Supporting Information

Classification

BIOLOGICAL SCIENCES: Microbiology

Title

Pathogen-mediated manipulation of arthropod microbiota to promote infection

Authors

Nabil M. Abraham [1,2Ŧ], Lei Liu [1Ŧ *], Brandon L. Jutras [2,3], Akhilesh K. Yadav [4], Sukanya Narasimhan [1], Vissagan Gopalakrishnan [1,2,5], Juliana M. Ansari [6], Kimberly K. Jefferson [7], Felipe Cava [4], Christine Jacobs-Wagner [2,3,8,9], Erol Fikrig [1,2,9 *]

Supporting Information- Materials and Methods

Mice and ticks

Four to 6 week old female C3H/HeJ and C3H/SCID mice were purchased from the Jackson Laboratory. Animals were housed and handled under the guide for the care and use of laboratory animals of the National Institutes of Health. The animal experimental protocol was approved by the Yale University Institutional Animal Care and Use Committee. All experiments were performed in a Biosafety Level 2 animal facility, according to the regulations of Yale University. *I. scapularis* nymphs were obtained from a tick colony maintained in the laboratory in an incubator at 23° C with 85% relative humidity and a 14/10 h light/dark photo period regimen.

Ethics Statement

All studies with mice were carried out following the animal protocol number 2014-07941 approved by Yale University's Institutional Animal Care and Use Committee (IACUC). The IACUC is governed by applicable Federal and State regulations, including those of the Animal Welfare Act (AWA), Public Health Service (PHS), NIH Office of Laboratory Animal Welfare Assurance Number A3230-01, and the United States Department of Agriculture (USDA), License and Registration Number 16-R-0001, and is guided by the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training.

Anaplasma phagocytophilum infection of mice

The *A. phagocytophilum* isolate NCH-1 (1) used in these studies was maintained through serial passage of infected blood in C3H/SCID mice (2). Quantitative PCR was performed on an aliquot of the blood collected from the SCID mice to quantify and standardize the infectious dose of *A. phagocytophilum*. In separate experiments, *A.*

phagocytophilum was similarly used to infect four to six week old female B6 mice deficient for IFN-γ or IFN-γ receptor (3) purchased from Jackson Laboratories.

Sample preparation and Illumina pyrosequencing of DNA from nymphal guts

Prior to dissection, ticks were surface sterilized with 70% ethanol, followed by a rinse with sterile water. Midguts were dissected with sterile blades, and transferred to 350 µl of RLT plus lysis buffer (Qiagen, Valencia, CA). DNA was extracted from guts of individual fed nymphs (12-16 nymphs) using a genomic DNA extraction kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The V4 variable region (V4) of the bacterial 16S rRNA was amplified from 1 µl of purified genomic DNA from each sample using 12-base barcoded primer sets that were previously described (4). Briefly, 16S rRNA genes were amplified from individual nymphal gut DNA samples, using the universal forward and reverse primers listed in SI Appendix, Table S6, and tagged with a unique 12-base barcode to mark PCR products from individual samples. PCR reactions were conducted using a Five Prime Hot Master Mix (MoBio Laboratories, Carlsbad, CA). PCR amplifications were performed in triplicate for each sample (4). Amplified bacterial 16S rRNA products were purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), quantified using the Nanodrop 2000C (Thermoscientific, Wilmington, DE), pooled together in equal molar concentrations, ethanol precipitated and suspended in 80 µl of sterile RNase and DNAse free water to a concentration of 8-10 ng /µl of each sample. The pooled DNA was submitted to KECK facility at Yale University. Amplicon libraries were sequenced by Illumina MiSeq platform and 250 base pair paired end sequence reads were obtained.

16S rDNA sequence analysis and selection criteria

Illumina paired-end reads were assembled by PANDAseq (5). The quality of sequences were analyzed by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequence with low quality scores (average quality score <0.25) was filtered out by PRINSEQ (6). Data were

analyzed using Quantitative Insights Into Microbial Ecology (QIIME) pipeline (v1.9.1) (7). Chimeric sequences were identified and removed using ChimeraSlayer. Open-reference picking strategies were performed, and sequences reads were clustered against the 2013 release of the Greengenes (8). Sequences with 97% similarity were clustered together and identified as different operational taxonomic units (OTUs), with one sequence per OTU being selected as a representative sequence for further downstream analysis. Each representative OTU sequence was aligned against a core set of reference sequences and taxonomy was assigned. OTUs which only occurring in 100% individual samples were included, in order to eliminate random occurred OTUs. Sequences that did not align to reference genes with 70% similarity threshold were assumed to be non-16S and removed from further analysis. Jackknifed beta diversity estimates (between-sample diversity comparisons) were calculated within QIIME using weighted and unweighted UniFrac (9). From these estimates, principal coordinates were computed to compress dimensionality into two-dimensional principal coordinate analysis (PCoA) plots. ADONIS was tested for statistical significance.

To best determine the bona fide microbiome of the nymphal tick we also included the following exclusion criteria-

1. To eliminate the possibility of contamination, we included a mock extraction of DNA under identical conditions using water, buffers and kits utilized for the experimental samples followed by Illumina Miseq analysis of 16S rRNA reads. The negative control sample provided only a handful of reads (about 413 reads) compared with experimental samples (>30,000 reads) (SI Appendix, Table S1 and Table S2) and did not provide reads corresponding to genera represented in the tick samples.

2. Genera which accounted for less than 1% of the total number of reads for both experimental groups was excluded i.e. If genera ‘X’ in both Ap and Clean represented <1% of OTUs within both groups, X was excluded.

3. Genera that were previously classified as “unknown” which have yet to be taxonomically classified were excluded from the final representation of genera.

4. Genera that were not consistently represented across all experiment groups- Ap, Clean, P1, sP1, D-serine, D-alanine were also excluded.

Antibiotic treatment of mice

One hundred μg of gentamicin was intraperitoneally injected at a concentration of 10 mg/ml (the equivalent of 1 mg/ 20 gm body weight of a mouse) 24 hours prior to placement of nymphs and subsequently once each day for the next 4 days until the nymphs had all fully engorged and detached. Control mice received PBS, following the same regimen.

Sectioning and staining of nymphs

Nymphs, fed for 72 hours, were fixed in Carnoy's fixative for 1 hour, then washed in histological grade alcohol followed by three washes in xylene, and then paraffin embedded. The samples were then sectioned at 4 μm, stained with PAS at the Yale Histology Core Facility as described previously (10) and visualized under a Zeiss Axio YLCW023212 microscope at 63x magnification with ZEN Lite (Carl Zeiss. Thornwood, NY). The thickness of the PAS-positive PM-like layer was assessed in 3 different regions of each microscopic field, and the arithmetic average was calculated. At least 3 fields per section were evaluated, and 10 individual sections were examined in each group. To visualize co-localization of IAFGP to biofilms within the tick guts, nymphs, fed on *Anaplasma* infected C3H/HeJ mice for 72 hours, fixed in Carnoy's fixative for 1 hour, washed in histological grade alcohol followed by three washes in xylene, and then paraffin embedded. The samples were then sectioned at 3 μm. Section pretreatment and immunostaining was performed as described (11). To detect IAFGP, mouse α-IAFGP polyclonal antibody was used (12), and rabbit α -PNAG polyclonal antibody was used to detect biofilm (13, 14). After incubation with primary antibodies, slides were washed and incubated with Alexa-488 labeled α -mouse or Alexa-555 labeled α -rabbit secondary antibodies (Invitrogen, Carlsbad, CA) for 1 hour. All incubation steps were completed in a humidified chamber. Cover slips were mounted along with a ProLong™ Gold antifade reagent (ThermoFisher Scientific, Waltham, MA). The slides were viewed under confocal microscopy using the Leica LSM510 confocal microscope at 63x magnification (Leica Microsystems, Buffalo Grove, IL).

Bacterial strains and growth conditions

Staphylococcus aureus strain SA113 (ATCC 35556), derived from laboratory strain NCTC 8325, and its isogenic mutants were maintained in tryptic soy broth (TSB) or on its respective agar plates. *S. aureus* Δ*icaADBC* isolate is a PNAG-deficient mutant (15). SA113 Δ*tagO* and SA113 pRBtagO is a wall teichoic acid (WTA) mutant and its isogenic WTA complemented strain respectively (16). *Enterococcus faecalis* strain MMH594 (NR-31975), vancomycin-resistant *Enterococcus faecalis* V587 (NR-31979) (BEI Resources, Manassas, VA) and *Enterococcus faecium* ATCC 49624 were maintained in brain heart infusion broth (BHI) or on its respective agar plates. Bacteria were cultured in their respective medium or supplemented with D-amino acids (D-serine or D-alanine) (Sigma, St. Louis, MO) to alter peptidoglycan composition.

Immunoblot analysis

PNAG detection was performed as described using rabbit antisera against PNAG (12). In brief, following incubation of *S. aureus* with proteins or peptides, PNAG was released into the supernatant by boiling in 0.5 M EDTA. Proteins were digested with Proteinase K treatment. Insoluble components were sedimented by centrifugation before loading the supernatant fraction on nitrocellulose membranes for detection by immunoblot. The guts from fed nymphs (*A. phagocytophilum*-infected versus uninfected, or sP1-injected versus P1-injected) were collected and PNAG was extracted as described above. Each sample was pooled from 2 guts and 20 μL from each sample was spotted onto the membrane. The dot blots were analyzed and semi-quantified by ImageJ™ software (National Institutes of Health, Bethesda MD).

