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Supplemental information

Interspecies recombination, not *de novo* mutation, maintains virulence after β-lactam resistance acquisition in *Streptococcus pneumoniae*

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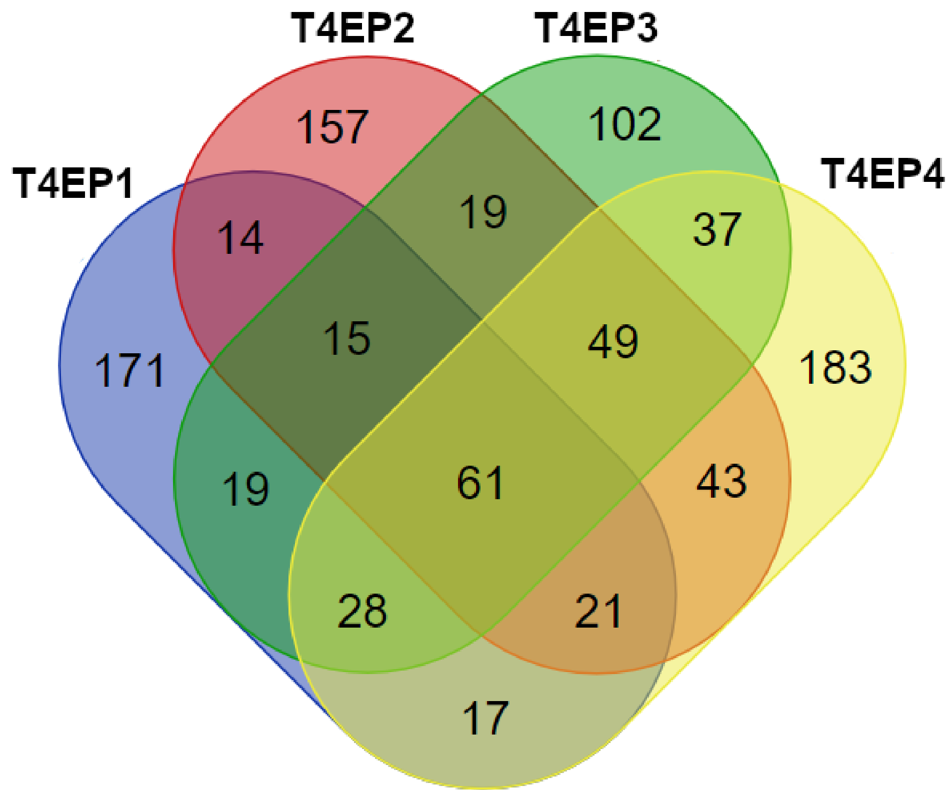


Figure S1. Genome variations between in vitro-evolved populations. Venn diagram illustrates unique versus shared positional variations in the TIGR4 genome between penicillin-passaged populations T4EP1 through T4EP4. Numbers in overlapped areas represent the number of shared positional variations (SNPs and indels) detected in the genome sequences of the overlapping populations at any of four distinct timepoints during passaging (Day 7, 14, 21, and 32). Unique variations are listed within the non-overlapping spaces of each population.

