In vitro reconstitution of peptidoglycan assembly from the Gram-positive pathogen *Streptococcus pneumoniae*

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Supporting Information

METHODS

Pneumococcal strains. Strains, plasmids and oligonucleotides are listed in supporting Tables. The R6 strain was used as wild type pneumococcus. The derivative spnLR29 was constructed by introducing at the *BgaA* locus the *murT*/*gatD* operon (spr1443/1444) under the control of a zinc-inducible promoter by way of the pCM38 plasmid (laboratory collection), a derivative of pJWV25 [1] with an introduced AgeI site upstream of the gfp gene that allows its replacement by other genes. The endogenous *murT*/*gatD* operon was then deleted by replacement with a chloramphenicol resistance cassette.

pCM38 was obtained by site-directed mutagenesis of pJWV25 using primers FORoCM44 and REVoCM45 with the . The *murT/gatD* operon was PCR amplified from R6 genomic DNA using primers AGEMTGDFW and NOTMTGDRV, and introduced as an *Age*I/*Not*I fragment into pCM38. The resulting plasmid pCM83-MTGD amplified in *Escherichia coli* DH5α under ampicillin selection was used to transform R6 pneumococcus under tetracycline selection (3.1 mg L^{-1}). Insertion at the *BgaA* locus in the resulting SpnLRyy strain was checked by PCR using primers FORoCM84 and REVoCM85, followed by digestion of the product by *Age*I.

For deletion of the *murT*/*gatD* operon, a chloramphenicol resistance cassette was amplified from plasmid pR326 [2] using primers MTGDCATFW and MTGDCATRV. The one-kbp upstream and downstream regions of the *murT*/*gatD* were PCR amplified from R6 genomic

DNA using the pairs of primers MTGDUPFW and MTGDUPR, and MTGDDNFW and MTGDDNRV. The upstream and downstream region thus amplified were appended on each side of the chloramphenicol resistance cassette by a reconstitutive PCR using the three purified PCR products as template with the MTGDUPFW and MTGDDNRV primers. The resulting purified product was used to transform spnLR29. Transformants were selected on chloramphenicol (3.4 mg L^{-1}) in the presence of 150 μM $ZnCl_2$.

Growth assays. The R6, spnLR29 and spnLR30 strains were grown in Todd-Hewitt broth (BD) supplemented with 150 μM $ZnCl_2$ without antibiotics. Cells were streaked on Columbia agar (BD) plates containing 5% (v/v) horse blood, supplemented or not with 150 μM $ZnCl_2$, and incubated at 37°C with 5% CO_2. Alternatively, cultures at an optical density at 600 nm of 0.3 were washed three times with an equal volume of Todd-Hewitt medium without added zinc, prior to inoculation (1:100) of the same medium, with or without 150 μM $ZnCl_2$, in 24-wells plate and incubation at 37°C in a FLUOstar plate reader (BMG Labtech). Optical density was measured at 595 nm.

Preparation of MurT/GatD. The *murT*/*gatD* operon (spr1443/1444) was amplified by PCR with primers MurTFOR and GatDREV from genomic DNA from the R6 *S. pneumoniae* strain and introduced in a modified pET-30 plasmid to allow co-expression in *E. coli* BL21 Star™ (DE3) of MurT with a N-terminal poly-histidine-tag and GatD without tag. After growth in Luria broth to saturation, expression was induced overnight at 20°C by the addition of 0.5 mM IPTG. Cells were resuspended in 50 mM HEPES, pH 7.5, 300 mM NaCl, 10 mM $MgCl_2$, 25 mM imidazole and Complete™ protease inhibitors. After cell breakage by sonication and removal of cellular debris and membranes by low speed and ultracentrifugation, the lysate was loaded onto a 1 mL HisTrap FF column (GE Healthcare) and proteins were eluted with imidazole concentration

steps at 50, 100 and 200 mM. Fractions of the 100 mM imidazole steps were pooled and diluted 10-fold in the same buffer without NaCl nor imidazole, and loaded onto a 6 ml ResourceQ column (GE Healthcare). Proteins were eluted with a 150-450 mM NaCl gradient over 70 mL. After concentration, proteins were further purified by size-exclusion chromatography on a Superdex200 (10X30) column (GE Healthcare) equilibrated with 50 mM HEPES, pH 7.5, 150 mM NaCl and 10 mM $MgCl_2$.

