Supporting Information-Tables

Table S1. Bacterial strains and plasmids used in this work

Bacterial strains	Description	Source of reference
Streptococcus pneumoniae		1
Cp1015	non-capsulated and βL susceptible laboratory strain	
D39	virulent βL-susceptible laboratory strain	NCTC 7466
R6	non-capsulated and βL susceptible laboratory strain	ATCC BAA-255
9V3	serotype-9V variant of the Spain^{9V}-3 international clone	ATCC 700671
Cba-12	βLR serotype-14 variant of the Spain^{9V}-3 clone	2
Cba-19	βLR serotype-14 variant of the Spain^{9V}-3 clone	2
Cba-28	βLR serotype-14 variant of the Spain^{9V}-3 clone	2
Cba-52	βLR serotype-14 variant of the Spain^{9V}-3 clone	2
Cba-54	βLR serotype-14 variant of the Spain^{9V}-3 clone	2
Cba-56	βLR serotype-14 variant of the Spain^{9V}-3 clone	2
Cba-62	βLR serotype-14 variant of the Spain^{9V}-3 clone	2
pbp2b^{19}	Cp1015 with *pbp2b* from Cba-19, PIP resistant	This work
pbp1a^{19}	Cp1015 with *pbp1a* from Cba-19, CTX resistant	This work
pbp2x^{19}	Cp1015 with *pbp2x* from Cba-19, CTX resistant	This work
pbp2b^{19}, *pbp1a*^{19}	Cp1015 with *pbp2b* and *pbp1a* from Cba-19, Pip and CTX resistant	This work
pbp2b^{19} *pbp2x*^{19}	Cp1015 with *pbp2b* and *pbp2x* from Cba-19, PIP and CTX resistant	This work
pbp2b^{19}*pbp2x*^{19} *pbp1a*^{19}	Cp1015 with *pbp2b*, *pbp1a* and *pbp2x* from Cba-19, PIP and CTX resistant	This work
pbp2b^{28}	Cp1015 with *pbp2b* from Cba-28 , PIP resistant	This work
pbp1a^{28}	Cp1015 with *pbp1a* from Cba-28, CTX resistant	This work
pbp2x^{28}	Cp1015 with *pbp2x* from Cba-28, CTX resistant	This work
pbp2b^{28} *pbp1a*^{28}	Cp1015 with *pbp2b* and *pbp1a* from Cba-28, PIP and CTX resistant	This work
pbp2b^{28} *pbp2x*^{28}	Cp1015 with *pbp2b* and *pbp2x* from Cba-28, PIP and CTX resistant	This work
pbp2b^{28} *pbp2x*^{28} *pbp1a*^{28}	Cp1015 with *pbp2b*, *pbp1a* and *pbp2x* from Cba-28, PIP and CTX resistant	This work
pbp2b^{9V3}	Cp1015 with *pbp2b* from 9V3, PIP resistant	This work
pbp2x^{9V3}	Cp1015 with *pbp2x* from 9V3, CTX resistant	This work
pbp2b^{9V3} *pbp2x*^{9V3}	Cp1015 with *pbp2b* and *pbp2x* from 9V3, Pip and CTX resistant	This work
pbp2b^{9V3} *pbp2x*^{9V3} p*bp1a*^{9V3}	Cp1015 with *pbp2b*, *pbp1a* and *pbp2x* from 9V-3, PIP and CTX resistant	This work
pbp2b^{28} *pbp2x*^{12}	Cp1015 with *pbp2b* from Cba-28, *pbp2x* from Cba-12, PIP and CTX resistant	This work
pbp2b^{28}*pbp2x*^{19}	Cp1015 with *pbp2b* from Cba-28, *pbp2x* from Cba-19, PIP and CTX resistant	This work
pbp2b^{28}*pbp2x*^{52}	Cp1015 with *pbp2b* from Cba-28, *pbp2x* from Cba-52, PIP and CTX resistant	This work
pbp2b^{28}*pbp2x*^{54}	Cp1015 with *pbp2b* from Cba-28, *pbp2x* from Cba-54, PIP and CTX resistant	This work
pbp2b^{28}*pbp2x*^{56}	Cp1015 with *pbp2b* from Cba-28, *pbp2x* from Cba-56, PIP and CTX resistant	This work
pbp2b^{28} *pbp2x*^{62}	Cp1015 with *pbp2b* from Cba-28, *pbp2x* from Cba-62, PIP and CTX resistant	This work
pbp2b^{28} *pbp2x*^{9V3}	Cp1015 with *pbp2b* from Cba-28, *pbp2x* from 9V-3, PIP and CTX resistant	This work
D39 *pbp2b*^{9V3}	D39 with *pbp2b* from 9V-3, virulent and PIP resistant	This work
D39 *pbp2b*^{9V3} *pbp2x*^{19}	D39 with *pbp2b* from 9V3 and *pbp2x* from Cba-19, virulent, PIP and CTX resistant	This work
D39 *pbp2b*^{9V3} *pbp2x*^{19} *pbp1a*^{19}	D39 with *pbp2b* from 9V-3 and *pbp2x* and *pbp1a* from Cba-19, virulent, PIP and CTX resistant	This work
pbp2b ^{28} *pbp1a* ^{28} A2	Cp1015 with *pbp2b* and an internal fragment (from 599 to 1002) of *pbp1a* from Cba-28 , PIP and CTX resistant	This work
pbp2b ^{28} *pbp1a* ^{28} A3	Cp1015 with *pbp2b* and an internal fragment (from 881 to 1294) of *pbp1a* from Cba-28 , PIP and CTX resistant	This work
pbp2b ^{28} *pbp2x* ^{28} X4	Cp1015 with *pbp2b* and an internal fragment (from 1286 to 2253) of *pbp2x* from Cba-28 , PIP and CTX resistant	This work
Cp1015 *pbp2bHA*::pEVP3::*pbp2b'*	CpCp1015 strain expressing PBP2b fused to the HA tag, CLM^{R}	This work
Cp1015 *pbp2xHA*::pEVP3::*pbp2x'*	Cp1015 strain expressing PBP2x fused to the HA tag, CLM^{R}	This work
Cp1015 *pbp1aHA*::pEVP3::*pbp1a'*	Cp1015 strain expressing PBP1a fused to the HA tag, CLM^{R}	This work
Cp1015 *pbp2b*^{28}*HA*::pEVP3::*pbp2b*^{28}, *Cp1015 pbp2b*^{28}	Cp1015 *pbp2b*^{28} strain expressing PBP2b^{28} fused to the HA tag, CLM^{R} Cp1015 *pbp2b*^{28} strain expressing PBP2x fused to the HA tag, CLM^{R}	This work This work

