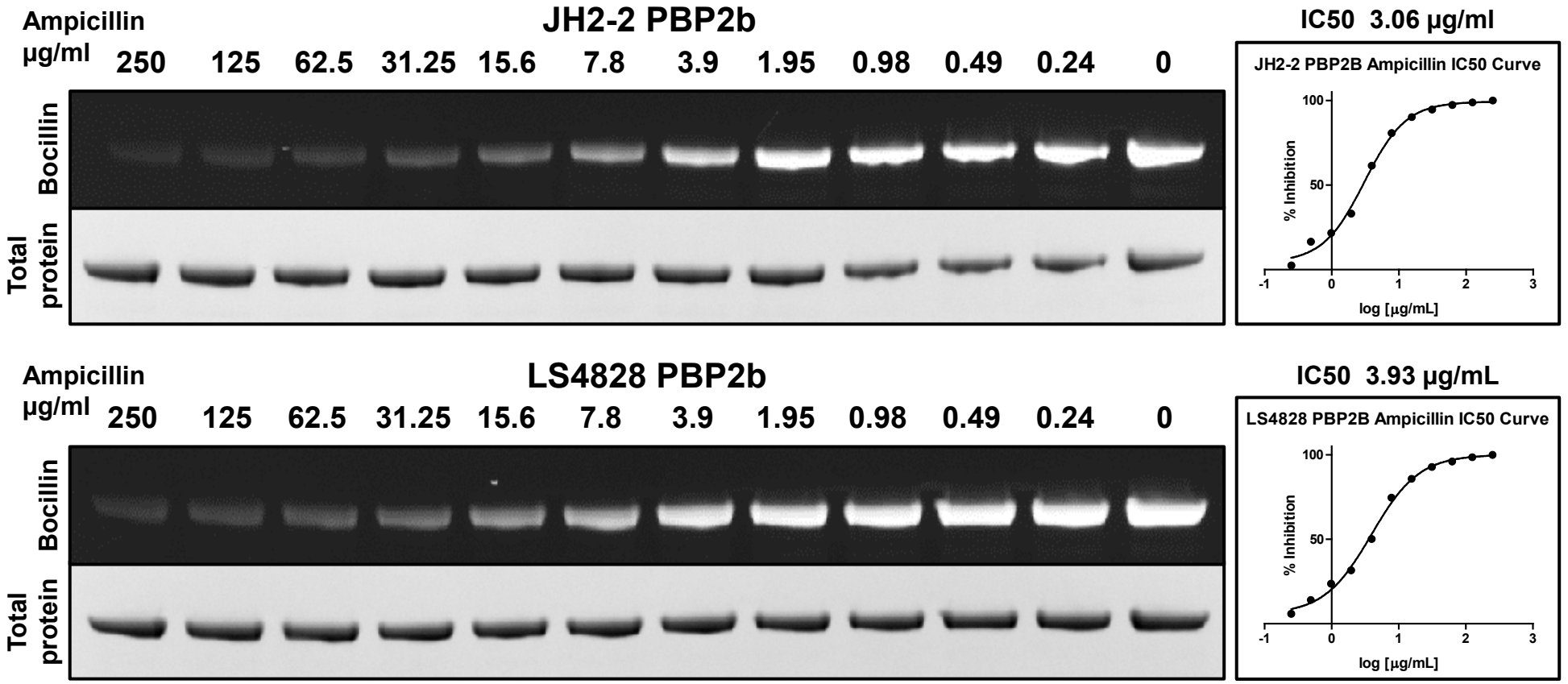
Supplementary Figure 1. Homology between *pbpA* genes of *E. faecalis* JH2-2 and *E. faecalis* LS4828