Preparation of PBP2a and PBP1a. Full length PBP2a and its S410A and E110Q variants were prepared as described previously [3]. The gene for full length PBP1a was PCR amplified with the primers PbP1aFor and PbP1aRev, and prepared for ligation-independent cloning into pET-46 (Novagen) as detailed in the manufacturer's instructions by treatment with T4 DNA polymerase. Following isolation of the correct recombinant plasmid verified by DNA sequencing, protein expression was achieved in *E. coli* BL21(DE3) Star grown overnight after induction at 25°C. After cell breakage by sonication and removal of cellular debris by low speed centrifugation, membranes were pelleted by ultracentrifugation and initially resuspended in PBS buffer containing 1% (w/v) sodium deoxycholate. Following removal of insoluble material, the membranes were diluted 5-fold with PBS containing 0.1% (w/v) DDM and mixed for 3 hours with Talon Cobalt resin pre-equilibrated in the same buffer. The slurry was then washed in a column format with 0.1% (w/v) DDM containing PBS buffer with 15 mM and 50 mM imidazole to remove contaminating proteins and the PBP1a eluted with 250 mM imidazole-containing buffer. Further purification of PBP1a was achieved by size-exclusion chromatography using a Superose 6 column equilibrated in 50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, and 0.02% (w/v) Triton X-100.

Preparation of PBP2x and PBP2b. After PCR amplification with primers Nde2x and 2xStopNheSpeBam, and Nde2b and 2bStopNheSpeBam, the genes encoding full length PBP2x or PBP2b were introduced into a modified pET-30 plasmid to permit expression of fusions with a C-terminal Strep-tag. Cultures and expression were as for MurT/GatD. Cells were resuspended in 50 mM HEPES, pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$ and Complete™ protease inhibitors. After cell breakage by sonication and removal of cellular debris by low speed centrifugation, membranes were pelleted by ultracentrifugation and resuspended in the same buffer but with 150 mM NaCl. Membranes were solubilized by the addition of 1% (w/v) of Triton X-100 and the solution was loaded onto a 1 ml of Strep-Tactin® resin (Iba). The detergent concentration was brought down gradually to 0.02% (w/v) during successive washes and the protein was eluted with 2.5 mM desthiobiotin in the same buffer. Pooled fraction were diluted to bring the NaCl to 15 mM in the same buffer and loaded onto a 1 ml ResourceQ column (GE Healthcare). Proteins were eluted with a 0–1 M NaCl gradient over 60 ml.

Supplementary Table 1. Primers used in this study

Name	Sequence
FORoCM44	GGAGGCAAATATGAAACATCTTACCGGTAGCAAAGGAGAAGAACTTTTCAC
REVoCM45	GTGAAAAGTTCTTCTCCTTTGCTACCGGTAAGATGTTTCATATTTGCCTCC
AGEMTGDFW	GATATAAT**ACCGGT**ATGAACTTAAAAACTACTTTGGG
NOTMTGDRV	TGATATCAT**GCGGCCGC**GTTTAAGAAAAGTCAGCCTTGC
MTGDCATFW	CTCTTTTGTGATATAATAGAAACGAAAATTTGTTTGATTTTTAATG
MTGDCATRV	GAAAAGTCAGCCTTGCTTTTGACGTCTAGAACTAGTGGATCCCC
MTGDUPFW	CGTCTTTGACTCAACAGGTATCC
MTGDUPRV	CATTAAAAATCAAACAAATTTTCGTTTCTATTATATCACAAAAGAG
MTGDDNFW	GGGGATCCACTAGTTCTAGACGTCAAAAGCAAGGCTGACTTTTC
MTGDDNRV	GAAGTACTCACCATAACCAGC
FORoCM84	CGCCCCAAGTTCATCACCAATGAC
REVoCM85	CTTTTTTACCTTTAGTAACTAC
MurTFOR	TTCAGGGGTCC**CATATG**AACTTAAAAACTACTTTGGG
GatDREV	CTCGAATTCGGATCCACTAGT**GCTAGC**TTAAGAAAAGTCAGCCTTG
PbP1aFor	GACGACGACAAGATGCATATGCACCATCACCATCACCATGTTTTTTTCTACTACGTTAGCAAG
PbP1aRev	GAGGAGAAGCCCGGTTTATGGTTGTGCTGGTTGAGGATTC
Nde2x	GAGAATTC**CATATG**AAGTGGACAAAAAGAGTAATC
2xStopNheSpeBam	GGTCGAC**GGATCC**ACTAGTGCTAGCTTAGTCTCCTAAAGTTAATG
Nde2b	GAGAATTC**CATATG**AGACTGATTTGTATGAGAAAATTTAAC
2bStopNheSpeBam	GGTCGAC**GGATCC**ACTAGTGCTAGCCTAATTCATTGGATGGTATTTTTG

