Supporting Information

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SI Materials and Methods

Bacterial Strains and Mutants. A *Streptococcus pneumoniae* TIGR4 mutant lacking *sp_1119* was constructed using overlap extension PCR. Primers koSP19-F3 (5′-GAC AAA AAT GAA GGA GTG ATT ACA TGA-3′) and koSP19-R2 (5′-GTT TTA CAC GAT TAT TTC CTC CCG TTA AAT AAT AG-3′) were used to amplify the erythromycin *erm* cassette from shuttle vector pMU1328 (1); nucleotides complementary to the *sp_1119*-flanking regions were added at the beginning and end of the cassette. Primer pairs koSP19-F1 (5′-GCC ATT AAC AGT GCT AGT TTT G-3′)/R1.2 (5′-CAC TGG TTC ATT TTT GTC CTC CTG AAA ATG-3′) and koSP19-F4 (5′-AGG AAA TAA TCG TGT AAA ACC AGG AAA TTG-3′)/R4.2 (5′-CCG AGA CGA GTT CCT TGC CCA-3′) were used to amplify up- and downstream flanking regions of *sp_1119* from TIGR4, deleting all of the gene and adding nucleotides complementary to the *erm* cassette. The three fragments were pieced together using the primers koSP19-F2 (5′-TTG GAT GTT GAA GAA GAT TTG G -3′) and koSP19-R3.2 (5′-CCC TTA CGA ATA AAG GAA AGA ACA CT-3′). This PCR construct, with the *erm* cassette replacing the *sp_1119* locus, was transformed into TIGR4 with selection for erythromycin resistance (1 μg/mL), creating strain TIGR4*sp_1119*. An unencapsulated TIGR4*sp_1119* mutant was created by transforming TIGR4*cps* (2) with genomic DNA from TIGR4*sp_1119* and selecting for erythromycin and kanamycin (200 μg/mL) resistance. All mutants were back-transformed and confirmed by PCR.

Staphylococcus aureus strains used in this study include 8325–4, 8325–4*spa* (a mutant lacking protein A, provided by Tim Foster, Trinity College, Dublin), 502A, Newman, MW2, SH1000, USA300, Reynolds, Becker, and COL (provided by Barry Kreiswirth, University of Medicine and Dentistry of New Jersey, Newark, NJ). An unmarked, in-frame deletion mutant of *rocA*, which encodes P5CDH, was constructed in strain 502A using methods previously described by Bae and Schneewind (3) using pKOR1-*rocA*. The resulting strain, 502A*rocA*, was complemented with the single-copy integration plasmid pCL55 using methods previously described by Lee et al. (4). The coding sequence and presumptive native promoter of *rocA* were amplified from the 502A chromosome for cloning into pCL55 using primer pairs 1845rocA-F (TAC TTC CAA TCC AAT GGA TAA TGA CCT ACC ATC CGA AAC)/1846rocA-R (TTA TCC ACT TCC AAT GTA GAT GAG AAA CTC ATG AGC G). This construct then was used to create strain 502A*rocA*::pCL55-*rocA*$^+$ using the pCL55-specific protocol (4). Deletion and complementation were confirmed by PCR and Western blot.

Mouse Model of Nasopharyngeal Colonization. Six-to-eight-week-old female C57BL/6 and B6.129-S2-Igh-6tm1Cgn/J (μMT; Jackson Laboratories) mice were housed in accordance with University of Pennsylvania Institutional Animal Care and Use Committee protocols. μMT mice do not produce specific antibody because of a targeted mutation in the heavy-chain locus of IgM (5). The murine model of pneumococcal nasopharyngeal colonization has been described previously (6). Mice were inoculated intranasally (i.n.) without anesthesia with 10 μL of PBS containing 10^7 cfu of PBS-washed, midlog-phase bacteria. Inocula were plated to confirm the dose. Where indicated, mice received a second inoculation at week 2 following the initial inoculation and were challenged at week 7, at which time no pneumococci remain in the nasopharynx (6). Control animals were subjected to the same protocol but were mock-colonized with 10 μL of PBS.

Intranasal challenge of *S. aureus* 502A consisted of 10^8 cfu of PBS-washed, midlog-phase bacteria in 10 μL PBS. Twenty-four hours after inoculation, animals were killed, sera were collected from cardiac punctures, and nasal lavages were obtained as previously described (6) for quantitative culture on BBL CHROMagar Staph aureus (BD Diagnostics). The lower limit of detection for bacteria in lavages was 10 cfu/mL Where indicated, preimmune sera were collected by tail bleed before bacterial colonization.