Tick RNA isolation, quantitative RT-PCR and Microinjection

RNA was extracted from individual guts by using an RNA isolation kit (Qiagen, Valencia, CA). cDNA was synthesized using the iScript RT-PCR kit (BioRad, Hercules,

CA). qRT-PCR for expression levels of *iafgp*, *peritrophin-1, 2, 3, 4,* and *5*, total bacterial burden using universal bacterial *16S rRNA*, or bacterial specific *16S rRNA* or *23S rRNA* were done using SYBR Green Master Mix (BioRad, Hercules, CA) and primers listed in SI Appendix, Table S6. These data were normalized to tick *actin* (t*Actin*) (17). For RNA interference, double stranded RNA targeting *iafgp* transcripts and dsRNA targeting *peritrophin-1, 2, 3, 4, 5*, and control *dsgfp* RNA were generated using primers listed in SI Appendix, Table S6. Fed nymphal gut cDNA or pCAG-GFP plasmid (www.addgene.org) was used as the template, respectively. dsRNA was injected into the anal pore of nymphal ticks as described (18). In separate experiments, a synthesized peptide derived from IAFGP, named P1, which retains the anti-biofilm properties of IAFGP, was also injected into the anal pore of nymphal ticks. A scrambled P1 (sP1) served as control (12). To examine the role of D-serine *in vivo*, 300 mM D-serine was injected into the anal pore of nymphal ticks; D-alanine served as control. To examine the role of INF-γ in *iafgp* expression, 10 nL of 100 nM mouse INF- γ (R&D Systems, Minneapolis, MN) was injected into anal pore of nymphal ticks and compared to PBS negative control.

Cloning, expression, and purification of IAFGP.

Purified IAFGP-GST was prepared as previously described (12). Briefly, *iafgp*, without its signal sequence, was PCR amplified from pGEMT*iafgp* (19) and cloned into pGEX-6p-3 (GE Healthcare, Piscataway, NJ). Protein expression was induced with 1 mM IPTG and bacteria were lysed using a French pressure cell press at 20,000 pounds per square inch. Following centrifugation, IAFGP-GST was affinity purified using GST Sepharose (GE Healthcare, Piscataway, NJ), eluted and stored in aliquots at −80 °C. Precision protease treatment or dialysis resulted in IAFGP degradation. Therefore, the GST-tagged protein in elution buffer was used for experiments. Recombinant GST was purified from pGEX-6p-3 following the same protocol and used as a control.

Extraction of peptidoglycan and muramidase digestion

Gram positive bacteria- Fifty-milliliter overnight cultures of bacteria- *S. aureus* strain SA113 grown in either tryptic soy broth (TSB) or medium supplemented with 125 mM D-serine (D-serine), 0.1 mg/mL GST-tagged IAFGP recombinant protein (IAFGP) or GST alone (GST) or each *Enterococcus* sp. grown in brain heart infusion broth alone was harvested by centrifugation and resuspended in the PBS. The samples were slowly dropped into an equal volume of boiling 10% (wt/vol) SDS and vigorously stirred ≥2 h and left stirring overnight. The insoluble fractions were recovered by high speed centrifugation (150,000 x g, 15 min, 25 °C) and washed until the fractions were free from SDS. The insoluble fractions were purified for peptidoglycan according to the protocol described by Jonge et al. (20). Purified peptidoglycan was re-suspended in 50 mM $NaPO_4$ buffer, pH 4.9 and treated with 80 µg/mL muramidase (Cellosyl) for 16 h at 37°C. Muramidase digestion was stopped by incubation in a boiling water bath, and coagulated proteins were removed by centrifugation (21,000 x g, 10 min). The supernatants were adjusted to pH 9.0 with sodium borate and reduced with sodium borohydride for 20 min at room temperature. Finally, samples were adjusted to pH 3.5 with orthophosphoric acid and filtered prior chromatographic analysis.

Gram negative bacteria- Five hundred-milliliter overnight cultures of bacteria- *P. aeruginosa* strains PA01 or PA14, and *E. coli* strains CS109 and DV900 were grown in LB media harvested by centrifugation and resuspended in the PBS. Muropeptides were isolated following previously described methods (21-23). In brief, samples were boiled in SDS 10% (wt/vol) for ≥2 h and left stirring overnight, and sacculi were repeatedly washed with MilliQ water by ultracentrifugation (150,000 × g, 15 min, 25 °C) until the fractions were free from SDS. Samples were treated with Pronase E (100 µg/mL, 1 h, 60 °C) for Braun's lipoprotein removal. The reaction was heat-inactivated, and sacculi were further washed by ultracentrifugation as described above. Purified peptidoglycan was re-suspended in 50 mM $NaPO_4$ buffer, pH 4.9 and digested with muramidase (80 µg/mL) for 16 h at 37 °C and heat-inactivated. Coagulated proteins were removed by 10 min of centrifugation at 21000 × g. The supernatants were adjusted to pH 9.0 with sodium borate and reduced with sodium borohydride for 20 min at room temperature. Finally, samples were adjusted to pH 3.5 with orthophosphoric acid.

UPLC and MS/MS peptidoglycan analysis.

The separation and identification of muropeptides, from gram positive bacteria, was performed on an UHPLC system (Agilent 1290 Infinity LC System) hyphenated to an Agilent Q-TOF LC/MS (6550 iFunnel) system (Agilent Technologies, Santa Clara, CA). The chromatographic separation was achieved on RP-18 (Kinetex® 1.7 µm, 100 Å, 150 x 2.1 mm id, Phenomenex) column. The eluents were A: 0.1% formic acid in Milli-Q water and B: 0.1% formic acid in acetonitrile. The gradient was set as follows: 0-3 min 2-5% B, 3-6 min 5-6.8% B, 6-7.5 min 6.8-9% B, 7.5-9 min 9-14% B, 9-10.5 min 14-18.8% B, 10.5-11 min 18.8-20% B, 11-12 min hold at 20% B, 12-13 min 20-40% B, 13-14 min 40-98% B with a flow rate of 0.175 ml/min; 14-17 min hold at 98% B, 17-17.5 min 98-2% B with a flow rate of 0.3 ml/min; and 17.5-21 min hold at 2% B with a flow rate of 0.175 ml/min. Chromatograms were recorded at 204 nm. The QTOF-MS instrument operated in positive ionization mode. For MS/MS experiments fixed collision energy of 40 V was applied. The system was operated in full scan mode from 50 to 1700 m/z with a scan rate of 6 spectra/s for both MS and MS/MS experiments. Data acquisition of MS and MS/MS spectra was performed in Auto-MS/MS mode selecting 3 precursors per cycle. The source conditions were: gas temperature 150 °C, gas flow 16 l/min, nebulizer pressure 35 psi, sheath gas temperature 350 °C, sheath gas flow 11 l/min, capillary voltage 3500 V, nozzle voltage 300 V, fragmentor voltage 380 V, skimmer 45 V and octopole radio frequency voltage 750 V. Structural characterization of muropeptides was done on the basis of their MS data and MS/MS fragmentation pattern which matched with the peptidoglycan composition and structure reported in previous publications (20, 24-26). Data analysis was performed with the software MassHunter Qualitative Analysis B.07.00 (Agilent Technologies). Molar fractions of oligomers (dimers, trimers, tetramers) were determined from the area under the peaks in the chromatogram, and the crosslinking percentage was calculated according to the formula: % Crosslinking = % molar fraction dimers + 2 x (% molar fraction trimers) + 3 x (% molar fraction tetramers). The multipliers in the formula above are used to account for the number of crosslinks per oligomer; for instance, a trimer contains two crosslinks and therefore is multiplied by 2 (27).

Muropeptide binding assay.

Magnetic, streptavidin-coated Dynabeads® (Life Technologies, Grand Island, NY) at 4 mg/mL were washed and separated using a Magnetic Particle Concentrator (DynaMag-Spin, Invitrogen, Carlsbad, CA). The solution was incubated with 1 mg/mL biotinylated recombinant IAFGP-GST or GST (12) protein or 1 mg/mL biotinylated synthetic muropeptides (ThermoFisher Scientific, Waltham, MA) at a 1:2 v/v ratio. Two synthetic muropeptides were chemically synthesized: [Biotin]−L-alanine−D-iso-glutamine−L-lysine−D-alanine−D-alanine and [Biotin]−L-alanine−D-iso-glutamine−L-lysine−D-alanine−D-serine with >86% purity. Subsequent to 30-minute incubation with gentle agitation, samples were separated into the unbound biotinylated protein or muropeptide supernatants and magnetically bound fraction. Samples were washed and resuspended in PBS. 100 µL of endogenous muropeptides isolated from either TSB or 125 mM D-serine supplemented medium were added to the magnetically bound protein-bead solution at a 1:2 volume ratio. Similarly, 1 mg/mL of IAFGP-GST, GST or PBS were added to muropeptide bound beads. When assessing pentapeptide-P1 binding, 1 mg/ml of 6x histidine-tagged P1 and 6x histidine-tagged scrambled-P1 (sP1) were added to the muropeptide-bead solution at a 1:2 volume ratio. After incubation at 4°C for 1 hour with gentle agitation, samples were separated with the magnet and the supernatants were collected. Beads were washed with PBS + 0.1% Bovine Serum Albumin solution and resuspended. Samples were heated at 65°C for 5 minutes to dissociate the streptavidin beads from the biotinylated muropeptide or protein. Supernatants were collected and blotted on a nitrocellulose membrane (Bio-Rad, Hercules, CA). IAFGP-GST was detected using a polyclonal murine primary antibody (12) and GST was detected using a monoclonal murine primary antibody (ThermoFisher Scientific, Waltham, MA). Histidine-tagged peptides were detected using a polyclonal murine primary antibody to the 6x-histidine tag (Clontech, Mountain View, CA). Biotinylated muropeptides or proteins were detected using an infrared (IR)-labeled streptavidin probe (LI-COR, Lincoln, NE). Endogenous muropeptides were detected using a polyclonal goat Wheat Germ Agglutinin antibody (Vector Laboratories, Burlingame, CA). Appropriate LICOR

secondary antibodies were used that would be detected using the LI-COR Odyssey system (LI-COR, Lincoln, NE). Dynabeads® His-Tag isolation beads (Life Technologies, Grand Island, NY) were used, according to manufacture recommended protocols, to assess interactions between chemically synthesized 6x-His tagged IAFGP or P1 peptide variants (ThermoFisher Scientific, Waltham, MA) (with >86% purity) (SI Appendix, Table S5) and muropeptides extracted from *S. aureus* or *E. faecalis* bacterial cells cultured in TSB.