pbp2xHA::pEVP3::*pbp2x'*		
Cp1015 pbp2b^{28}	Cp1015 *pbp2b*^{28} strain expressing PBP1a fused to the HA tag, CLM^{R}	This work
pbp1aHA::pEVP3::*pbp1a'*		
Cp1015 pbp2b^{28} *pbp2x*^{2} *pbp1a*^{28} *HA*::pEVP3::*pbp1a*^{28}'	Cp1015 triple *pbp* mutant expressing PBP1a^{28} fused to the HA tag, CLM^{R}	This work
Cp1015 pbp2b^{28} *pbp2x*^{28} *pbp1a*^{28} *HA*::pEVP3::*pbp2b*^{28}'	Cp1015 triple *pbp* mutant expressing PBP2b^{28} fused to the HA tag, CLM^{R}	This work
Cp1015 pbp2b^{28} *pbp2x*^{28} *pbp1a*^{28} *HA*::pEVP3::*pbp2x*^{28}'	Cp1015 triple *pbp* mutant expressing PBP2x^{28} fused to the HA tag, CLM^{R}	This work
Escherichia coli		
DH5α	used to propagate the pGEM-pbp plasmid	Invitrogen
Top10	used to propagate the pGFPTOPO plasmid	Invitrogen
XL1 Blue MR	used to co-transformed pBT and pTRG recombinant plasmids in order to test protein-protein interactions.	Stratagene