Western Blot Analysis. Cell lysates of *S. aureus* were prepared by incubation with lysostaphin (0.1 mg/mL) (Sigma) for 20 min at 37 °C, followed by boiling at 100 °C for 10 min in Laemmli sample buffer. Equal loading was confirmed by measurement of total protein with the Micro BCA protein assay (Pierce Chemical Co.) and staining by Ponseau S (0.2%, Sigma). Proteins were separated by 1D SDS/PAGE on a 10% Tris·HCl gel (Bio-Rad) and then were transferred to a PVDF membrane (Thermo Scientific) by using the Trans-Blot SD semidry transfer system (Bio-Rad) at 18 V. For 2D SDS/PAGE, isoelectric focusing (pI 4.7–5.9) was carried first out in a Protean IEF cell (Bio-Rad), using 7-mm ReadyStrips, according to the manufacturer's instructions. Gels from 2D SDS/PAGE were partially transferred (18 V for 18 min, compared with 36 min for 1D SDS/PAGE), and after transfer the remaining gel was stained using Coomassie brilliant blue R-250 (Fisher Scientific) to obtain a stained gel and membrane pair. Membranes were blocked with either 1% BSA (Sigma) or 0.5% goat serum (Sigma) before incubation with primary antibody, either mouse sera (1:500 dilution) or rabbit antisera (1:30,000 dilution), as indicated. Bound antibody was detected by using anti-mouse or anti-rabbit secondary antibody conjugated to alkaline phosphatase (Sigma) and 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (Fisher) development.

Mass Spectrometry. Cross-reactive spots identified by Western blot analysis were excised from the corresponding Coomassie-stained gel for mass spectrometry. Following digestion with trypsin, sample peptides were separated using an HPLCy C_{18} column and a linear trap quadrupole ion-trap mass spectrometer (Thermo Scientific). Mascot software was used to search bacterial databases for sequence similarities. Cutoffs were assigned as a protein score of >70 with a unique peptide value of >2.

Measurement of Serum Antibody Binding by Flow Cytometry. Similar to previously described assays (7), 200 μL of midlog-phase bacteria were pelleted and washed in Hanks buffer (Invitrogen) supplemented with 5% (vol/vol) FCS (HFCS; HyClone). For *S. aureus*, a protein A mutant, 8325–4*spa*, was used to prevent nonspecific antibody binding by protein A. Primary antibodies (diluted 1:20 for sera from *S. pneumoniae*-colonized mice or 1:200 for rabbit-specific antisera) were added to the reaction mixtures and incubated at 37 °C for 60 min with rotation. After washing in HFCS, the cells were incubated with FITC-conjugated secondary antibody against either mouse or rabbit IgG (Sigma) for 60 min at 4 °C in the dark at a dilution of 1:200. After washing, the cells were fixed in 200 μL of 1% paraformaldehyde for flow cytometric analysis. A total of 50,000 bacterial cells per sample were analyzed on a BD FACS Calibur flow cytometer (BD Biosciences), and groups were compared using FlowJo software (Tree Star). The percentage of FITC-positive cells was calculated by subtracting background from a no-primary antibody control.

Detection of Antigen-Specific Serum IgG by ELISA. Immulon 2HB 96-well plates (Thermo Scientific) were coated in buffer containing 0.015 M Na_2CO_3 and 0.035 M $NaHCO_3$ overnight at 4 °C with either midlog phase *S. pneumoniae* TIGR4 at OD_{620nm} = 0.1 or purified protein antigens at 0.5 μg/mL Plates were blocked with 1% BSA (Sigma-Aldrich) in PBS and washed between steps with PBS containing Brij-35 (0.05%). Serum samples were added in doubling serial dilutions and incubated overnight at 4 °C. Bound antibody was detected by anti-mouse IgG alkaline phosphatase-conjugated secondary antibody and *p*-nitrophenyl phosphatase (Sigma-Aldrich) development. The absorbance was read at 415 nm, and geometric mean titers were calculated based on the sample dilution at which A_{415nm} = 0.1.