Static biofilm assay.

Biofilm assays were performed as previously described by Christensen et al. (28). Briefly, planktonic cultures of *S. aureus* SA113, *E. faecalis* MMH594, *Pseudomonas aeruginosa* strains PA01 or PA14, and *Escherichia coli* CS109 were diluted in glucose-supplemented tryptic soy broth (1% glucose; TSB/G), brain heart infusion broth (1% glucose; BHI/G) alone or Luria-Bertani broth (1% glucose; LBG) respectively or media supplemented with the appropriate treatment condition to an OD_{600}= 0.015 and distributed into 96 well plates (Corning, NY). Plates were incubated for 18 h at 37°C and bacterial growth in each well was confirmed by the measurement of OD_{600} using a spectrophotometer (BioTek, Winooski, VT). The supernatant was discarded and the wells were washed with water twice. Bacteria associated with the well surface were dried and stained with red-colored safranin dye (for *S. aureus*) or crystal violet dye (for *E. faecalis*, *P. aeruginosa* or *E. coli*). The dye was then dissolved in 33% acetic acid and the absorbance quantified at 415 nm (for *S. aureus*) or 550 nm (for *E. faecalis*, *P. aeruginosa* or *E. coli*) using a spectrophotometer (BioTek, Winooski, VT).

Artificial feeding of nymphs.

Capillary feeding was performed as described earlier (29) with a solution of fluorescein-conjugated 500,000 MW dextran (Molecular Probes, ThermoFisher Scientific, Waltham, MA) (2 μM in PBS/0.2 % sucrose) for 1 h. Whole ticks were then dissected, and guts placed in a drop of ProLong™ Gold antifade reagent (ThermoFisher

Scientific, Waltham, MA), cover-slipped and visualized immediately under a Leica LSM510 confocal microscope.

Scanning electron microscopy

Tick guts were dissected to reveal luminal surface out and were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M sodium cacodylate buffer for 1 hour. The samples were rinsed well in 0.1M sodium cacodylate and were postfixed in 2% osmium tetroxide for 1 hour, then rinsed, dehydrated in an ethanol series 30% to 100% and were critical point dried using Leica 300 with liquid carbon dioxide as transitional fluid. The samples were glued to aluminum stubs, and sputter coated with 15nm gold using an EMS (Electron Microscopy Science, Hatfield, PA) sputter coater. The samples were viewed and digital images acquired in an FEI ESEM (FEI, Hillsboro OR) using 5 - 10kV at a working distance of 12mm.

Transmission electron microscopy.

Bacteria were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH7.4 for 1 hour. Buffer rinsed cells were spun down in 2% agar in 0.1M sodium cacodylate. Chilled blocks were trimmed and post fixed in 1% osmium tetroxide for 1 hour, en bloc stained in aqueous 2% uranyl acetate for another hour, and then rinsed in buffer and dehydrated in an ethanol series from 50% to 100%. The samples were infiltrated with Embed 812 (Electron Microscopy Sciences, Hatfield, PA) epoxy resin and baked overnight at 60°C. Hardened blocks were cut using a Leica UltraCut UC7. 60nm sections were collected on formvar/carbon coated grids and contrast stained using 2% uranyl acetate and lead citrate. Grids were viewed using a FEI Tencai Biotwin TEM at 80Kv. Images were taken using Morada CCD and iTEM software (Olympus, Center Valley, PA).

Following imaging, area measurements of the peptidoglycan layer were conducted on the GNU Image Manipulation Program (GIMP). The program was used to

demarcate two circles- one around each bacterial cell including the peptidoglycan layer and the other around the cell membrane (without the peptidoglycan). The difference in the total number of pixels from the two circles yielded the number of pixels within the peptidoglycan layer. This area, of the peptidoglycan layer, was converted into a percentage. Identical measurements were made across all experimental groups. Analyzed images were of equal magnification.

Fluorescence microscopy experiments.

Overnight cultures of *S. aureus* SA113 bacteria were subcultured for 2 hours in fresh TSB or medium supplemented with GST alone, IAFGP-GST (each at 0.1 mg/mL), 125 mM D-alanine or D-serine or an equivalent volume of PBS control. Cells were harvested and washed with PBS to prevent further treatment. Each sample was resuspended in PBS supplemented with 1 mM $CaCl_2$ and incubated with 2 μg/mL of Wheat-Germ Agglutinin conjugated to TexasRed (ThermoScientific, Waltham, MA) or 5 μg/mL of the lipophilic membrane binding probe- FM® 4-64 (ThermoScientific, Waltham, MA) at room temperature for 10 minutes. Excess stain was removed by washing twice with, and resuspended in PBS. Cell suspensions were spotted on 2% agarose pads containing PBS and phase/epifluorescent microscopy images were acquired using an Eclipse Ti-U microscope (Nikon, Japan) outfitted with an Orca-ER camera (Hamamatsu, Japan). Image processing and quantitative analysis were performed using MATLAB (MathWorks, Natick, MA). Each cell included in the analysis were detected using a modified version of MATLAB scripts described elsewhere (30). Total signal intensity was normalized by the total area of the cell and graphed as histograms using MATLAB.

Statistical analysis

All experiments were repeated independently at least 3 times, if not noted otherwise. One representative experiment or pooled data are shown. Statistical differences between experimental and control groups were analyzed using GraphPad

385 Prism version 6.0 software (GraphPad Software, La Jolla, CA). Significance was
386 determined using two-tailed nonparametric Mann-Whitney test, Students T-Test, or One-
387 Way ANOVA with Dunnett's multiple comparison post-test. A p value < 0.05 was
388 considered significant.
389

Supporting Information- Figure Legends

Figure S1.

(A, B). *Anaplasma phagocytophilum* changes the tick microbiota.

Comparison of the gut microbiota composition, at the taxonomic rank of order, of uninfected and *A. phagocytophilum*-infected fed nymphs. (**A**) Total bacterial composition (in percent) and (**B**) fold change ratio of *A. phagocytophilum*-infected (Ap) to uninfected (Clean) fed nymphs.

(C, D, E). Gentamicin treatment alters the tick microbiome.

qRT-PCR assessment of (**A**) total bacterial burden, (**B**) *Pseudomonas* sp., and (**C**) *Enterococcus* sp. of nymphal tick midguts (MG) upon feeding with gentamicin (Gen) treated mice. PBS treated clean mice were used as a negative control. Horizontal bars represent the median. Each dot represents one nymph. Results were pooled from two independent experiments and statistical significance was calculated using a two-tailed nonparametric Mann-Whitney test (** $p=0.052$, *** $p<0.0002$)

Figure S2.

(A). PNAG antibody detects native biofilms within tick guts

Clean tick guts extracted for biofilm-associated PNAG polysaccharide was spotted directly onto nitrocellulose membranes and probed with a goat a-PNAG antibody. Spots were compared to PNAG produced by *Staphylococcus aureus* strain SA113 wildtype and its isogenic PNAG deficient mutant (SA113 Δ*ica*).

(B). IAFGP co-localizes with biofilms within the tick gut.

A. phagocytophilum-infected ticks were paraffin embedded and sectioned for immunofluorescence staining using the α-PNAG antibody to detect biofilms and the α-IAFGP-GST antibody to detect IAFGP. In contrast, we used the α-GST antibody as a control. Brightfield images shows gut epithelium cells surrounding the lumen "L". Co-localization was observed between IAFGP and the biofilms in merged compared to the α-GST antibody control antibody (arrows). Scale bar = 10μm.

(C, D, E). *Anaplasma phagocytophilum* infection diminishes *Enterococci* burden within the tick gut.

Comparison of the levels of *Enterococci* within the tick gut microbiota between uninfected (Clean) and *A. phagocytophilum*-infected (Ap) fed nymphs. Relative abundance of *Enterococci* among (**B**) individual samples and (**C**) across all samples (pooled). (**D**) *A. phagocytophilum*-infected fed nymphs reduces levels of *Enterococci* compared to uninfected nymphal ticks as determined by qRT-PCR using *Enterococcus* specific primers. Statistical significance was calculated using a two-tailed nonparametric Mann-Whitney test (* $p<0.05$, **** $p<0.0001$).

Figure S3.