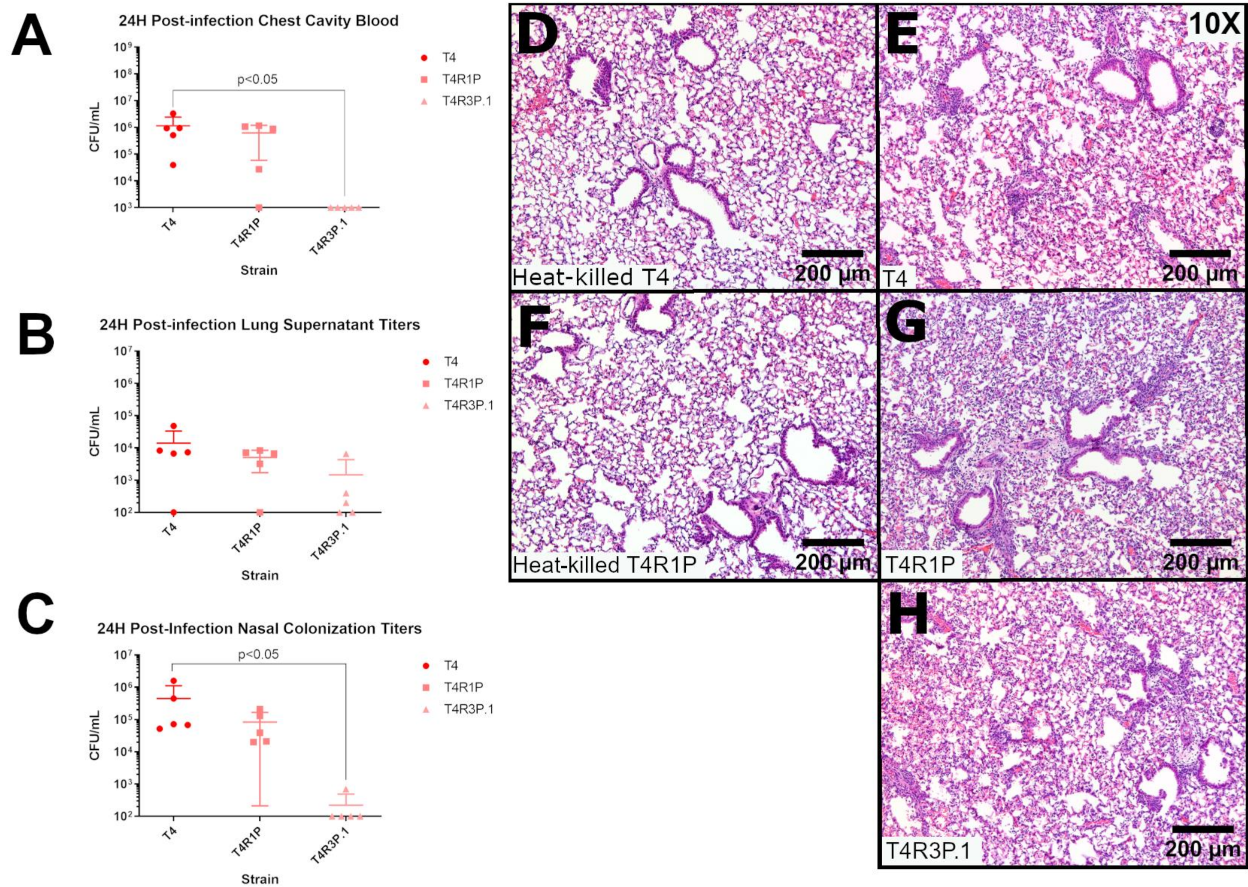


Figure S2. Bacterial burden in the blood and lungs and nasal colonization 24-hours post-intranasal challenge and pulmonary histopathology of murine lungs infected with recombinant mutants vs. respective wild-type strains. Pneumococcal titers from chest cavity blood (A), lung tissue (B), or nasal passages (C) were obtained 24 hours after intranasal infection with T4, the first-round recombinant T4R1P, or the third-round recombinant T4R3P.1 (n=5 mice/group). (D-H) Representative images of right lobar sections 24 hours post-infection are shown at 10× magnification for heat-killed T4 (D), T4 (E), heat-killed T4R1P (F), T4R1P (G), and T4R3P.1 (H) strains used in the respective infected mice (n=5 mice/ non–heat-killed strains, and n=3 mice/heat-killed strains).