Plasmids

pGEMT-easy	cloning vector	Promega
pCRTOPO 2.1	cloning vector	Invitrogen
pBT-LGF2	interaction control plasmid encoding the dimerization domain (40 amino acids) of the Gal4 transcriptional activator protein	
pTRG-Gal11	interaction control plasmid encoding a domain (90 amino acids) of the mutant form of the Gal11 protein.	Stratagene
pBT	contains a 3.2 kb-size bait plasmid that carries a low-copy p15A replication origin and confers chloramphenicol resistance. The plasmid encodes the full-length bacterial phage λ cI protein under the control of the IPTG-inducible lac-UV5 promoter.	Stratagene
pTRG	contains a 4.4 kb-size target plasmid that carries the low-copy ColE1 replication origin and confers tetracycline resistance. The plasmid directs transcription of the amino-terminal domain of RNA polymerase α subunit through a multiple cloning site at the 3′ end of the α subunit gene and is under the control of the IPTG-inducible, tandem promoter lpp/lac-UV5.	Stratagene
pGEM-pbp	contains the *pbp2b* coding sequence cloned into the pGEMT-easy vector	This work
pGFPTOPO	contains a *gfpmut3* copy under control of a pneumococcal promoter into the pCRTOPO 2.1 vector	This work
pBT-PBP2bwt	interaction plasmid encoding the full-length coding sequence of pbp2b gene obtained from Cp1015 wt strain	This work
pBT-PBP2b*	interaction plasmid encoding the full-length coding sequence of pbp2b gene obtained from Cba-28 clinical strain	This work
pTRG-ftsZ	interaction plasmid encoding the full-length coding sequence of ftsZ gene obtained from Cp1015 wt strain	This work
pTRG-PBP2xwt	interaction plasmid encoding the full-length coding sequence of pbp2x gene obtained from Cp1015 wt strain	This work
pTRG-PBP2x*:	interaction plasmid encoding the full-length coding sequence of pbp2x gene obtained from Cba-28 clinical strain	This work
pAT18	*E.coli-S.pneumoniae* shuttle vector, LacZα, SPC^{R}	3
pEVP3	*S. pneumoniae* insertion vector, CLM^{R}	4
pCM18	source of the *gfpmut3* gene	5
pAT18 *pbp2b*^{28}*-gfp*	pAT18 containing *pbp2b*^{28} fused to the *gfp* gene	This study
pAT18 *pbp-gfp*	pAT18 containing *pbp2b* fused to the *gfp* gene	This study
pEVP32bHA	pEVP3 containing the last 420 bp of *pbp2b* fused to the HA sequence tag	This study
pEVP32xHA	pEVP3 containing the last 342 bp of *pbp2x* fused to the HA sequence tag	This study
pEVP31aHA	pEVP3 containing the last 317 bp of *pbp1a* fused to the HA sequence tag	This study
pEVP32b^{28}HA	pEVP3 containing the last 420 bp of *pbp2b*^{28} fused to the HA sequence tag	This study
pEVP32x^{28}HA	pEVP3 containing the last 342 bp of *pbp2x*^{28} fused to the HA sequence tag	This study
pEVP31a^{28}HA	pEVP3 containing the last 317 bp of *pbp1a*^{28} fused to the HA sequence tag	This study

References: βLR, β-lactam resistant; CTX, cefotaxime; PIP, piperacillin; CLM: chloramphenicol; SPC, spectinomycin; Cba, Córdoba; 9V3, Spain^{9V}-3 clone.

1) Morrison, D. A., Lacks, S. A., Guild, W. R. and Hageman, J. M. (1983). Isolation and characterization of three new classes of transformation-deficient mutants of Streptococcus pneumoniae that are defective in DNA transport and genetic recombination. J. Bacteriol.156, 281-290.

2) Albarracin Orio AG, Cortes PR, Tregnaghi M, Pinas GE, & Echenique JR (2008) A new serotype 14 variant of the pneumococcal Spain9V-3 international clone detected in the central region of Argentina. J Med Microbiol 57(Pt 8):992-999.

3) Trieu-Cuot, P., et al., Shuttle vectors containing a multiple cloning site and a *lacZ* alpha gene for conjugal transfer of DNA from *Escherichia coli* to gram-positive bacteria. Gene, 1991. 102(1): p. 99-104.
4) Claverys JP, Dintilhac A, Pestova EV, Martin B, Morrison DA. 1995. Construction and evaluation of new drug-resistance cassettes for gene disruption mutagenesis in *Streptococcus pneumoniae*, using an *ami* test platform. Gene 164: 123-8
5) Hansen, M.C., et al., Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. Microbiology, 2001. 147(Pt 5): p. 1383-91.

Table S2. Fitness cost analysis of double *pbp2b pbp2x* mutants obtained by transformation of the pbp2b28 with different *pbp2x* alleles from clinical strains

pbp mutant	*pbp2x* donor clinical strain	cefotaxime MIC (µg/ml)	relative fitness in vitro[a,b]	SD	95% CI
pbp2b^{28}	28	0.015	0.65	0.02	0.62-0.68
pbp2b^{28} *pbp2x*^{12}	12	0,05	0,81	0,05	0.69-0.93
pbp2b^{28} *pbp2x*^{19}	19	0,15	0,86	0,05	0.74-0.98
pbp2b^{28} *pbp2x*^{28}	28	0,15	0,86	0.01	0.84-0.87
pbp2b^{28} *pbp2x*^{52}	52	0,08	0,64	0,05	0.52-0.76
pbp2b^{28} *pbp2x*^{54}	54	0,1	0,74	0,04	0.64-0.84
pbp2b^{28} *pbp2x*^{56}	56	0,05	0,72	0,05	0,60-0,84
pbp2b^{28} *pbp2x*^{62}	62	0,15	0,85	0,05	0.73-0.97
pbp2b^{28} *pbp2x*^{9V3}	Spain^{9V}-3	0,1	0,85	0,05	0.73-0.97