(A, B). Variable binding of peptide P1 to *Enterococci* bacteria and their peptidoglycan.

Enterococcus faecalis (E.fs), vancomycin-resistant *Enterococcus faecalis* (VRE), and *Enterococcus faecium* (E.fm) (**A**) whole bacterial cells were incubated with His-tagged sP1 or P1 peptide and assessed for binding. Bound (Associated) or unbound (Supernatant) peptide was detected by immunoblot analysis using a monoclonal His-tagged antibody. Cell wall bound fraction (Associated) versus unbound (Supernatant) bacterial fractions were distinguished by using a polyclonal anti-Wheat Germ Agglutinin (α-WGA) that detects bacterial peptidoglycan. (**B**) Streptavidin-coated magnetic Dynabeads bound to biotinylated P1 (b-P1) or sP1 (b-sP1) were incubated with purified muropeptide extracts obtained from the bacterial cells grown in brain heart infusion broth. Biotinylated peptides bound to beads were detected using an infrared (IR)-labeled streptavidin probe. Muropeptides from cells were detected using a polyclonal Wheat Germ Agglutinin antibody (α-WGA). Unbound peptide was also collected and spotted. Associated or Supernatant fractions represent the fractions that were either pulled down with the magnetic bead or remained unbound. Strongest binding to whole cells or purified peptidoglycan was demonstrated between P1 and *E. faecalis*.

(C). P1 binds *Staphylococcus aureus* peptidoglycan.

Insoluble *S. aureus* peptidoglycan was diluted to 0.5 mg/mL in PBS/ 0.5% Tween/ 5% fetal bovine serum solution and washed for 40 minutes at 4°C with gentle agitation. 1 mg/mL biotinylated peptide- P1 (b-P1) or scrambled peptide- sP1 (b-sP1) was added to the peptidoglycan samples at 1:1 v/v ratio and incubated for 60 minutes at 4°C with

gentle agitation. Samples were washed in PBS and immunoblot assays were performed on a nitrocellulose membrane spotted with the respective samples. Bacterial peptidoglycan was detected using a polyclonal anti-Wheat Germ Agglutinin antibody (α-WGA) and biotinylated peptides were detected using an infrared-labeled streptavidin probe.

(D). P1 binds to the terminal D-alanine residue of the native *Staphylococcus aureus* muropeptide chain.

1 mg/ml synthetic biotinylated muropeptides containing either D-alanine or D-serine were immobilized to streptavidin-coated magnetic dynabeads. Muropeptide-peptide binding was determined by incubating 1 mg/ml of 6x histidine-tagged peptide P1 or 6x histidine-tagged scrambled-P1 (sP1) to the muropeptide-bead solution at a 1:2 volume ratio. Bead-muropeptide-peptide complexes were washed in a PBS/0.1% Bovine Serum Albumin (BSA), dissociated from one another and spotted directly on a nitrocellulose membrane. Histidine-tagged peptides were detected using a polyclonal murine primary antibody to the 6x-histidine tag. Biotinylated muropeptides were detected using an infrared (IR)-labeled streptavidin probe. Unbound muropeptide (UB) collected prior to incubation with His-tagged peptides was also spotted. PBS was used as a peptide binding buffer control. Associated or Supernatant fractions represent the fractions that were either pulled down with the magnetic bead or remained unbound.

(E, F). IAFGP and P1 do not bind *Enterococci* grown in D-serine

(**E**) Recombinant GST-tagged IAFGP or GST alone and (**F**) His-tagged sP1 or P1 peptide were tested for binding to *E. faecalis* (E.fs) or *E. faecium* (E.fm) whole bacteria grown with 125mM D-serine supplemented to the culture medium. (**E**) Lane 1. Positive control- 1 mg/mL IAFGP or GST alone; Lanes 2, 3- Protein bound to *E. faecalis* (E.fs) or *E. faecium* (E.fm) bacterial surface (Associated); Lanes 4, 5- Protein found in the unbound fraction (Supernatant) for *E. faecalis* (E.fs) or *E. faecium* (E.fm). (**F**) Peptide was detected using a monoclonal His-tagged antibody (α-His). Cell wall bound fraction (Associated) versus unbound (Supernatant) bacterial fractions were distinguished by using a polyclonal anti-Wheat Germ Agglutinin antibody (α-WGA).

(G). Bacterial wall teichoic acids are not involved in mediating P1 binding to bacterial cells.

Approximately 1.5x107 *S. aureus* SA113 wildtype (WT) bacteria, isogenic wall teichoic acid (WTA) deletion mutant (Δ*tagO*), and its complemented strain (+TagO) were incubated with His-tagged sP1 or P1 peptide and assessed for binding. Bound (Associated) or unbound (Supernatant) peptide was detected by immunoblot analysis using a monoclonal His-tagged antibody. Cell wall bound fraction (Associated) versus unbound (Supernatant) bacterial fractions were distinguished by using a polyclonal anti-Wheat Germ Agglutinin antibody (α-WGA) that detects bacterial peptidoglycan. P1 binds WT bacteria and its isogenic WTA deletion and complementation mutants without any distinct variability.

(H). IAFGP displays very weak binding to lipoteichoic acid.

Streptavidin-coated magnetic Dynabeads bound to biotinylated IAFGP-GST (b-IAFGP) or GST alone (b-GST) were incubated with purified lipoteichoic acid (LTA). Biotinylated proteins were detected using an infrared (IR)-labeled streptavidin probe. IAFGP-GST was detected using a polyclonal murine primary antibody (α-IAFGP-GST) and GST was detected using a monoclonal murine primary antibody (α-GST). LTA was detected using a monoclonal murine antibody directed against LTA (α-LTA) from gram positive bacteria. Unbound IAFGP or GST protein (UB) and unbound LTA (UB-LTA) was also collected and spotted. Associated represent the fractions that was pulled down with the magnetic bead and remained bound to the biotinylated protein. IAFGP displays only a modicum increase in binding to LTA compared to GST alone control protein.

Figure S4.

Muropeptide composition and profile of *Staphylococcus aureus* SA113 obtained by UPLC-QTOF-MS/MS.

(**A**, **B**, **C**). Muropeptide profile of *S. aureus* SA113 cultured in either TSB or medium supplemented with 125 mM D-serine. (**A**) Total Ion Current (TIC) chromatogram obtained by reversed phase UPLC-QTOF-coupled to MS/MS is shown. Peaks are represented as numbers throughout the chromatogram. (**B**) Histogram profile representing peak intensity of individual peaks obtained from (**A**). Results were pooled from 3 independent experiments ± SEM. (**C**) Proposed structures of the indicated peaks that were successfully identified.

(**D**). Total Ion Current (TIC) chromatogram of muropeptides extracted from *S. aureus* SA113 cultured in either TSB or medium supplemented with 0.1 mg/mL GST alone or GST-IAFGP (IAFGP) protein.
(**E**). Crosslinking percentage between the glycan strands from cells grown in the respective media. Results were pooled from 3 independent experiments ± SEM. Statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparison posttest (***p<0.001, ****p<0.0001).

Figure S5.

IAFGP and P1 does not bind gram negative peptidoglycan or inhibit their biofilms.
(**A**) Biotinylated IAFGP (b-IAFGP) or control protein biotinylated GST (b-GST) or (**B**) biotinylated P1 peptide (b-P1) or biotinylated control scramble peptide sP1 (b-sP1) were immobilized onto Streptavidin coated magnetic dynabeads and incubated with purified muropeptide extracts obtained from gram negative bacteria. Gram negative bacteria tested included 2 different *Pseudomonas aeruginosa* strains (PA01 and PA14) and 2 *Escherichia coli* strains- parental strain CS109 (Ec) and its isogenic DD-carboxypeptidase enzyme mutant strain DV900 (EcΔ) which accumulates pentapeptides-due accumulative mutations in DD-carboxypeptidase activities: Δ*ponB*, Δ*dacA*, Δ*dacB*, Δ*dacC*, Δ*dacD*, Δ*pbpG*, Δ*ampH*, Δ*ampC*, and Δ*pbp4b*. Biotinylated protein (**A**) or peptide (**B**) was detected using an infrared (IR)-labeled streptavidin probe. Muropeptides from bacteria was detected using a polyclonal Wheat Germ Agglutinin antibody (α-WGA). Unbound protein or peptide (UB) was also collected and spotted. Associated or Supernatant fractions represent the fraction that were either pulled down with the magnetic bead or remained unbound. Strongest binding interaction was observed with either b-IAFGP (**A**) or b-P1 (**B**) and the *E. coli* mutant DV900. Only trace amounts of binding were detected with the other bacterial strains. Neither control protein or peptide (b-GST or b-sP1) was found to interact with any of the muropeptide extracts.
(**C**, **D**, **E**) Wildtype *P. aeruginosa* strains PA01 (**C**), PA14 (**D**) and *E. coli* parental strain CS109 (**E**) were tested for growth and biofilm formation in their LBG (LB supplemented with 1% glucose) medium or medium supplemented with 0.1 mg/mL GST alone (GST), recombinant GST-tagged IAGFP (IAFGP), P1 or sP1 control scramble peptide. Biofilm

associated growth was assessed after static incubation at 37°C for 24 h and measured at OD_{600} (top graph). Biofilm associated bacteria were stained with crystal violet, imaged, and dissolved for quantification by measuring absorbance at 550nm (bottom graph). Results were pooled from 3 independent experiments, each with four technical replicate, ± SEM. *P. aeruginosa* strains biofilms are formed the air-liquid interface. No significant difference was noted in biofilms across all three bacteria when treated with either IAFGP or GST.