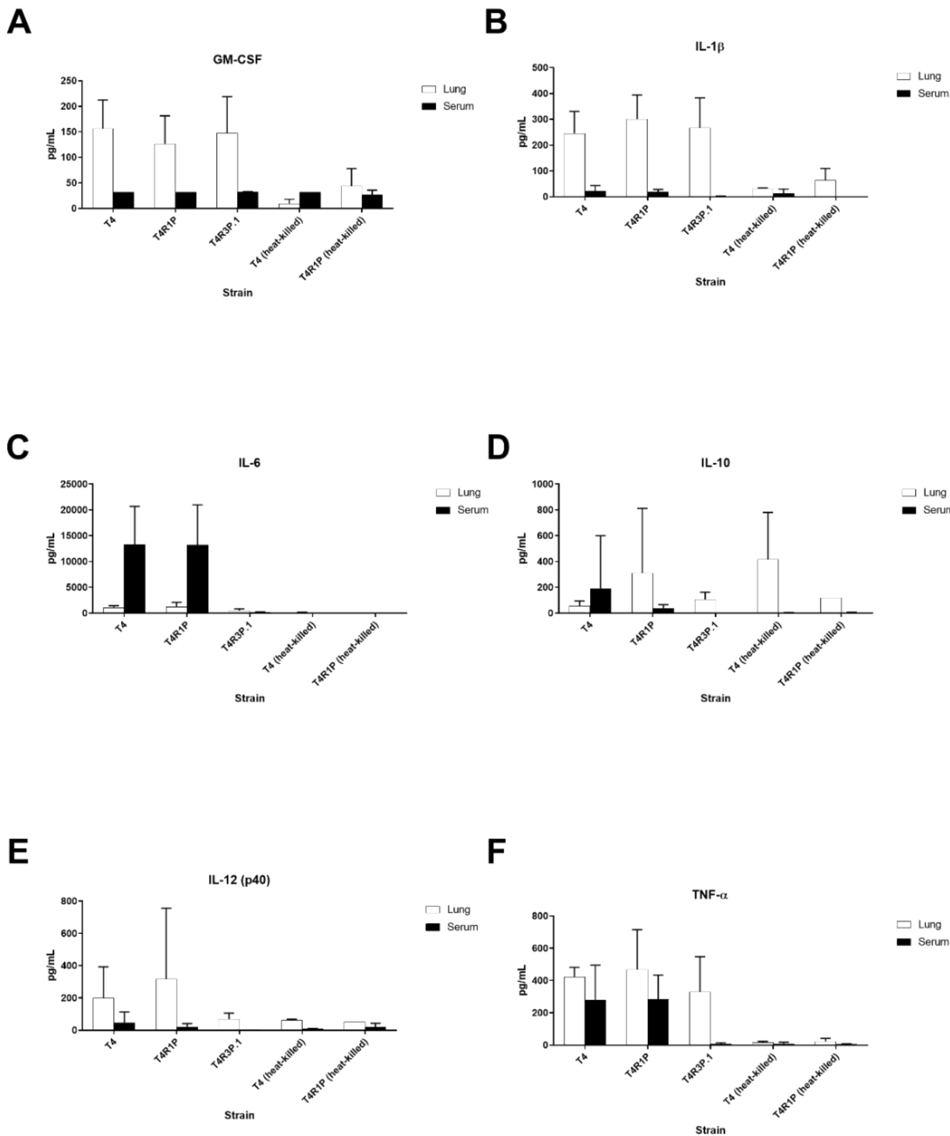


Figure S3. Cytokine profiles in collected serum and lung tissue in a murine intranasal infection model 24 hours post-infection. Concentrations in the serum or lung tissue of infected mice for GM-CSF (A), IL-1β (B), IL-6 (A), IL-10 (A), IL-12 (p40) (E), and TNF-α (F) are shown for T4 and recombinant pneumococcal strains T4R1P and T4R3P.1, as well as heat-killed counterparts. Results are based on five mice (biological replicates) per non–heat-killed strain, with serum and lung samples run in technical duplicate. Results for heat-killed strains were performed in biological triplicate.