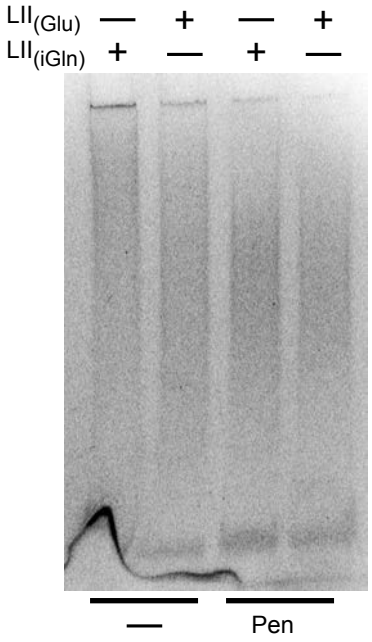
Supplementary Table 2. Plasmids used in this study

Name	Description	Source
pJWV25	Allows insertion of *gfp* under the control at the *BgaA* locus under the control of a zinc-inducible promoter. Tetracycline resistance in *S. pneumoniae*. Ampicillin resistance in *E. coli* *amp, tet, bgaA,* P_{czcD}*-gfp+*	[1]
pCM38	Derivative of pJWV25 with *Age*I site between promoter and *gfp* gene. Allows replacement of gfp by other genes. *amp, tet, bgaA,* P_{czcD}*-gfp+*	This study
pCM83-MTGD	Derivative of pCM38 to introduce *murT*/*gatD* at the *BgaA* locus under the control of a zinc-inducible promoter. *amp, tet, bgaA,* P_{czcD}*-murT*/*gatD*	This study
pR326	Used as template to amplify the chloramphenicol resistance cassette *cat*. *amp, cat*	[2]
pET30b	Allows expression of exogenous genes in *E. coli* (DE3). *kan*, *lacI,* P_{T7}*-+*	Novagen
pET46(LIC)	Allows expression of exogenous genes in *E. coli* (DE3) and cloning via the ligation independent cloning method. *amp*, *lacI*, P_{T7}*-+*	Novagen
pET30-H8N	Derivative of pET30, encodes a N-terminal octo-histidine-tag. Allows insertion of a gene at a *Nde*I site downstream of the tag sequence. *kan*, *lacI,* P_{T7}*-H8+*	[3]
pET30-StpC	Derivative of pET30, encodes a C-terminal Strep-tag. Allows insertion of a gene at a *Nde*I site. *kan*, *lacI,* P_{T7}*-+Stp*	This study
pET30-HAT	Derivative of pET30-H8N. Allows expression in *E. coli* (DE3) of pneumococcal MurT and GatD with an N-terminal His-tag. *kan*, *lacI,* P_{T7}*-H8murT*/*gatD*	This study
pET30-2a	Derivative of pET30b. Allows expression in *E. coli* (DE3) of pneumococcal PBP2a. *kan*, *lacI,* P_{T7}*-pbp2a*	[3]
pET30-2aΔTP	Derivative of pET30-2a. Allows expression in *E. coli* (DE3) of pneumococcal PBP2a-S410A mutant. *kan*, *lacI,* P_{T7}*-pbp2a-S410A*	This study
pET30-2aΔGT	Derivative of pET30-2a. Allows expression in *E. coli* (DE3) of pneumococcal PBP2a-E110Q. *kan*, *lacI,* P_{T7}*-pbp2a-E110Q*	[3]
pET30-2x-StpC	Derivative of pET30-H8N. Allows expression in *E. coli* (DE3) of pneumococcal PBP2x with a C-terminal Strep-tag. *kan*, *lacI,* P_{T7}*-pbp2x-Stp*	This study
pET30-2b-StpC	Derivative of pET30-H8N. Allows expression in *E. coli* (DE3) of pneumococcal PBP2b with a C-terminal Strep-tag. *kan*, *lacI,* P_{T7}*-pbp2b-Stp*	This study
pET46-1a	Derivative of pET46(LIC). Allows expression in E. coli (DE3) of pneumococcal PBP1a extracellular region with an N-terminal His-tag. *amp*, *lacI*, P_{T7}*-H6pbp1a*	This study