Figure S6.

Biofilm reduction with 125 mM D-serine does not influence *Staphylococcus aureus* or *Enterococcus faecalis* growth or viability.

Overnight cultures of *S. aureus* SA113 (**A**, **C**, **E**) or *E. faecalis* MMH594 (**B**, **D**) were diluted in TSBG or BHIG respectively (medium) or medium supplemented with 125 mM D-alanine, 125 mM D-serine or an equivalent volume of PBS control and inoculated into a 96-well microtiter plate.

(**A**, **B**). Biofilm associated growth was assessed after static incubation at 37°C for 24 h. Bacterial density was measured at OD_{600} using a microtiter ELISA plate reader. Data was compiled from three independent experiments, each experiment comprising four technical replicates, and pooled ± SEM.

(**C**, **D**). Bacterial viability was quantified after sonication of each well and plating serial dilutions. Average CFU values pooled from three independent experiments ± SEM is shown.

(**E**). Planktonic growth was measured at 30-minute intervals for 24 hours at 600nm using a spectrophotometer. Growth curves from three independent experiments, each with four technical replicates, were pooled ± SEM. Bacteria free media is represented as the negative control.

Figure S7.

(A – D). D-serine changes the tick microbiota.

Gut microbiota composition, at the taxonomic rank of genus, for D-alanine or D-serine injected nymphs. (**A**) Total bacterial abundance (in percent) and (**B**) fold change ratio of

D-serine treated nymphs relative to D-alanine injection of fed nymphs. Green and red box outlines represent genera with increased or decreased abundance upon D-serine treatment. (**C**, **D**) Comparison between the levels of *Enterococci* within the tick gut microbiota between D-alanine and D-serine injection of ticks. Relative abundance of *Enterococci* among (**C**) individual samples and (**D**) across all samples (pooled).

(E). P1 inhibits biofilm formation of different gram-positive bacteria.

Cultures of *Staphylococcus aureus* SA113 (S. a.), *Staphylococcus epidermidis* (S. e.), *Staphylococcus saprophyticus* (S. s.), *Streptococcus mutans* (S. m.), *Corynebacterium pseudodiptheriticum* (C. p.), and *Enterococcus faecalis* (E. f.) were grown in TSB-glucose or BHI-sucrose medium supplemented with 0.1 mg/mL P1 or sP1 control peptide in microtiter plates. Plates were incubated for 18 h at 37 °C stationarily, washed for non-adherent bacteria and stained for biofilms with crystal violet. Results were pooled from 3 independent experiments ± SEM and are shown as the total biofilm biomass normalized to sP1 control peptide. Statistical significance between each peptide for a given bacterial species was calculated using unpaired Student t-test with Welch's correction (*** $p<0.0001$, *** $p<0.0008$).

Figure S8.

(A – C). Expression pattern of IAFGP.

(**A**). qRT-PCR examination of the *iafgp* expression level between uninfected (Clean) fed ticks versus ticks fed after treatment with P1 peptide, D-serine or gentamicin. Each dot represents one nymph.

(**B**). Uninfected nymphal ticks (n=10) were fed on *A. phagocytophilum* infected IFN−γ knockout mice. qRT-PCR was performed to determine the expression of *iafgp* from tick midguts (MG) 72 hours post feeding. Uninfected (Clean) IFN−γ knockout mice were used as a control.

(**C**). qRT-PCR assessment of *iafgp* expression from nymphal ticks upon injection of 100 ng/mL recombinant mouse IFN−γ protein of nymphal ticks. PBS injection was used as a control. For both experiments, each dot represented one nymph and results are pooled from one experiment; statistical significance was calculated using a two-tailed nonparametric Mann-Whitney test (*** $p=0.0008$).

Figure S9.

(A, B). Impact of IAFGP binding on bacterial peptidoglycan.

(**A**). The area of the peptidoglycan layer was calculated for *S. aureus* SA113 grown planktonically in either TSB (n = 53 cells) or medium supplemented with 125 mM D-alanine (n = 55 cells), 125 mM D-serine (n = 61 cells), 0.1 mg/mL GST-IAFGP (n = 49 cells) or 0.1mg/mL GST alone (n = 53 cells). Cultures were also grown in an equivalent volume of PBS as a buffer control (n = 24 cells). The data is represented as a percentage normalized to the TSB control. Results were pooled from images taken with the transmission electron microscope ± SEM. Statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparison posttest (**** $p<0.0001$, ** $p<0.001$).

(**B**). Bacterial cells cultured in TSB or medium supplemented with PBS, D-alanine, D-serine, GST, or IAFGP were stained with the membrane-binding probe FM® 4-64 (**J**) and imaged by Phase-contrast (left-phase) and epifluorescent microscopy (middle). Population analysis (graph), at the single cell level, demonstrate that IAFGP and D-serine treatments result in an increase in fluorescent signal intensity. Total fluorescent signal intensities were normalized by cell area and histograms depicted as a function of population frequency. n > 500 cells for each treatment was pooled from 2 independent experiments. Scale bar = 2 μm.

Figure S10.

(A, B). Binding by IAFGP can only be outcompeted with high concentrations of free D-alanine.

(**A**) Synthetic biotinylated pentapeptide containing D-alanine immobilized to streptavidin-coated magnetic dynabeads were incubated with recombinant GST-IAFGP (IAFGP). IAFGP was eluted with varying concentrations of free D-amino acids (D-alanine [D-ala] or D-serine [D-ser]). The Bound or Eluted protein was spotted directly onto a nitrocellulose membrane for immunoblot analysis. Unbound or Bound biotinylated pentapeptide (bMA) was detected using an infrared (IR)-labeled streptavidin probe. IAFGP protein that was eluted or remained bound was detected using a polyclonal

murine primary antibody. D-serine was used as a negative control. (**B**) The relative intensity of the eluted or bound IAFGP treated with varying concentrations of free D-alanine was quantified. Results were pooled from 3 independent experiments ± SEM and analyzed using a 2-way ANOVA with Sidak's multiple comparison posttest (*p<0.1, **p<0.01, ***p<0.001, ****p<0.0001).

(C, D). Excess free D-alanine prevents IAFGP and P1 from inhibiting biofilms. (**C**) *S. aureus* SA113 and (**D**) *E. faecalis* MMH594 were grown for biofilms in either TSBG or BHIG respectively, or medium supplemented with 0.1 mg/mL GST alone, GST-IAGFP or GST-IAGFP treated with 125 mM (high) or 10 mM (low) D-alanine. Experiments were similarly conducted with 0.1 mg/mL sP1 and P1 peptide. *S. aureus* biofilms were stained with safranin (red color) and *E. faecalis* biofilms were stained with Crystal violet (purple); a representative biofilm stain is shown for each. Dissolved stains were pooled from 3 independent experiments ± SEM. Statistical significance was calculated using 2-way ANOVA with Sidak's multiple comparison posttest (****p<0.0001).

Figure S11.

(A, B). Growth and biofilm formation for IAFGP and P1 peptide variants. 1.5 x10^6 cfu/mL (**A**) *S. aureus* (strain SA113) or (**B**) *E. faecalis* (strain MMH594) were incubated with 5 different IAFGP peptide variants (i0, i1, i2, i3, and i4) (blue borders) or four P1 peptide variants (P1a, P1b, P1c, and P1d) (green borders) (SI Appendix, Table S5) in 96-well microtiter plates stationarily for 24 hours. All peptides were used at 0.1 mg/mL (40 μM). Peptide variants were generated based off their original sequence (12). Growth (top graph) was assessed by measuring absorbance at 600nm. The wells were washed for non-adherent bacteria and resident biofilm associated cells were stained with safranin (*S. aureus*) or crystal violet (*E. faecalis*). The stained cells were dried and dissolved in 33% acetic acid and read at 415nm (safranin) or 550 nm (crystal violet) to assess biofilm formation (bottom graph). Growth and biofilm in unsupplemented TSBG or BHIG medium for each bacterium was used as control. Additionally, 0.1 mg/mL GST-tagged IAFGP protein, GST alone, P1 peptide or scramble control peptide- sP1 were also used as controls. The results were pooled from three independent experiments each with