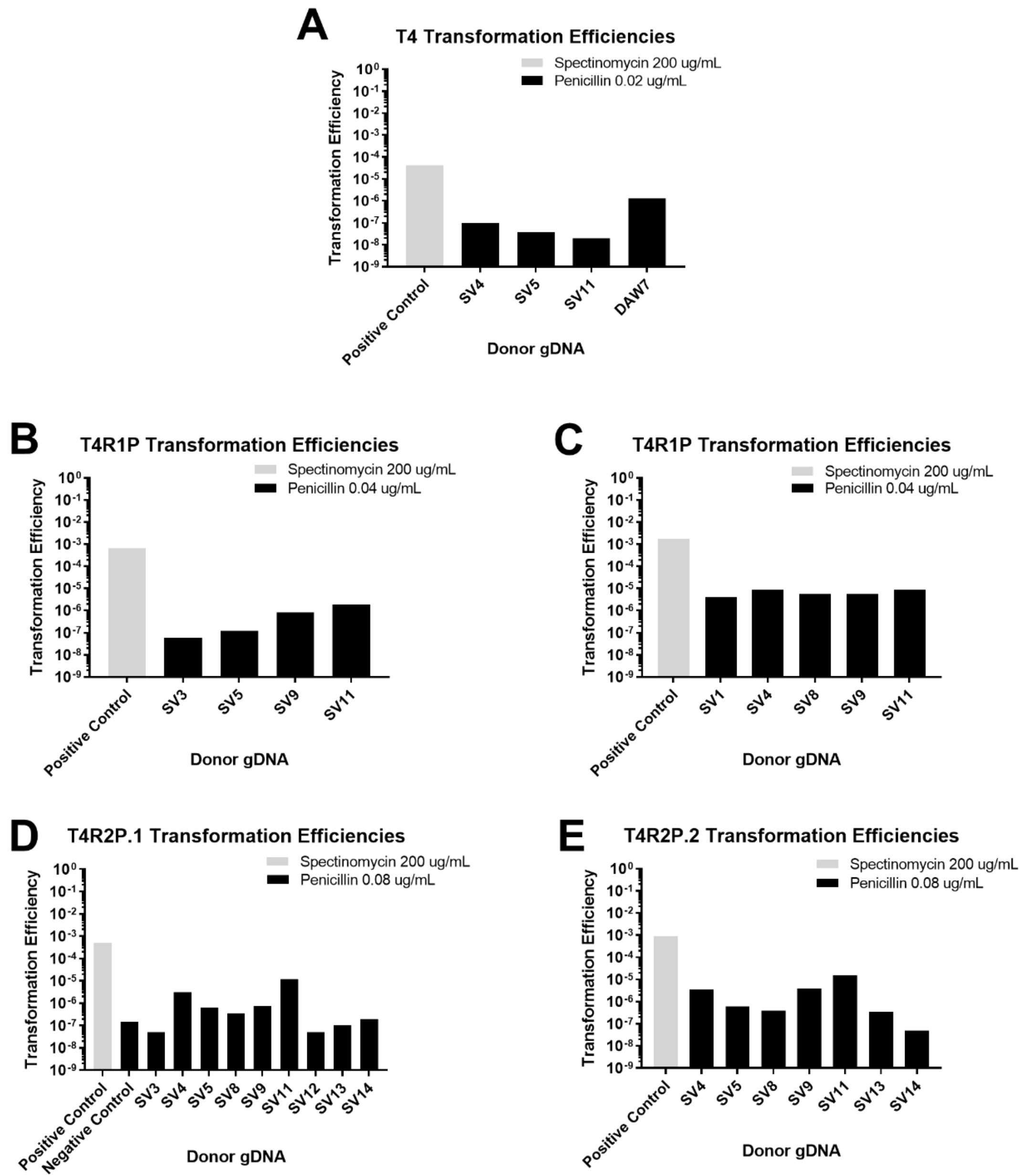
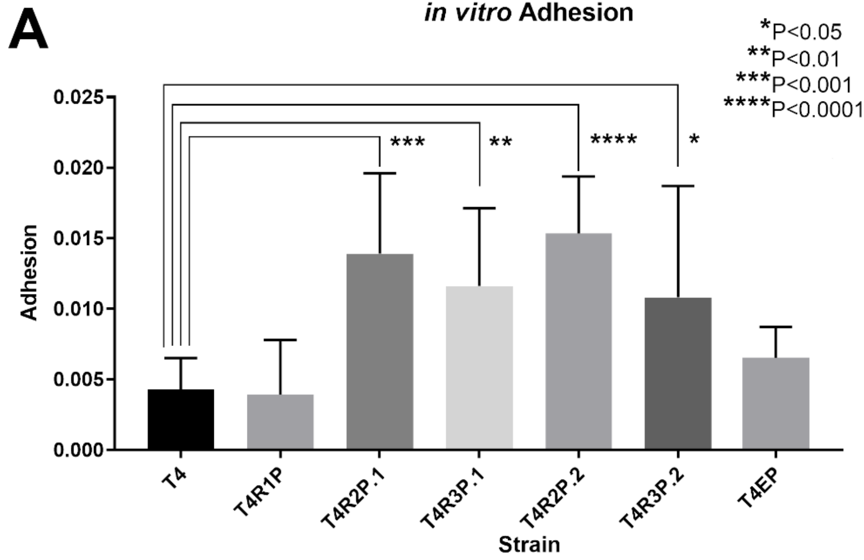
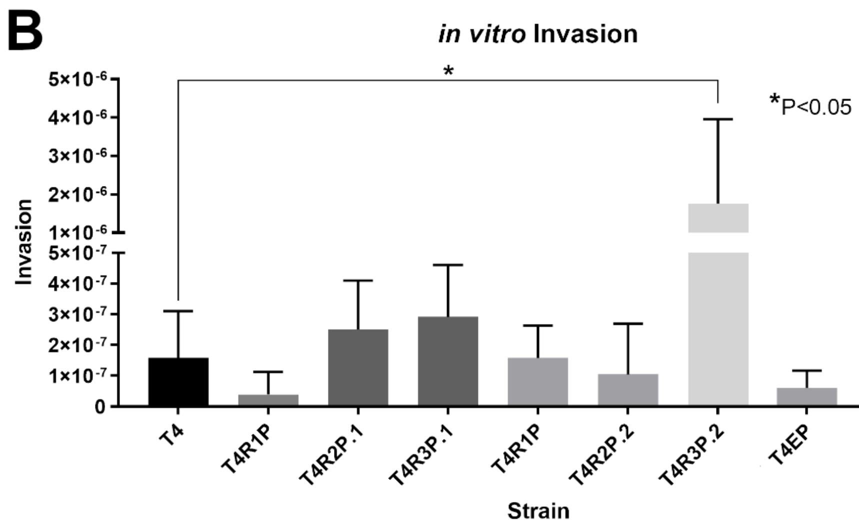


Figure S4. Transformation efficiencies of the parental TIGR4 strain with genomic DNA from viridans group streptococci. Transformation efficiency was calculated as the ratio of the number of recombinant CFUs selected on penicillin-containing blood agar plates to the total number of CFUs. (A) First-round transformation efficiencies, second-round transformation efficiencies (B, C), and third-round transformation efficiencies of the T4 parental strain were calculated. Positive-control transformation efficiencies were generated from Tn-Seq library gDNA.