Recombinant Antigen Purification and Generation of Specific Antisera. The coding sequences for each of the four candidate antigens were amplified from the chromosomal DNA of *S. aureus* 8325–4 or *S. pneumoniae* TIGR4, as appropriate, using the following primer pairs:

P5CDH-F (5′-TAT ACA TAT GGT AGT AGA ATT TAA AAA TGA ACC TGG-3′) and P5CDH-R (5′-TAT AGG TAC CGA ACA TTT CTG AAA CAA CCT TTT GTT CTA AG-3)

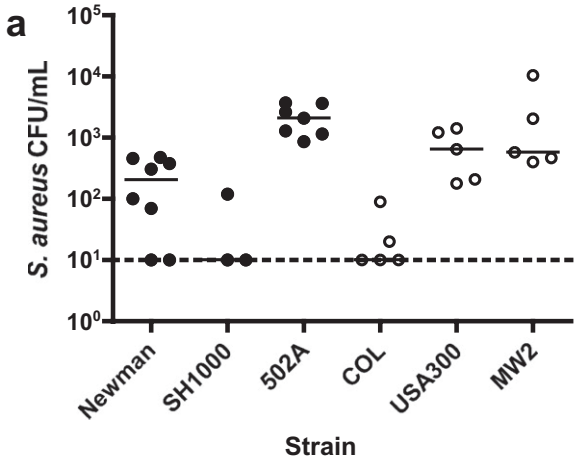
SP19-F (5′-TAT ACA TAT GAC AAG ATA TCA GAA TTT AGT AAA TGG AAA ATG GA-3′) and SP19-R (5′-TAT AGG TAC CTT TCA CAT CAA AAA CAA TGG ATT TGA CAT TTG TCA TC-3′)

DLDH-F (5′-TAT ACA TAT GGT AGT TGG AGA TTT CCC AAT TGA AAC-3′) and DLDH-R (5′-TAT AGG TAC CCA TTG TAT GGA TTG GGT ATC CGA TAG C-3′)

SP61-F (5′-TAT ACA TAT GGC CTT AGA AGT AAT TAT GCC AAA AGC C-3′) and SP61-R (5′-TAT AGG TAC CTT TTT TCT TAG GTG AAT GGA TGG CCA TTC C-3′)

Gel-purified PCR fragments were ligated into the pET29b expression vector (Novagen). Recombinant antigens then were expressed in *E. coli* BL21(DE3) and purified under native conditions through Ni^{2+} affinity chromatography as described by the manufacturer (Qiagen). As appropriate, his-tags were removed by a thrombin cleavage capture system (Novagen) and dialysis. Polyclonal rabbit sera to each purified recombinant antigen were prepared commercially (Cocalico Biologicals).

1. Achen MG, Davidson BE, Hillier AJ (1986) Construction of plasmid vectors for the detection of streptococcal promoters. *Gene* 45:45–49.
2. Nelson AL, et al. (2007) Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. *Infect Immun* 75:83–90.
3. Bae T, Schneewind O (2006) Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. *Plasmid* 55:58–63.
4. Lee CY, Buranen SL, Ye Z-H (1991) Construction of single-copy integration vectors for *Staphylococcus aureus*. *Gene* 103:101–105.
5. Kitamura D, Roes J, Kühn R, Rajewsky K (1991) A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene. *Nature* 350: 423–426.
6. Roche AM, King SJ, Weiser JN (2007) Live attenuated *Streptococcus pneumoniae* strains induce serotype-independent mucosal and systemic protection in mice. *Infect Immun* 75:2469–2475.
7. Roche AM, Weiser JN (2010) Identification of the targets of cross-reactive antibodies induced by *Streptococcus pneumoniae* colonization. *Infect Immun* 78:2231–2239.



b

S. aureus strain	Median (CFU/mL)	Approx. Range (log_{10})	Percent colonized
Newman	205	2	75%
SH1000	10	1	33%
502A	2100	1	100%
COL	10	1	40%
USA300	650	1.5	100%
MW2	580	2	100%