669 4 technical replicates ± SEM; representative images of the biofilm plates with two
670 representative wells per condition are shown below the graph. Statistical significance was
671 calculated using one-way ANOVA with Dunnett's multiple comparison (**** $p<0.0001$).
672
673 **Figure S12.**
674 (**A**) **Select IAFGP and P1 peptide variants bind *S. aureus* and *E. faecalis* bacteria.**
675 Five His-tagged IAFGP peptide variants (i0, i1, i2, i3, and i4) (top blot) and four His-
676 tagged P1 peptide variants (P1a, P1b, P1c, and P1d) (bottom blot) were generated based
677 off their original sequence (SI Appendix, Table S5) (12) and tested for binding to 1.5-
678 $2x10^7$ cells of *S. aureus* (Sa) or *E. faecalis* (E.fs) whole bacteria grown in TSB or BHI
679 medium respectively. Bound (Associated) or unbound (Supernatant) peptides were
680 detected by immunoblot analysis using a monoclonal His-tagged antibody (α-His). Cell
681 wall bound fraction (Associated) versus unbound (Supernatant) bacterial fractions were
682 distinguished by using a polyclonal Wheat Germ Agglutinin antibody (α-WGA) that
683 detects bacterial peptidoglycan. Among the IAFGP peptide variants only i0 showed most
684 significant binding to both *S. aureus* and *E. faecalis*. P1a and P1d were the only P1
685 peptide variants that bound significantly to both *S. aureus* and *E. faecalis*.
686 **(B, C). Only IAFGP or P1 peptide variants that inhibit biofilm bind *S. aureus* and *E.***
687 ***faecalis* peptidoglycan.**
688 (**B**) His-tagged IAFGP variants (i0, i1, i2, i3, and i4) (top blot) and His-tagged P1 peptide
689 variants (P1a, P1b, P1c, and P1d) (bottom blot) used in (**A**) were immobilized onto
690 magnetic His-Tag isolation beads and incubated with muropeptides extracted from *S.*
691 *aureus* (Sa) or *E. faecalis* (E.fs) grown in TSB or BHI medium respectively. (**C**)
692 Experiment was similarly performed as in (**B**) but in reverse using biotinylated synthetic
693 pentapeptides terminating in D-alanine immobilized onto streptavidin-coated magnetic
694 beads and probed for binding the His-tagged IAFGP (top blot) or P1 peptide variants
695 (bottom blot). His-tagged IAFGP or P1 peptide variants were detected using a
696 monoclonal His-tagged antibody (α-His). Extracted muropeptides from both bacteria was
697 detected using a polyclonal Wheat Germ Agglutinin antibody (α-WGA). Biotinylated
698 synthetic pentapeptide was detected using an infrared (IR)-labeled streptavidin probe.
699 Associated or Supernatant fractions represent the fractions that were either pulled down

with the magnetic bead or remained unbound. Across both panels we observe the IAFGP peptide variants- i0 and P1 peptide variants- P1a and P1d to significantly bind native muropeptides extracted from *S. aureus* or *E. faecalis* (**B**) or to bind the D-alanine terminating synthetic pentapeptide (**C**).

Supporting Information- References

1. Telford SR, 3rd, *et al.* (1996) Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. *Proceedings of the National Academy of Sciences of the United States of America* 93(12):6209-6214.
2. Hodzic E, *et al.* (1998) Granulocytic ehrlichiosis in the laboratory mouse. *The Journal of infectious diseases* 177(3):737-745.
3. Smith AA, *et al.* (2016) Cross-Species Interferon Signaling Boosts Microbicidal Activity within the Tick Vector. *Cell host & microbe* 20(1):91-98.
4. Caporaso JG, *et al.* (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal* 6(8):1621-1624.
5. Masella AP, Bartram AK, Truszkowski JM, Brown DG, & Neufeld JD (2012) PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics* 13:31.
6. Schmieder R & Edwards R (2011) Quality control and preprocessing of metagenomic datasets. *Bioinformatics* 27(6):863-864.
7. Caporaso JG, *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature methods* 7(5):335-336.
8. McDonald D, *et al.* (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME journal* 6(3):610-618.
9. Lozupone C & Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and environmental microbiology* 71(12):8228-8235.
10. Narasimhan S, *et al.* (2014) Gut microbiota of the tick vector *Ixodes scapularis* modulate colonization of the Lyme disease spirochete. *Cell host & microbe* 15(1):58-71.
11. Thepparit C, *et al.* (2011) Isolation of a rickettsial pathogen from a non-hematophagous arthropod. *PloS one* 6(1):e16396.
12. Heisig M, *et al.* (2014) Antivirulence properties of an antifreeze protein. *Cell reports* 9(2):417-424.

13. Abraham NM & Jefferson KK (2012) *Staphylococcus aureus* clumping factor B mediates biofilm formation in the absence of calcium. *Microbiology* 158(Pt 6):1504-1512.
14. Cywes-Bentley C*, et al.* (2013) Antibody to a conserved antigenic target is protective against diverse prokaryotic and eukaryotic pathogens. *Proceedings of the National Academy of Sciences of the United States of America* 110(24):E2209-2218.
15. Cue D, Lei MG, & Lee CY (2012) Genetic regulation of the intercellular adhesion locus in staphylococci. *Frontiers in cellular and infection microbiology* 2:38.
16. Weidenmaier C*, et al.* (2004) Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. *Nat Med* 10(3):243-245.
17. Liu L*, et al.* (2012) *Ixodes scapularis* JAK-STAT pathway regulates tick antimicrobial peptides, thereby controlling the agent of human granulocytic anaplasmosis. *The Journal of infectious diseases* 206(8):1233-1241.
18. Narasimhan S*, et al.* (2007) A tick antioxidant facilitates the Lyme disease agent's successful migration from the mammalian host to the arthropod vector. *Cell host & microbe* 2(1):7-18.
19. Neelakanta G, Sultana H, Fish D, Anderson JF, & Fikrig E (2010) *Anaplasma phagocytophilum* induces *Ixodes scapularis* ticks to express an antifreeze glycoprotein gene that enhances their survival in the cold. *The Journal of clinical investigation* 120(9):3179-3190.
20. de Jonge BL, Chang YS, Gage D, & Tomasz A (1992) Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. The role of penicillin binding protein 2A. *The Journal of biological chemistry* 267(16):11248-11254.
21. Desmarais SM, De Pedro MA, Cava F, & Huang KC (2013) Peptidoglycan at its peaks: how chromatographic analyses can reveal bacterial cell wall structure and assembly. *Mol Microbiol* 89(1):1-13.
22. Dorr T*, et al.* (2014) A novel peptidoglycan binding protein crucial for PBP1A-mediated cell wall biogenesis in *Vibrio cholerae*. *PLoS Genet* 10(6):e1004433.

23. Dorr T, *et al.* (2016) A cell wall damage response mediated by a sensor kinase/response regulator pair enables beta-lactam tolerance. *Proceedings of the National Academy of Sciences of the United States of America* 113(2):404-409.
24. Kuhner D, Stahl M, Demircioglu DD, & Bertsche U (2014) From cells to muropeptide structures in 24 h: peptidoglycan mapping by UPLC-MS. *Scientific reports* 4:7494.
25. Boneca IG, Xu N, Gage DA, de Jonge BL, & Tomasz A (1997) Structural characterization of an abnormally cross-linked muropeptide dimer that is accumulated in the peptidoglycan of methicillin- and cefotaxime-resistant mutants of *Staphylococcus aureus*. *The Journal of biological chemistry* 272(46):29053-29059.
26. De Jonge BL, Gage D, & Xu N (2002) The carboxyl terminus of peptidoglycan stem peptides is a determinant for methicillin resistance in *Staphylococcus aureus*. *Antimicrobial agents and chemotherapy* 46(10):3151-3155.
27. Desmarais SM, *et al.* (2015) High-throughput, Highly Sensitive Analyses of Bacterial Morphogenesis Using Ultra Performance Liquid Chromatography. *The Journal of biological chemistry* 290(52):31090-31100.
28. Christensen GD, *et al.* (1985) Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *Journal of clinical microbiology* 22(6):996-1006.
29. Soares CA, *et al.* (2005) Capillary feeding of specific dsRNA induces silencing of the isac gene in nymphal *Ixodes scapularis* ticks. *Insect Mol Biol* 14(4):443-452.
30. Parry BR, *et al.* (2014) The bacterial cytoplasm has glass-like properties and is fluidized by metabolic activity. *Cell* 156(1-2):183-194.

Table S1. Average number of reads across each experimental cohort

Sample type	Number of ticks evaluated	Average number of reads (pre-exclusion)	Average number of reads (post-exclusion)
Clean	12	325220.25	241211.7389
Anaplasma infected	11	429221.8	225764.5943
sP1 injected	13	501411.231	336372.5214
P1 injected	16	391913.438	259347.0567
Negative control	-	413	

Table S2. Relative abundance of genera within negative controls (413 reads)

Genera	Relative abundance (reads)	Relative abundance (%)
Other	407	98.5%
Serratia	4	1%
Staphylococcus	*2*	0.5%

"Other" bacterial genera are those that have yet to be taxonomically defined

Table S3. Genera with >1% relative abundance that were only observed within some experimental cohorts

Sample pair	Genera not included in current analysis
Clean or *Anaplasma* infected	*Streptococcus*, *Elizabethkingia**, *Serratia**, *Enhydrobacter**, *Hyrogenophaga**, *Geobacillus**, *Tepidimonas***
sP1 or P1 injected	*Anaerococcus*, *Peptoniphilus*, *Dermacoccus*, *Escherichia*, *Stenotrophomonas*, *Tepidimonas***
D-alanine or D-serine injected	*Elizabethkingia**, *Serratia**, *Enhydrobacter**, *Hyrogenophaga**, *Geobacillus**, *Enterobacteriaceae*

"Genera not currently included in the figures since they did not adhere to one criteria- consistency across all experimental groups (see Material and Methods). However, these genera were found at >1% within each treatment pair and could contribute to the diversity.* Common to Clean/Ap and D-alanine/D-serine sample pairs; ** Common to Clean/Ap and sP1/P1 sample pairs. All other genera were only found once within their respective sample pair.

Table S4. Muropeptides of *S. aureus* SA113 isolated from TSB or 125mM D-serine cultures and analysed by UPLC-MS/MS.