A
in vitro Adhesion
*P<0.05
**P<0.01
***P<0.001
****P<0.0001
Adhesion
0.025
0.020
0.015
0.010
0.005
0.000
***
**
****
*
T4
T4R1P
T4R2P.1
T4R3P.1
T4R2P.2
T4R3P.2
T4EP
Strain


B
in vitro Invasion
*
*P<0.05
Invasion
5×10⁻⁶
4×10⁻⁶
3×10⁻⁶
2×10⁻⁶
1×10⁻⁶
5×10⁻⁷
4×10⁻⁷
3×10⁻⁷
2×10⁻⁷
1×10⁻⁷
0
T4
T4R1P
T4R2P.1
T4R3P.1
T4R1P
T4R2P.2
T4R3P.2
T4EP
Strain

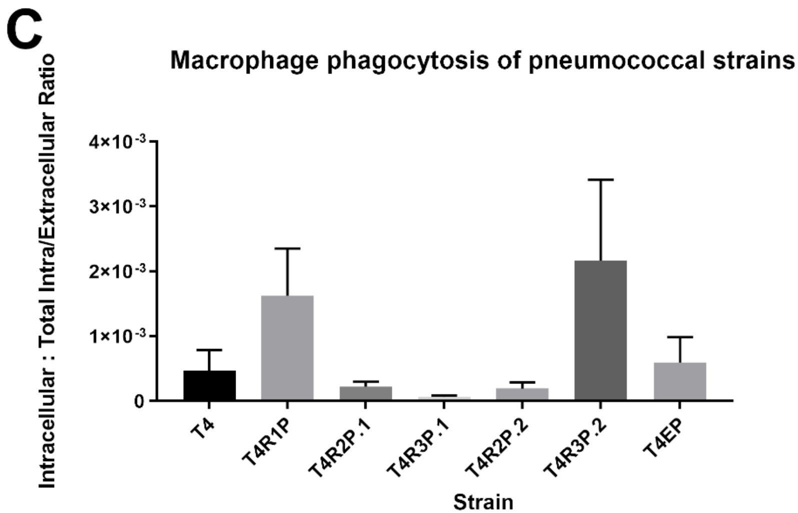

C
Macrophage phagocytosis of pneumococcal strains
Intracellular : Total Intra/Extracellular Ratio
4×10⁻³
3×10⁻³
2×10⁻³
1×10⁻³
0
T4
T4R1P
T4R2P.1
T4R3P.1
T4R2P.2
T4R3P.2
T4EP
Strain

Figure S5. Adhesion, invasion, and macrophage phagocytosis assays for recombinant pneumococcal strains versus parental strains and experimentally evolved populations. (A) Adhesion assays using A549 murine lung epithelial cells were performed to examine the adhesion of recombinant, *in vitro*–evolved, and WT strains. Adhesion ratio was calculated as the ratio of the number of adhered CFUs to the total number of CFUs, which was the sum of adherent and nonadherent CFUs. (B) Invasion assays using A549 murine lung epithelial cells were performed to examine the invasive ability of the same strains as in (A). Invasion ratio was calculated as the ratio of the number of adhered CFUs to the total number of CFUs at the time of plating. The total bacterial number expressed as CFUs was the sum of invaded (engulfed) and remaining bacteria in the supernatant. In both the adhesion and invasion assays, each strain was measured in 3 biological replicates. (C) Recombinants and their respective WT strains were incubated with activated J774A.1 murine macrophage cells at an approximate MOI of 50. Macrophage-killing assays were conducted in three biological replicates. The recombinants and experimentally evolved strains were compared to their respective parental strains via the Mann-Whitney test.