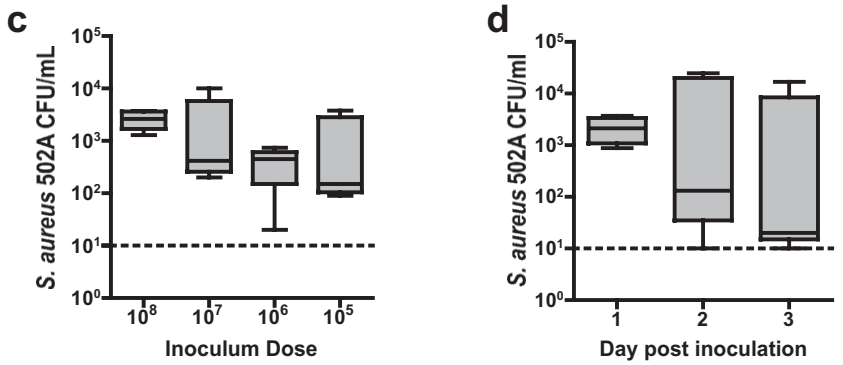


Fig. S1. Optimization of the *S. aureus* 502A colonization model in C57BL/6 mice. (*A*) Colonization density of the *S. aureus* strains indicated at day 1 after intranasal inoculation with 10^8 cfu in 10 μL PBS. Closed circles represent methicillin-sensitive strains. Open circles represent methicillin-resistant strains. Solid horizontal lines indicate medians. The dotted horizontal line indicates the lower limit of detection. (*B*) Table of column statistics for each *S. aureus* strain shown in (*A*). (*C*) Optimization of inoculum dose (indicated in cfu) of *S. aureus* 502A nasal colonization in naive C57BL/6 mice assessed in lavages of the upper respiratory tract 1 d after inoculation. (*D*) Time course of *S. aureus* 502A nasal colonization after inoculation with 10^8 cfu. In *C* and *D* box and whiskers plots are for $n \geq 5$ mice per condition, and the horizontal black dotted line indicates the limit of detection.

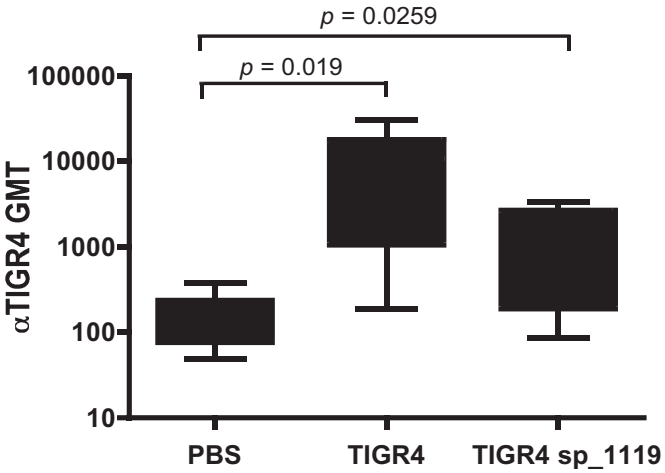


Fig. S2. Box and whiskers plot showing increased anti-pneumococcal antibody titers following colonization with TIGR4 and TIGR4*sp_1119*. Detection of IgG titers (GMT) to whole-cell *S. pneumoniae* TIGR4 in mouse sera after colonization with *S. pneumoniae* TIGR4, *S. pneumoniae* TIGR4*sp_1119*, or sham colonization (PBS), as indicated; $n = 10$ mice per group.

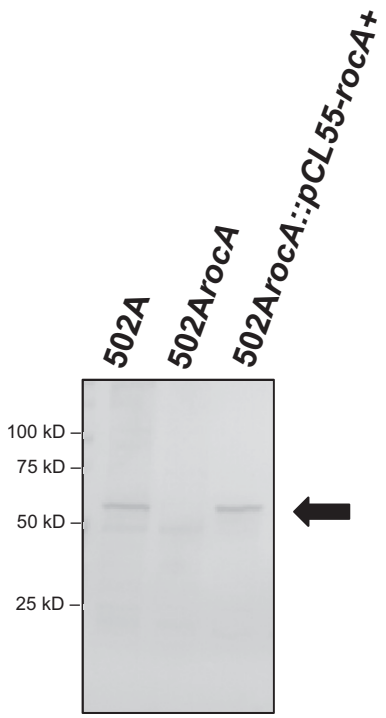


Fig. S3. Deletion and complementation of 1-pyrroline-5-carboxylate dehydrogenase (P5CDH) in *S. aureus* 502A. Detection of P5CDH (arrow) by specific anti-P5CDH sera in a Western blot of whole-cell lysates of the indicated *S. aureus* strains. The wild-type (502A) and complemented strains (502A*rocA*::pCL55-rocA^{+}) express approximately the same level of P5CDH, but the deletion mutant (502A*rocA*) does not express P5CDH.