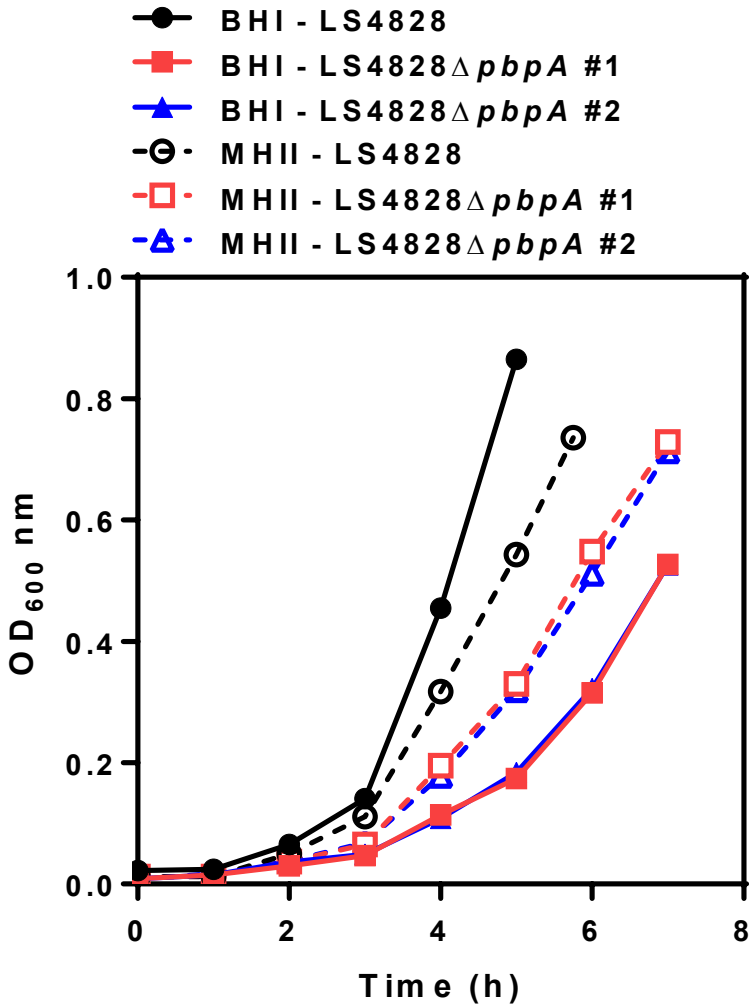
Supplementary Figure 2. L2068 *pbp4* gene does not have changes in its structural gene, but it has the same promoter deletion as seen on the LS4828 *pbp4* promoter



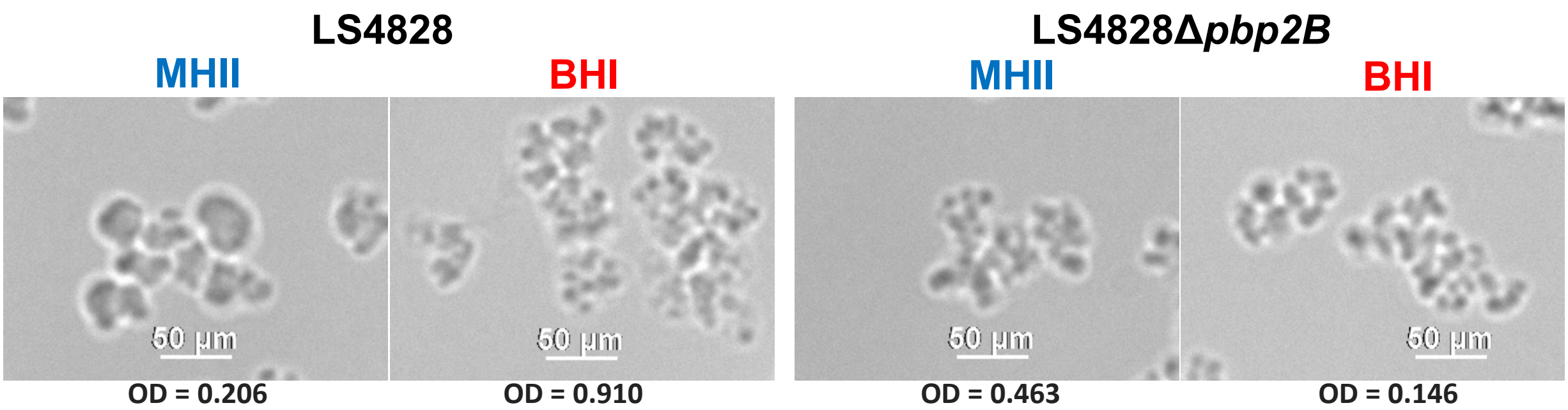
Supplementary Figure 3. JH2-2 and LS4828 PBP2b proteins show equal BOCILLIN FL™ binding affinities. Reactions of 3.3 μM purified PBP2b protein, no transmembrane domain, were treated with BOCILLIN FL™ for 20 min. at 37°C then processed for SDS-PAGE and fluorescence was imaged. The gels were subsequently stained for total protein using SimplyBlue SafeStain. These data are representative of 2 independent experiments.