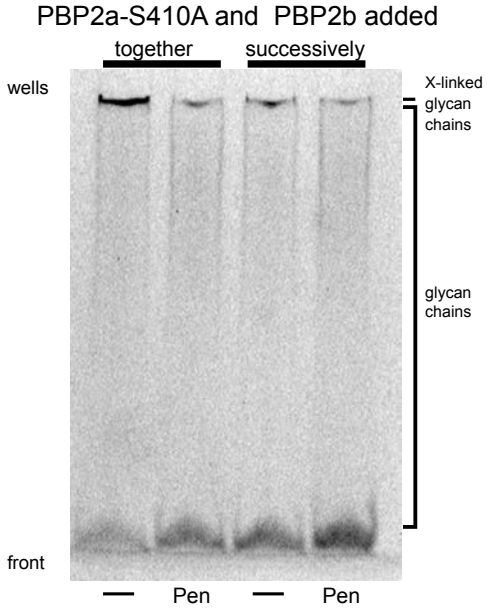
Supplementary Table 3. Strains used in this study

Name	Description	Source
S. pneumoniae R6	Unencapsulated laboratory strain. Used as wild type.	Lab collection
SpnLR29	Derivative of R6 with pCM83-MTGD introduced at the *BgaA* locus. *tet, P_{czcD}-murT/gatD*	This study
SpnLR30	Derivative of SpnLRxx with endogenous *murT/gatD* replaced by a chroramphenicol resistance cassette. *tet, cat, P_{czcD}-murT/gatD*	This study
E. coli BL21star	*E. coli* (DE3) strain used for heterologous protein expression from pET vectors	Novagen

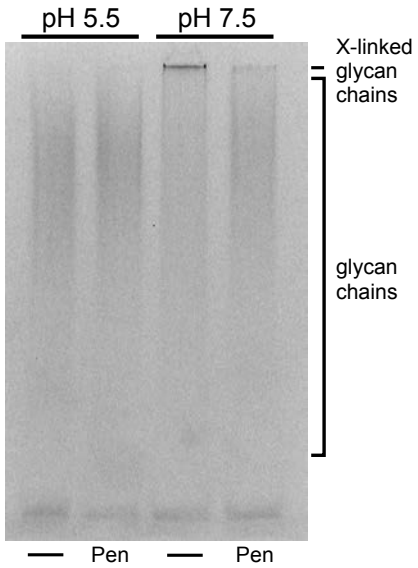
1. Eberhardt, A., Wu, L. J., Errington, J., Vollmer, W., and Veening, J. W. (2009) Cellular localization of choline-utilization proteins in *Streptococcus pneumoniae* using novel fluorescent reporter systems, *Mol. Microbiol. 74*, 395-408.
2. Claverys, J. P., Dintilhac, A., Pestova, E. V., Martin, B., and Morrison, D. A. (1995) Construction and evaluation of new drug-resistance cassettes for gene disruption mutagenesis in *Streptococcus pneumoniae*, using an ami test platform, *Gene 164*, 123-128.
3. Helassa, N., Vollmer, W., Breukink, E., Vernet, T., and Zapun, A. (2012) The membrane anchor of penicillin-binding protein PBP2a from *Streptococcus pneumoniae* influences peptidoglycan chain length, *FEBS J. 279*, 2071-2081.