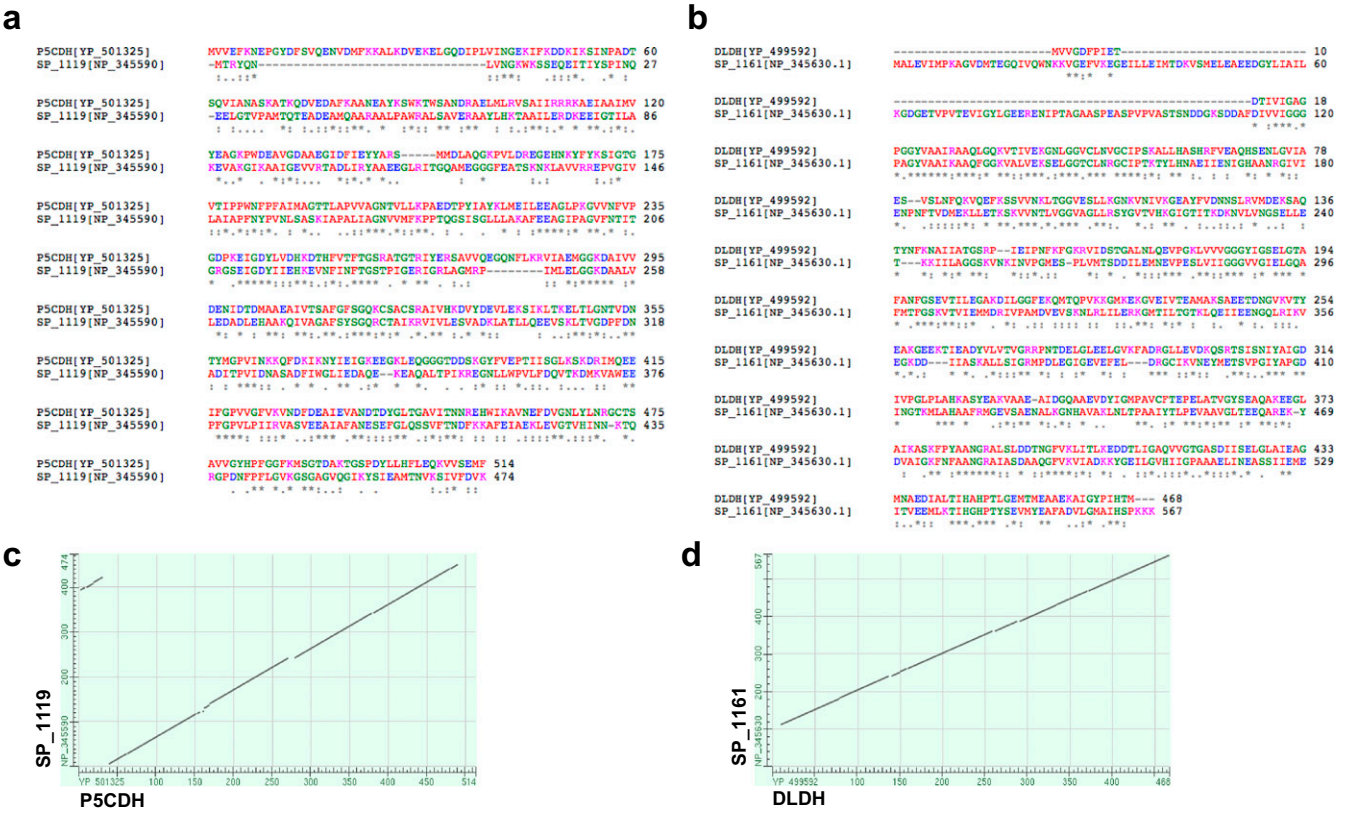


Fig. S4. Amino acid alignments of P5CDH with SP_1119 and DLDH with SP_1161. Amino acid alignments of *S. aureus* P5CDH with *S. pneumoniae* SP_1119 (*A*) and *S. aureus* DLDH with *S. pneumoniae* SP_1161 (*B*), by ClustalW2 (http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=clustalw2). Amino acid color code from ClustalW2 is as follows: red, small; blue, acidic; magenta, basic; green, hydroxyl or sulfhydryl or amine; gray, unusual. (*C* and *D*) Dot matrix amino acid alignments of *S. aureus* P5CDH with *S. pneumoniae* SP_1119 (*C*) and *S. aureus* DLDH with *S. pneumoniae* SP_1161 (*D*) compiled using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).