Peak	Observed Masses ($M+H^+$)		Muropeptide composition		
	TSB	D-Serine	Stem peptide	D-ala	D-ser
1	984.4727	984.4734	Penta	1	1
2	-	1057.4890	Penta (1-Gly)		2
3	-	1285.5750	Penta (5-Gly)		2
4	-	1041.4968	Penta (1-Gly)	1	1
5	-	1098.5220	Penta (2-Gly)	1	1
	-	1155.5456	Penta (3-Gly)	1	1
	-	1269.5810	Penta (5-Gly)	1	1
6	1182.5515	1182.5552	Tetra (5-Gly)	1	
	1025.5025	1025.4993	Penta (1-Gly)	2	
7	1253.5923	1253.5813	Penta (5-Gly)	2	
9	-	2466.1132	Penta-tetra (10-Gly)		3
10	-	2222.2082	Penta-tetra (6-Gly)	1	2
11	-	2450.1260	Penta-tetra (10-Gly)	1	2
12	-	2206.2204	Penta-tetra (6-Gly)	2	1
13	-	2434.1262	Penta-tetra (10-Gly)	2	1
14	-	2346.0880	ND		
15	-	3613.9879	Penta-tetra-tetra (15-Gly)	2	2
16	-	3597.9757	Penta-tetra-tetra (15-Gly)	3	1
2'	897.4406	-	Tetra	1	
3'	968.4808	-	Penta	2	
4'	1068.5111	-	Tetra (3-Gly)	1	
	1182.5528	-	Tetra (5-Gly)	1	
5'	1239.5702	-	Tetra (6-Gly)	1	
5a'	954.4628	-	Tetra (1-Gly)	1	
8'	1039.5063	-	Penta	3	
9'	1183.5802	-	ND		
	1297.6155	-	ND		
10'	1164.5397	-	ND		
11'	2347.0940	-	Tetra-tetra (10-Gly)	2	
12'	2418.2708	-	Penta-tetra (10-Gly)	3	
13'	2418.3446	-	Penta-tetra (10-Gly)	3	
14'	2328.2710	-	Tetra-tetra (cyclic) (10-Gly)	2	
15'	3581.8495	-	Penta-tetra-tetra (15-Gly)	4	

Peak numbers are defined according to **Figure S4A**; Tetra or Penta stem peptide refers to: L-Ala−D-Gln −L-Lys−D-Ala(−D-Ala) attached to GluNAc-MurNAc. The structures are depicted in **Figure S4C**. The Glycine (Gly) residues, represented in the parenthesis), are involved in the interpeptide bridge and are attached to D-Lys residue in the stem peptide and is represented as sum of all Gly residues present in each murapeptide. D-alanine (D-ala) and D-Serine (D-ser) columns represents total number of D-alanine or D-serine residues present in the peptide moieties; ND- structures not defined.

Table S5. IAFGP and P1 peptide variants utilized for bacterial and peptidoglycan binding and biofilm assays.

	Peptide Name	Notes	Sequence
IAFGP variants	i0	Native IAFGP sequence aa 111-134	PARKAR AATPATPATAATPATAAT
	i1	'AAT' triplets of P0 replaced by 'PAT' triplets	PARKAR PATPATPATPATPATPAT
	i2	'PARKAR' leader with double 'AAT/PAT' repeats	PARKAR AATPATPATAATPATAATAATPATPATAATPATAAT
	i3	'AAT & PAT' triplets without 'PARKAR' leader	AATPATPATA ATPATAAT
	i4	'PARKAR' in the middle of peptide	AATPATAATP ARKARAATPATPAT
P1 variants	P1a	P2 with four 'AAT' repeats	PARKAR AATAATAATAAT
	P1b	P2 with two 'AAT' repeats	PARKAR AATAAT
	P1c	two 'AAT', 'PARKAR', two 'AAT'	AATAATPARKARAATAAT
	P1d	six 'AAT' triplets without 'PARKAR'	AATAATAATAATAATAAT

IAFGP or P1 peptide variants were generated based off the original full length IAFGP or P1 peptide sequence (6). His-tagged variants were also genereated with the 6x-His tag at the N terminus of each peptide.

Table S6. Primers utilized in this study

Quantitative PCR primers

Gene Name	Forward primer sequence	Reverse primer sequence
iafgp	ggatacggaagcagtctg	ttcatttggatttaaaccatagaag
peritrophin-1	atgccgaataaggtcgactg	agtggaatctggacggaatg
peritrophin-2	agaagggattggccttcaac	tacaggctacgcaacaaacg
peritrophin-3	tgtgacaagacgacctccag	catcgagttcttggcctgt
peritrophin-4	acctgtcgacggatgtgact	cctcgcacgtgtagctgtag
peritrophin-5	acctgggattccagtgtcc	ccagccgtgcacgttgag
Anaplasma 16S rRNA	ggtgagtaatgcataggaatc	gctcatctaatagcgataaatc
Pseudomonas 16S rRNA	actttaagttgggaggaaggg	acacaggaaattccaccaccc
Enterococcus 23S rRNA	agaaattccaaacgaacttg	cagtgctctacctccatcatt
Universal bacterial *16S rRNA*	agagtttgatcctggctcag	catgctgcctcccgtaggagt
Tick *actin*	ggcgacgtagcag	ggtatcgtgctcgactc

Double stranded RNA Primers

Gene Name	Forward primer sequence	Reverse primer sequence
iafgp	taatacgactcactatagggctacaccagctaggaagg	taatacgactcactataggggtgtccaccgtagaatcc
peritrophin-1	taatacgactcactatagggcgtgtcttattcctgtc	taatacgactcactatagggcgtaatggtcgtgatgtc
peritrophin-2	taatacgactcactatagggttgttcctcagcatctctag	taatacgactcactatagggaatcccttctggcagttg
peritrophin-4	taatacgactcactatagggacgatgtctaccagaactc	taatacgactcactatagggaccctccagttgtagttg
gfp	taatacgactcactataggggcgacgtaaacggccacaagtt	taatacgactcactatagggcgcgggtcttgtagttgccgtc

Figure S1.

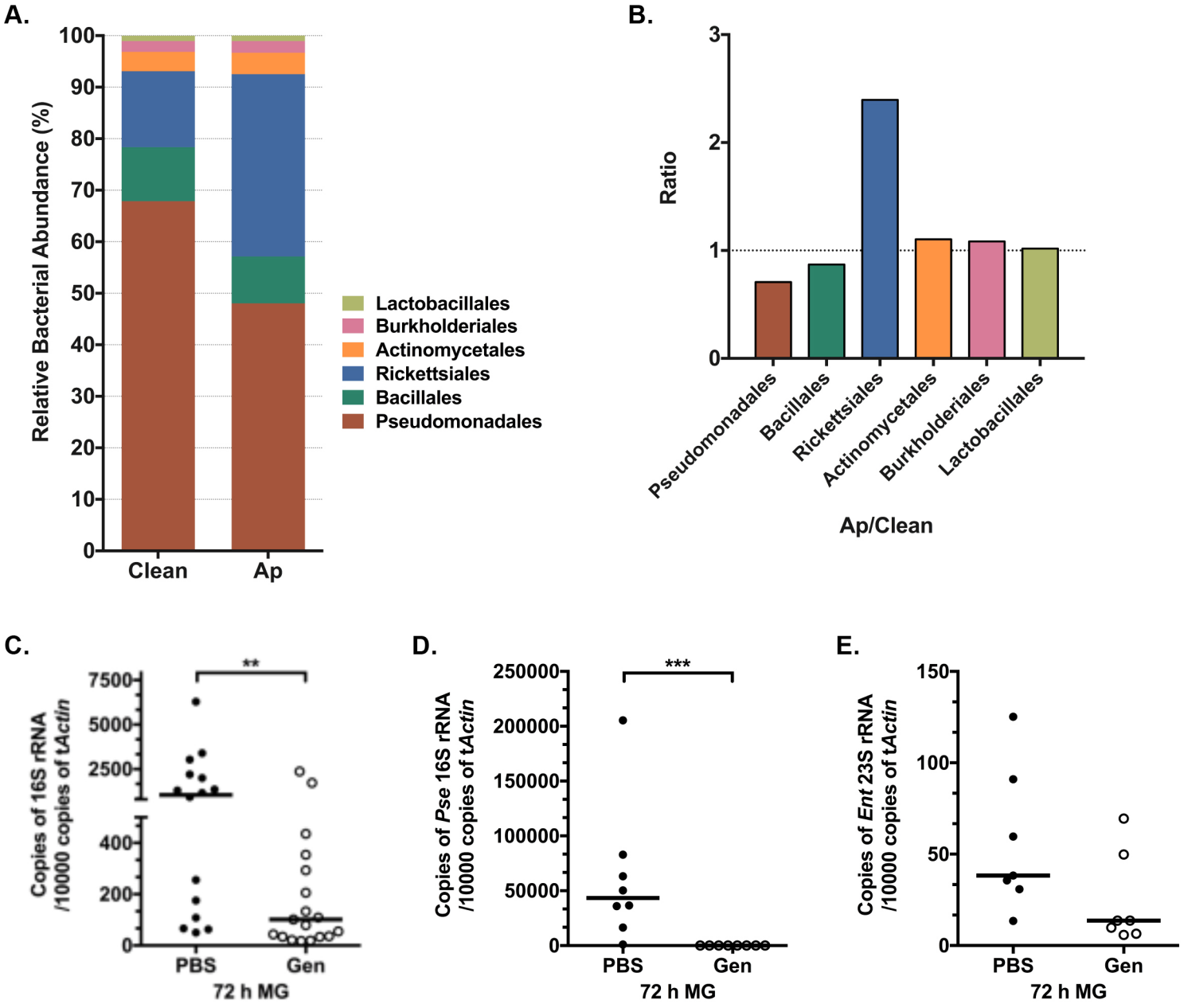

A.
Relative Bacterial Abundance (%)
100
90
80
70
60
50
40
30
20
10
0
Clean
Ap
Lactobacillales
Burkholderiales
Actinomycetales
Rickettsiales
Bacillales
Pseudomonadales
B.
Ratio
3
2
1
0
Pseudomonadales
Bacillales
Rickettsiales
Actinomycetales
Burkholderiales
Lactobacillales
Ap/Clean
C.
Copies of 16S rRNA /10000 copies of tActin
7500
5000
2500
400
200
0
**
PBS
Gen
72 h MG
D.
Copies of Pse 16S rRNA /10000 copies of tActin
250000
200000
150000
100000
50000
0
***
PBS
Gen
72 h MG
E.
Copies of Ent 23S rRNA /10000 copies of tActin
150
100
50
0
PBS
Gen
72 h MG

Figure S2.