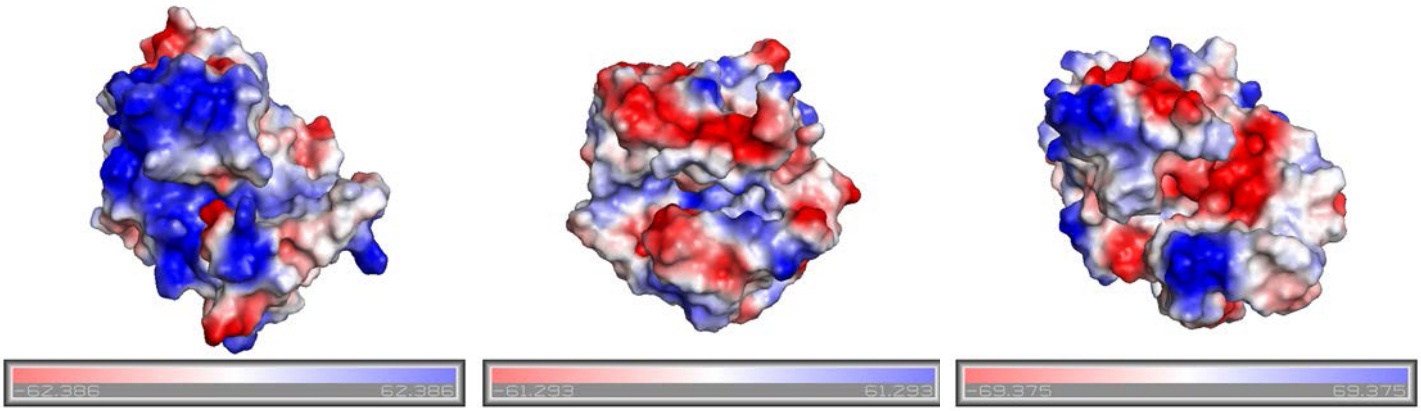
Supplementary Figure 1. Cross-linking of peptidoglycan by PBP2x. Mixtures of 50 µM amidated (LII(iGln)) or non-amidated lipid II (LII(Glu)) and 5 µM non-amidated dansylated lipid II were incubated with 1 µM of transpeptidase-inactivated PBP2a-S410A and 1 µM of PBP2x in the absence or presence of penicillin G. All reactions were overnight at 30°C. Samples were analysed by SDS-PAGE, and the dansyl fluorescence was imaged by blue or UV trans-illumination.



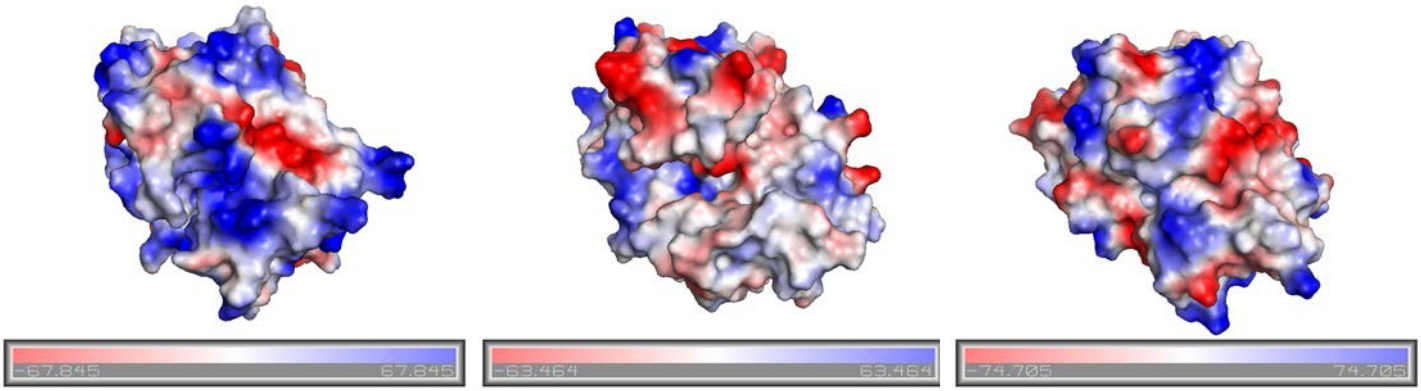
Supplementary Figure 2. A mixture of 50 µM amidated lipid II and 5 µM non-amidated dansylated lipid II was incubated overnight at 30°C with 1 µM of transpeptidase-inactivated PBP2a-S410A, with or without 1 µM PBP2b. After addition of buffer or PBP2b to the samples that already contained PBP2b or not, respectively, incubation was continued overnight. All reactions were with or without 1 mM penicillin G. Samples were analysed by SDS-PAGE, and the dansyl fluorescence was imaged by blue or UV trans-illumination.