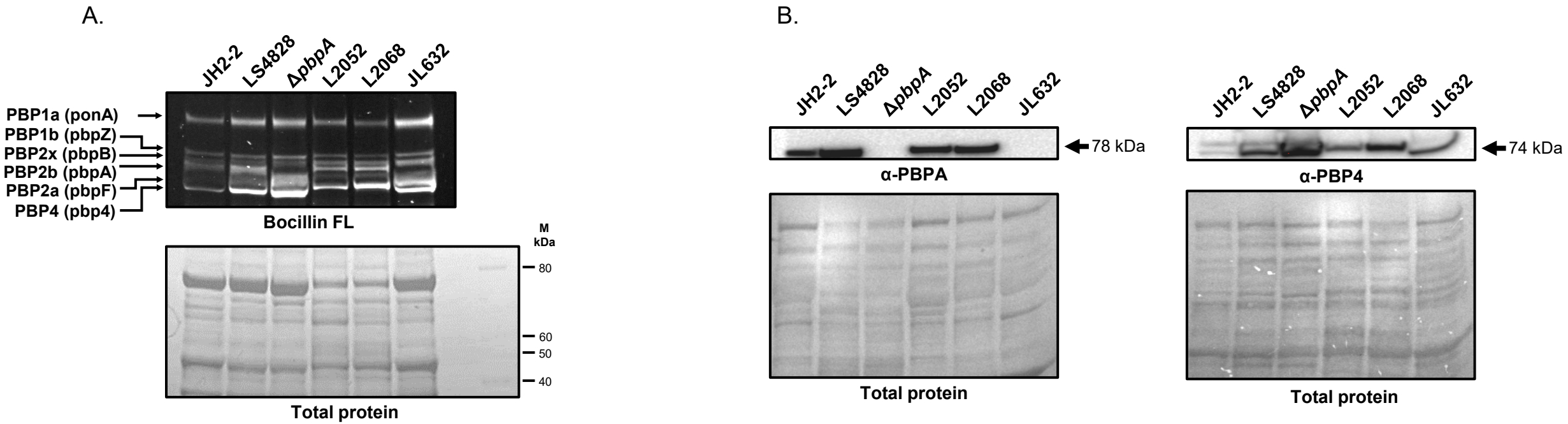
Supplementary Figure 4. Ampicillin competition using BOCILLIN FL™ for JH2-2 and LS4828 PBP2b proteins. Reactions of 3.3 µM of purified protein with titrated ampicillin concentrations for 10 minutes were followed by BOCILLIN FL™ labeling. These reactions were subjected to SDS-PAGE, the gel was imaged for fluorescent binding, then stained for total protein using SimplyBlue SafeStain. IC50 curves were generated in Graphpad Prism using percent inhibition data and represent two independent experiments.



Supplementary Figure 5. Growth curves for LS4828 and the Δ*pbpA* mutant in BHI or MHII broth. LS4828 and two independent colonies of the Δ*pbpA* mutant were inoculated from overnight cultures and followed hourly through early exponential growth at 37°C with shaking at 180 rpm. These data represent at least 2 independent experiments.



Supplementary Figure 6. Light microscopy imaging of LS4828 and the ΔpbpAmutant in Brain-Heart Infusion (BHI) or Mueller-Hinton II (MHII) broth. The mutant grew slower and to a lower density than the parent strain and there was a size difference with LS4828 grown in MHII appearing larger than the ΔpbpA mutant when grown in MHII broth and larger than either isolate grown in BHI. Optical density (OD) values from supplementary figure 5 are included on the bottom for reference.