[a] Relative competitive fitness is given by the ratio of the number of generations of the resistant and susceptible CP1015 strains.
[b] Relative fitness in vitro was determined as described in Material and Methods.
References: 9V3; Spain^{9V}-3 international clone. CI, confidence intervals. SD: standard deviation

Table S3. Contribution of internal fragments of *pbp1a* and *pbp2x* to resistance and fitness compensation

pbp mutant	*pbp1a* fragment transformed	*pbp2x* fragment transformed	cefotaxime MIC (µg/ml)	relative fitness in vitro[b,c]	SD	95% CI
pbp2b^{28}	-	-	0,015	0.65	0.02	0.62-0.68
pbp2b^{28} *pbp1a*^{28}*A2*	599 to 1002	-	0,12	0,96	0,05	0,83-1.08
pbp2b^{28} *pbp1a*^{28}*A3*	881 to 1294	-	0,15	0,70	0,05	0,58-0.82
pbp2b^{28} *pbp2x*^{28}*X4*	-	1286 to 2253	0,15	0,64	0,05	0,52-0.76

Table S4. Primers used in this work

Primer Sequence	Target gene	Primer function
FX1 5'-CTTTCATAAGTATCTGGACATGG	*pbp2x*	Amplifying fragment X1 of *pbp2x* to transform Cp1015*pbp2b*[28]
RX1 5'-AGGACTGGAGGGGGCTGGAA	*pbp2x*	Amplifying fragment X1 of *pbp2x* to transform Cp1015*pbp2b*[28]
FX2 5'-AGGATCGTCTGGGTAATATTGT	*pbp2x*	Amplifying fragment X2 of *pbp2x* to transform Cp1015*pbp2b*[28]
Rx2 5'-GTCGCATCCGCTATTTTGAAT	*pbp2x*	Amplifying fragment X2 of *pbp2x* to transform Cp1015*pbp2b*[28]
Fx3 5'-GACTTTGTTTGGCGTGATATCC	*pbp2x*	Amplifying fragment X3 of *pbp2x* to transform Cp1015*pbp2b*[28]
Rx3 5'-GCAATAGCTGTAAAGGCACG	*pbp2x*	Amplifying fragment X3 of *pbp2x* to transform Cp1015*pbp2b*[28]
Fx4 5'-TGTTTATGATTCCACTGCAA	*pbp2x*	Amplifying fragment X4 of *pbp2x* to transform Cp1015*pbp2b*[28]
RX4 5'- CTTCTCTCGAGTTATTAGTCTCCTAAAGTTAAT GTAATTTTTTTA	*pbp2x*	Amplifying fragment X4 of *pbp2x* to transform Cp1015*pbp2b*[28]
Fa1 5'-GCTTCTTCGACCACAGGGGG	*pbp1a*	Amplifying fragment A1 of *pbp1a* to transform Cp1015*pbp2b*[28]
Ra1 5'-CAGATAAGACCAAGTTTCGG	*pbp1a*	Amplifying fragment A1 of *pbp1a* to transform Cp1015*pbp2b*[28]
Fa2 5'-TACCTCAGTTAGCCTTGCTG	*pbp1a*	Amplifying fragment A2 of *pbp1a* to transform Cp1015*pbp2b*[28]
Ra2 5'-TACGACCGTAGATGCGACTT	*pbp1a*	Amplifying fragment A2 of *pbp1a* to transform Cp1015*pbp2b*[28]
Fa3 5'-CTGGGATGGATGTTTACACA	*pbp1a*	Amplifying fragment A3 of *pbp1a* to transform Cp1015*pbp2b*[28]
Ra3 5'-TGACATTTCGTGATTGTTGA	*pbp1a*	Amplifying fragment A3 of *pbp1a* to transform Cp1015*pbp2b*[28]
Fa4 5'-TGTTTATGATTCCACTGCAA	*pbp1a*	Amplifying fragment A4 of *pbp1a* to transform Cp1015*pbp2b*[28]
Ra4 5'-CGTGTACCTACATCTGAAAATTC	*pbp1a*	Amplifying fragment A4 of *pbp1a* to transform Cp1015*pbp2b*[28]
Fa5 5'-TACGGAGCAAGTAGTGAAAAAAT	*pbp1a*	Amplifying fragment A5 of *pbp1a* to transform Cp1015*pbp2b*[28]
Ra5 5'-GCTTCCTTCAGACAGATACG	*pbp1a*	Amplifying fragment A5 of *pbp1a* to transform Cp1015*pbp2b*[28]
F2bdh 5'-CGGGATCCATGAGACTGATTTGTATGAGAA	*pbp2b*	Two hybrid system assay
R2bdh 5'-ATCACCTCGAGATTCATTGGATGGTATTTT	*pbp2b*	Two hybrid system assay
F2xdh 5'-GAGGATCTATGAAGTGGACAAAAAGAG	*pbp2x*	Two hybrid system assay
R2xdh 5'-GGATCTATGAAGTGGACAAAAAGAG	*pbp2x*	Two hybrid system assay
FftsZdh 5'-CGGGATCCATGACATTTTCATTTGATACAG	*ftsZ*	Two hybrid system assay
RftsZdh 5'-ATCACCTCGAGACGATTTTTGAAAAATGGAGGT	*ftsZ*	Two hybrid system assay
FB1 5'- TGCGATATCTGGTTTGTCCA	*pbp2b*	*pbp2b* gene sequencing
RB1 5'- TTGGAGAACTGATGCTCACA	*pbp2b*	*pbp2b* gene sequencing
FB2 5'- TGATGCTAGTGGAAAACCTT	*pbp2b*	*pbp2b* gene sequencing
RB2 5'- GGCTTCTGCTTCTTCCGCTG	*pbp2b*	*pbp2b* gene sequencing
FB3 5'- TGCCTGGCATTAGTATTTCT	*pbp2b*	*pbp2b* gene sequencing
RB3 5'- ATAGGGAATGAACCGTAAGC	*pbp2b*	*pbp2b* gene sequencing
FB4 5'- GTTGACGCCTGATTCCTTGG	*pbp2b*	*pbp2b* gene sequencing
RB4 5'- CCATAAATGCCTTCAACAAT	*pbp2b*	*pbp2b* gene sequencing
FB5 5'-TGAATCTACTGGATTTGTTCCC	*pbp2b*	*pbp2b* gene sequencing
RB5 5'- TCAAACGTCCACAAATAGAACAA	*pbp2b*	*pbp2b* gene sequencing