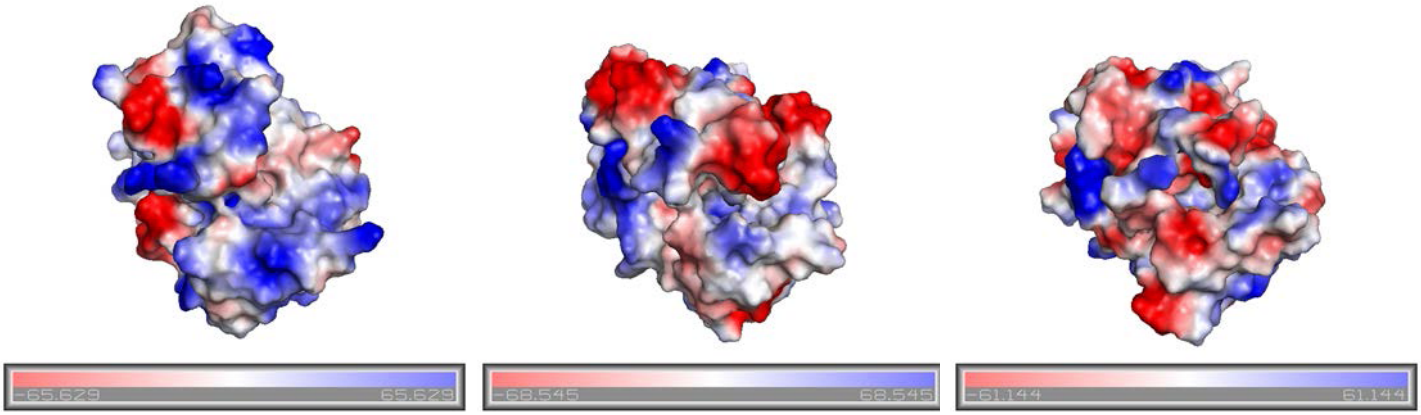
Supplementary Figure 3. Effect of the pH on the TP activity of PBP2a. A mixture of 50 µM amidated lipid II and 5 µM non-amidated dansylated lipid II was incubated at pH 5.5 and pH 7.5 with 1 µM PBP2a, in the presence or absence of or penicillin G (Pen). Buffers were 50 mM MOPS and HEPES, respectively. Reactions were overnight at 30°C. Samples were analysed by SDS-PAGE, and the dansyl fluorescence was imaged by UV trans-illumination.



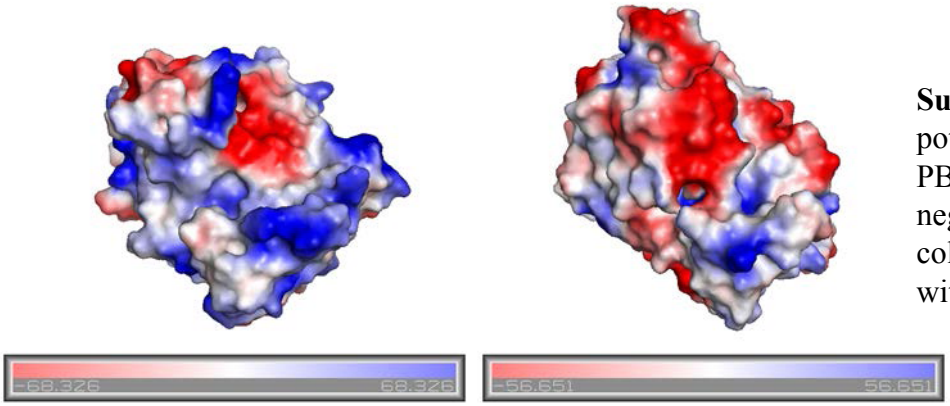
PBP1b *Escherichia coli* | PBP2x *Streptococcus pneumoniae* | PBP3 *Staphylococcus aureus*



PBP3 *Pseudomonas aeruginosa* | PBP2b *Streptococcus pneumoniae* | PBP2a *Staphylococcus aureus*



PBP1a *Acinetobacter baumanii* | PBP1a *Streptococcus pneumoniae* | PBP3 *Mycobacterium tuberculosis*



PBP2 *Neisseria gonorrhea* | PBP1b *Streptococcus pneumoniae*

Supplementary Figure 4. Electrostatic potential surface of TP domains of PBPs from various species (Gram-negative organisms are in the left column). Orientations are the same with the active site facing.