Supplementary Figure 7. Bocillin FL Binding of Membrane Proteins and Immunodetection of PBP4 and PBPA expression by Western Blot Analysis. (A) *E. faecalis* purified membrane proteins were incubated at 37°C with 50 µM Bocillin FL for 30 minutes and were processed for SDS-PAGE to identify all PBPs. The Bocillin FL gel was subsequently stained with SimplyBlue SafeStain for total protein. (B) Fifteen micrograms of membrane proteins were electrophoresed in 8% Bis-Tris Plus Bolt gels and transferred to PVDF membranes for immunoblot detection of PBP4 or PBPA using custom polyclonal antibody. The PVDF blots were then stained with Coomassie blue R-250 for total proteins transferred.

Note: The PBPs in the Bocillin FL gel were identified according to the pattern observed by Djoric et al (2020) using an 8% SDS-PAGE gel
Membrane protein isolation for Bocillin FL and Western blot protocols described in reference (2)

TABLE S1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic or description	Reference or source
***Enterococcus faecalis* strains**		
JH2-2	Penicillin-sensitive isolate; Pen^s, Amp^s, Cro^r	(1)
LS4828	Penicillin-resistant clinical isolate; Pen^r, Amp^r, Cro^r	(2)
L2052	Penicillin-susceptible clinical strain	Kerry LaPlante
L2068	Penicillin-elevated clinical strain	Kerry LaPlante
LS319	LS4828 *ΔpbpA*; Pen^r, Amp^r, Cro^r	This study
JL632	OG1RF *ΔpbpA*(2b); Cro^s	(3)
LS310	LS4828 *ΔpbpA* with LS4828 pbp2B complement	This study
LS311	LS4828 *ΔpbpA* with JH2-2 pbp2B complement	This study
***Escherichia coli* strains**		
NEB 10 Beta	routine cloning host	NE Biolabs
NEB 5 alpha	routine cloning host	NE Biolabs
TOP10	routine cloning host	Invitrogen
BL21 Star (DE3)	protein expression host	Invitrogen
EC1000	*E. coli* cloning host, provides RepA in trans	(4)
Plasmids		
pBSU100	pAT28 derivative carrying *egfp*; Spe^r	(5)
pRIH310	LS4828 *pbpA* ORF and promoter cloned into pHOU1; Gen^r	This study
pRIH311	JH2-2 *pbpA* ORF and promoter cloned into pHOU1; Gen^r	This study
pET-RP1B	Expression vector for amino-terminal His tag fusions	Wolfgang Peti

pRIH316	JH2-2 *pbpA* coding region, no TM domain cloned into pET-RP1B for overexpression in *E. coli*; Kan^r	This study
pRIH317	LS4828 *pbpA* coding region, no TM domain cloned into pET-RP1B for overexpression in *E. coli*; Kan^r	This study
pHOU1	Allelic exchange vector; Gen^r, *pheS** counterselection	(6)
pRIH319	ΔpbpA complementation construct cloned into pHOU1; Gen^r	This study

Abbreviations: Pen penicillin; Amp, ampicillin; Cro, ceftriaxone; Kan, kanamycin; Gen, gentamicin; Spe, spectinomycin.

References

1. Jacob AE, Hobbs SJ. 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. Journal of Bacteriology 117:360-372.
2. Rice LB, Desbonnet C, Tait-Kamradt A, Garcia-Solache M, Lonks J, Moon TM, D'Andrea ED, Page R, Peti W. 2018. Structural and Regulatory Changes in PBP4 Trigger Decreased beta-Lactam Susceptibility in Enterococcus faecalis. MBio 9.
3. Djoric D, Little JL, Kristich CJ. 2020. Multiple Low-Reactivity Class B Penicillin-Binding Proteins Are Required for Cephalosporin Resistance in Enterococci. Antimicrob Agents Chemother 64.

4. Leenhouts K, Buist G, Bolhuis A, ten Berge A, Kiel J, Mierau I, Dabrowska M, Venema G, Kok J. 1996. A general system for generating unlabelled gene replacements in bacterial chromosomes. Mol Gen Genet 253:217-24.
5. Aymanns S, Mauerer S, van Zandbergen G, Wolz C, Spellerberg B. 2011. High-level fluorescence labeling of gram-positive pathogens. PLoS One 6:e19822.
6. Panesso D, Montealegre MC, Rincon S, Mojica MF, Rice LB, Singh KV, Murray BE, Arias CA. 2011. The hylEfm gene in pHylEfm of Enterococcus faecium is not required in pathogenesis of murine peritonitis. BMC Microbiol 11:20.