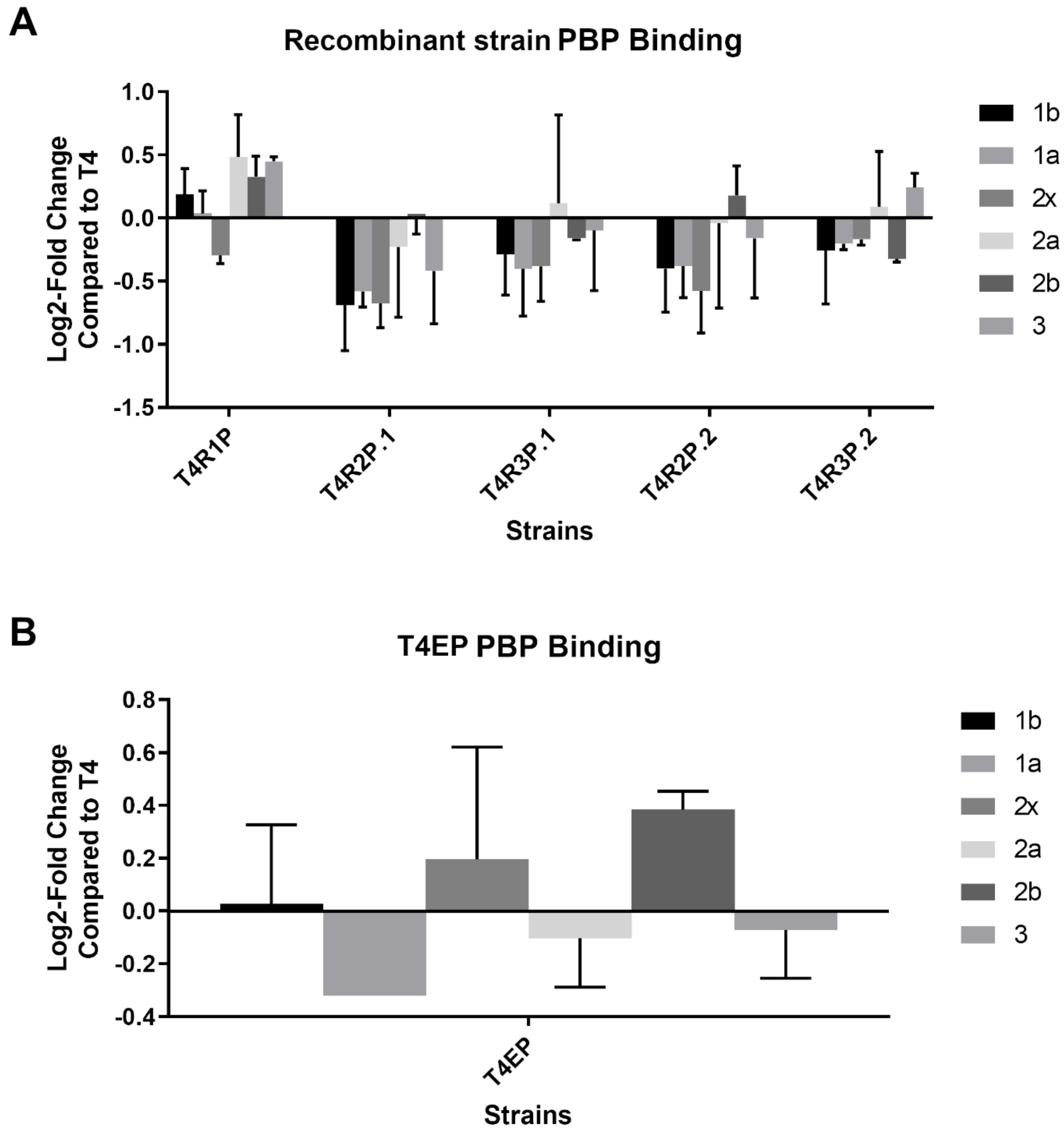


Figure S6. **BOCILLIN FL–binding assay in recombinant strains, parental strains, and experimentally evolved pneumococci**. The T4-derived recombinants (A) and experimentally evolved T4EP (B) were transiently exposed to BOCILLIN FL, and the fluorescent band intensities for six PBPs were visualized and quantified. Differences in the affinity of BOCILLIN FL for PBPs from the derivatives was compared to that for the parental strain and is expressed as log-fold change in band intensity.