F2bf 5'-CTCGAGATGAGACTGATTTGTATGAGAA	*pbp2b*	*construction of pbp2b-GFP*
R2bf 5'-ATCACCTCGAGATTCATTGGATGGTATTTT	*pbp2b*	*construction of pbp2b-GFP*
Fgfp3 5'-CTATCCCGGGCCAAAATTTGTTTGATTTGTA TCTTAAAATTTTGTATAATAGGTAGAAAAGAGGAAGGAA ATAATAAATGGTCGACATGCGTAAAAGGAGAAGAACTTTTCAC	*gfp*	*construction of pbp2b-GFP*
Rgfp3 5'-CCTACCCGGGATTATTAAGGCCTTTTGTATAGTTCA TCCATGCCATG	*gfp*	*construction of pbp2b-GFP*
F2xtag 5`-CTAGTCTAGACTAGCCTATGCCCAGTGTCAAGGA	*pbp2x*	*construction of pbp2x-HA*
R2xHA 5-TATTATCCGCTCGAGTCAGTTAAGCATAATCTGGA ACATCGTAAGGATAGTCTCCTAAAGTTAATGTAATTTTTTTAATG	*pbp2x*	*construction of pbp2x-HA*
F1a-tag 5-CTAGTCTAGACTAGCGCTGCCAAAGTTTACCGCTCT	*pbp1a*	*construction of pbp1a-HA*
R1aHA 5- ATTATCCGCTCGAGTCAGTTAAGCATAATCTGG AACATCGTAAGGATATGGTTGTGCTGGTTGAGGATT	*pbp1a*	*construction of pbp1a-HA*
R2bHA 5-TATTATCCGCTCGAGTCAGTTAAGCATAATCTGGAA CATCGTAAGGATAATTCATTGGATGGTATTTTTGATACAG	*pbp2b*	*construction of pbp2b-HA*
F2btag 5-CTAGTCTAGACTAGTCGTGTGGCTCCTCGTATTG-3	*pbp2b*	*construction of pbp2b-HA*