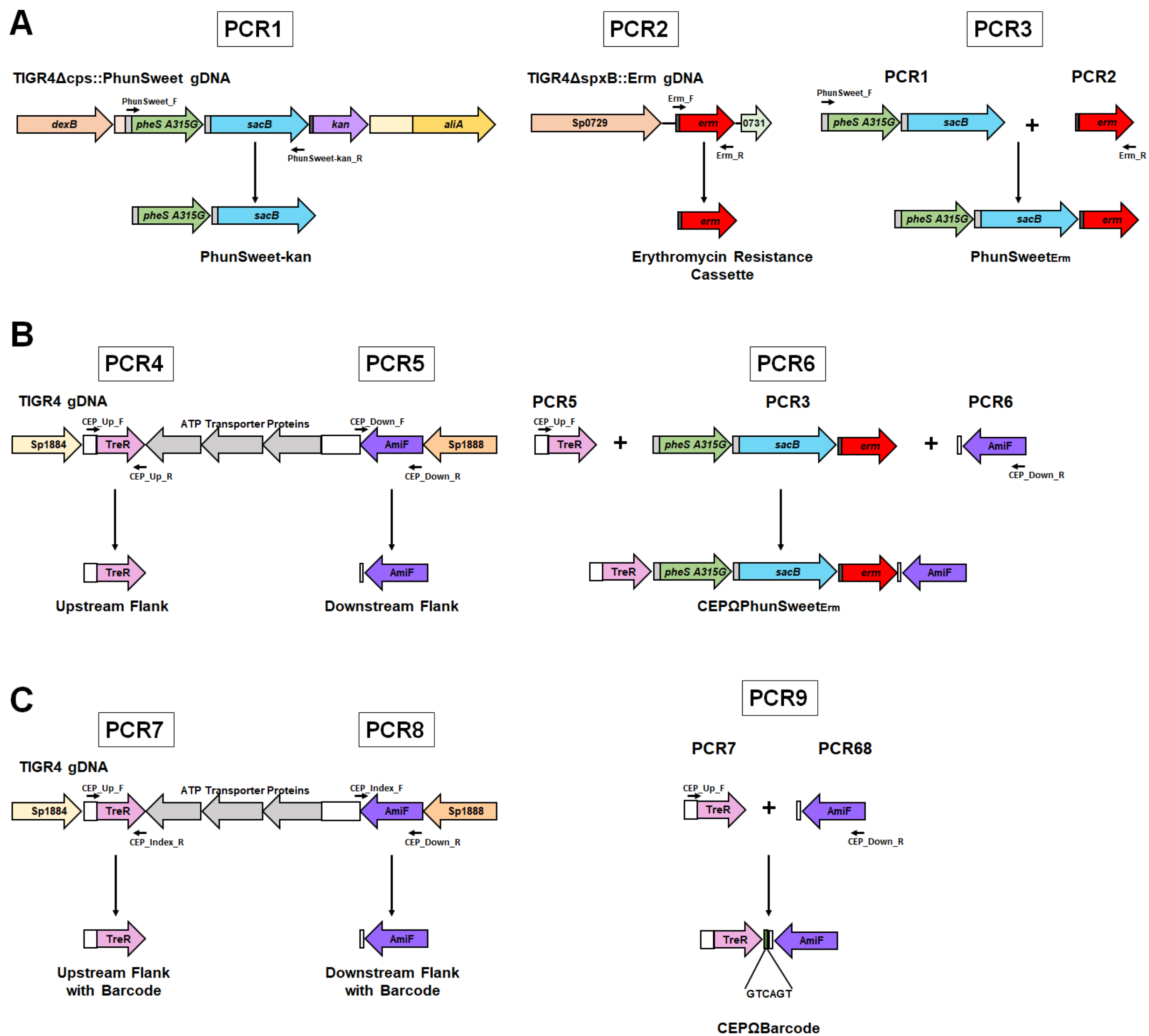


Figure S7. PCR reactions used in the construction of barcoded pneumococcal T4 derivative strains. PCR products corresponding to (A) the PhunSweet-kan and erythromycin resistance fragments were amplified and fused via SOE reaction generating the PhunSweetErm cassette. (B) Upstream and downstream segments flanking the functionally inert CEP locus were PCR amplified and fused to the PhunSweetErm cassette for use as donor fragment in pneumococcal transformation. (C) The SOE PCR product containing the unique sequence identifiers flanked by TreR and AmiF CEP-adjacent genes was generated after amplification utilizing primers with unique index sequences.

Table S1. Penicillin minimum inhibitory concentration to strains used in this study.

Strain	ID	Description	MIC (µg/mL)
			Penicillin
T4	T4	Parent	0.010
T4R1P	T4+SV5	1st recombinant	0.032
T4R2P.1	T4+SV5+SV3	2nd recombinant	0.047
T4R3P.1	T4+SV5+SV3+SV11	3rd recombinant	0.19
T4R2P.2	T4+SV5+SV8	2nd recombinant	0.047
T4R3P.2	T4+SV5+SV8+SV4	3rd recombinant	0.38
T4trDAW7	T4trDAW7	Pneumococcal recombinant	0.19
T4EP1	T4 Pen Pop1	Experimentally-evolved population	0.023
T4EP2	T4 Pen Pop2	Experimentally-evolved population	0.012
T4EP3	T4 Pen Pop3	Experimentally-evolved population	0.016
T4EP4	T4 Pen Pop4	Experimentally-evolved population	0.016
T4EP5	P132C	Experimentally-evolved population	0.032
T4EP6	P232C	Experimentally-evolved population	0.032
T4EP7	P332C	Experimentally-evolved population	0.023
T4EP8	P432C	Experimentally-evolved population	0.032
N132C	N132C	Penicillin-free evolved population	0.012
N232C	N232C	Penicillin-free evolved population	0.012
N332C	N332C	Penicillin-free evolved population	0.006
N432C	N432C	Penicillin-free evolved population	0.012
DAW7	DAW7	Pneumococcal clinical isolate	1
SV2	SV2	VGS clinical isolate	0.75
SV3	SV3	VGS clinical isolate	1.5
SV4	SV4	VGS clinical isolate	2
SV5	SV5	VGS clinical isolate	4
SV8	SV8	VGS clinical isolate	>32
SV11	SV11	VGS clinical isolate	6

Table S2. Primers used in the construction of barcoded T4 derivative strains.[a]

Name	Sequence
PhunSweet_F	CAATTAACTTTACAAATTCCCACTATTAAGG
PhunSweet-kan_R	GTTTGCTTCTAAGTCTTATTTCCACTTTTGTGCCCGTGCTTATAAGGG
Erm_F	GGAAATAAGACTTAGAAGCAAAC
Erm_R	CCAAATTTACAAAAGCGACTC
CEP_Up_F	GCAAATCTTTGGCTTCTTGTTCAAATTTTC
CEP_Up_R	ATAGTGGGAATTTGTAAAGTTAATTGGATCTGGTGTCTCAGTCTTTTATTTCTTGCG
CEP_Down_F	GAGTCGCTTTTGTAAATTTGGGGTCGCTGAAACTTCTATCGTCAAGAAG
CEP_Down_R	CGTCCTTTCTTTTTGATGTTCAAAGC
CEP_Index1_F	AAAGACTGAGACACCAGATC**GTCAGT**TAATTCCCATAAAAATTGACATGGAAATTATAAA
CEP_Index1_R	CATGTCAATTTTTATGGGAATTA**ACTGAC**GATCTGGTGTCTCAGTCTTTTATTTCTTGCG
CEP_Index2_F	AAAGACTGAGACACCAGATC**CCAGCA**TAATTCCCATAAAAATTGACATGGAAATTATAAA
CEP_Index2_R	CATGTCAATTTTTATGGGAATTA**TGCTGG**GATCTGGTGTCTCAGTCTTTTATTTCTTGCG
CEP_Index3_F	AAAGACTGAGACACCAGATC**CAGTCA**TAATTCCCATAAAAATTGACATGGAAATTATAAA
CEP_Index3_R	CATGTCAATTTTTATGGGAATTA**TGACTG**GATCTGGTGTCTCAGTCTTTTATTTCTTGCG
CEP_Index4_F	AAAGACTGAGACACCAGATC**AGTAGT**TAATTCCCATAAAAATTGACATGGAAATTATAAA
CEP_Index4_R	CATGTCAATTTTTATGGGAATTA**ACTACT**GATCTGGTGTCTCAGTCTTTTATTTCTTGCG
CEP_Index5_F	AAAGACTGAGACACCAGATC**TCATCA**TAATTCCCATAAAAATTGACATGGAAATTATAAA
CEP_Index5_R	CATGTCAATTTTTATGGGAATTA**TGATGA**GATCTGGTGTCTCAGTCTTTTATTTCTTGCG
CEP_Index6_F	AAAGACTGAGACACCAGATC**GTTCAT**TAATTCCCATAAAAATTGACATGGAAATTATAAA
CEP_Index6_R	CATGTCAATTTTTATGGGAATTA**ATGAAC**GATCTGGTGTCTCAGTCTTTTATTTCTTGCG
CEP_Index7_F	AAAGACTGAGACACCAGATC**AGCAGG**TAATTCCCATAAAAATTGACATGGAAATTATAAA
CEP_Index7_R	CATGTCAATTTTTATGGGAATTA**CCTGCT**GATCTGGTGTCTCAGTCTTTTATTTCTTGCG
CEP_Index8_F	AAAGACTGAGACACCAGATC**CAAGTA**TAATTCCCATAAAAATTGACATGGAAATTATAAA
CEP_Index8_R	CATGTCAATTTTTATGGGAATTA**TACTTG**GATCTGGTGTCTCAGTCTTTTATTTCTTGCG
CEP_Index9_F	AAAGACTGAGACACCAGATC**TCGTCC**TAATTCCCATAAAAATTGACATGGAAATTATAAA
CEP_Index9_R	CATGTCAATTTTTATGGGAATTA**GGACGA**GATCTGGTGTCTCAGTCTTTTATTTCTTGCG
CEP_Index10_F	AAAGACTGAGACACCAGATC**GTTAAG**TAATTCCCATAAAAATTGACATGGAAATTATAAA
CEP_Index10_R	CATGTCAATTTTTATGGGAATTA**CTTAAC**GATCTGGTGTCTCAGTCTTTTATTTCTTGCG

[a]nucleotides in blue are a unique 6-nucleotide index per primer set

Table S3. Competency and β-lactam Resistance in select Streptococcal species

Species	Natural Competence	β-lactam Resistance	Reference
S. pneumoniae	Yes	Yes	(Centers for Disease Control and Prevention, 2019; Jensen *et al.*, 2015)[6,15]
S. pyogenes	No	No	(Mashburn-Warren *et al.*, 2012; Oppegaard *et al.*, 2020)[20,22]
S. agalactiae	No	No	(Berg et al., 2012; Fluegge *et al.*, 2004)[1,25]
Viridans group streptococci			
S. sanguinis	Yes	Yes	(Gaustad, 1985; Pasquantonio et al., 2012)[2,3]
S. salivarius	Yes	Yes	(Fontaine et al., 2010; Pasquantonio *et al.*, 2012)[3,4]
S. mutans	Yes	Yes	(Li et al., 2001; Pasquantonio *et al.*, 2012)[4,5]
S. mitis	Yes	Yes	(Konig et al., 1998; Nakayama and Takao, 2003; Pasquantonio *et al.*, 2012; Salvadori et al., 2018)[4,6,7,16]
S. oralis	Yes	Yes	(Pasquantonio *et al.*, 2012; Ronda et al., 1988; Sibold *et al.*, 1994; Todorova *et al.*, 2015)[4,8,13,29]

SUPPLEMENTAL REFERENCES

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