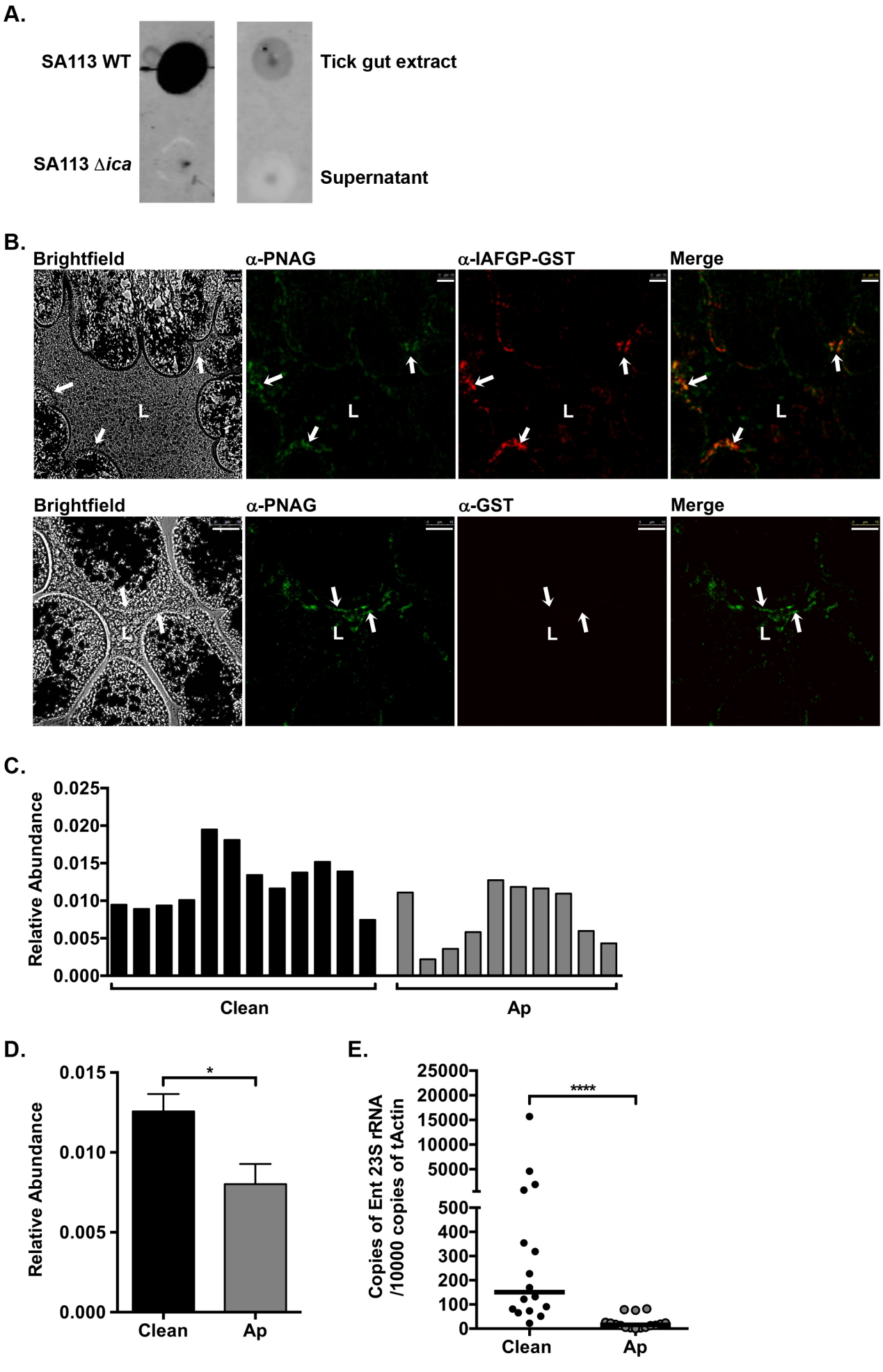

A.
SA113 WT
SA113 Δica
Tick gut extract
Supernatant
B.
Brightfield
α-PNAG
α-IAFGP-GST
Merge
Brightfield
α-PNAG
α-GST
Merge
L
C.
Relative Abundance
0.025
0.020
0.015
0.010
0.005
0.000
Clean
Ap
D.
Relative Abundance
0.015
0.010
0.005
0.000
*
Clean
Ap
E.
Copies of Ent 23S rRNA /10000 copies of tActin
25000
20000
15000
10000
5000
500
400
300
200
100
0
****
Clean
Ap

Figure S3.

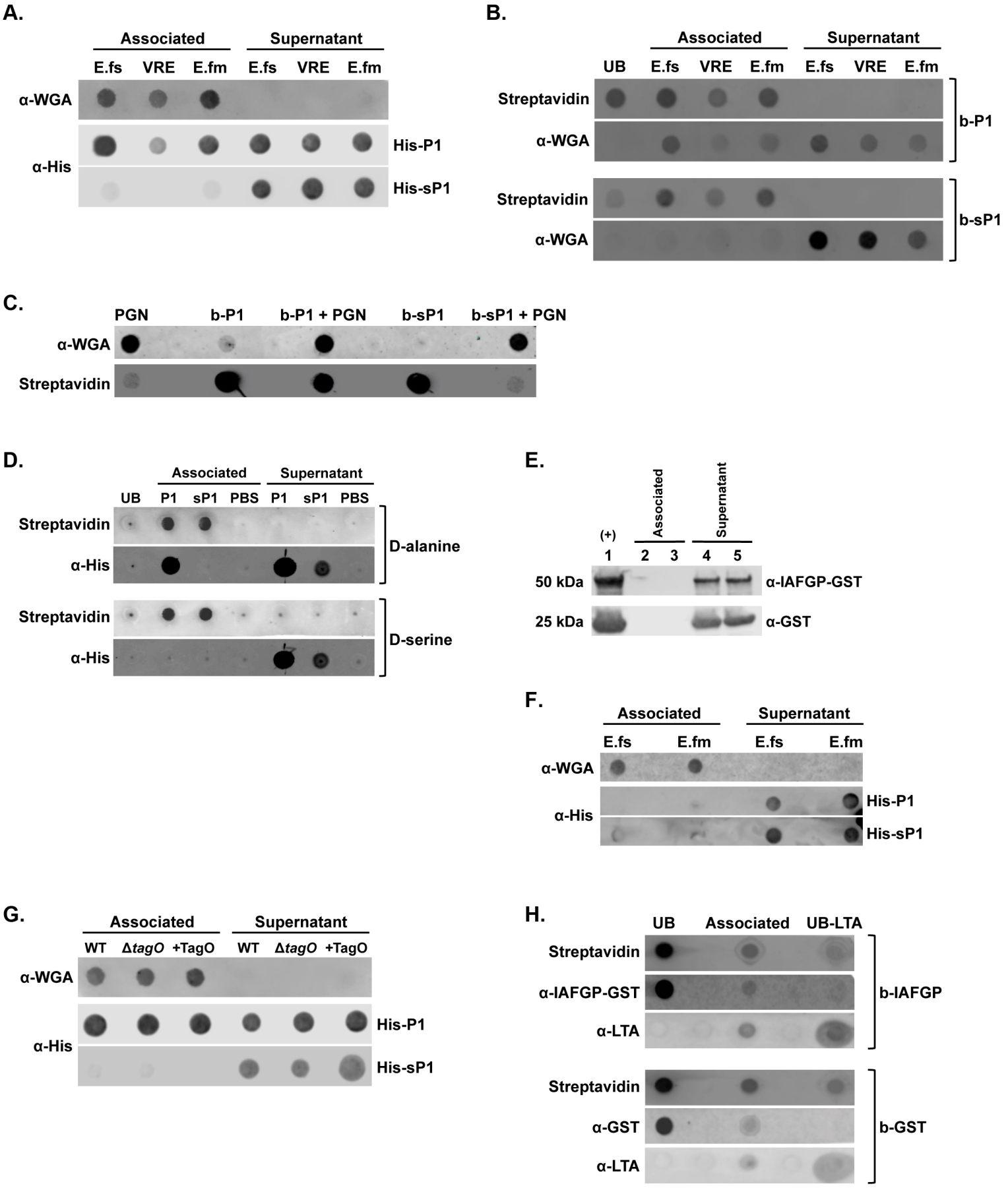
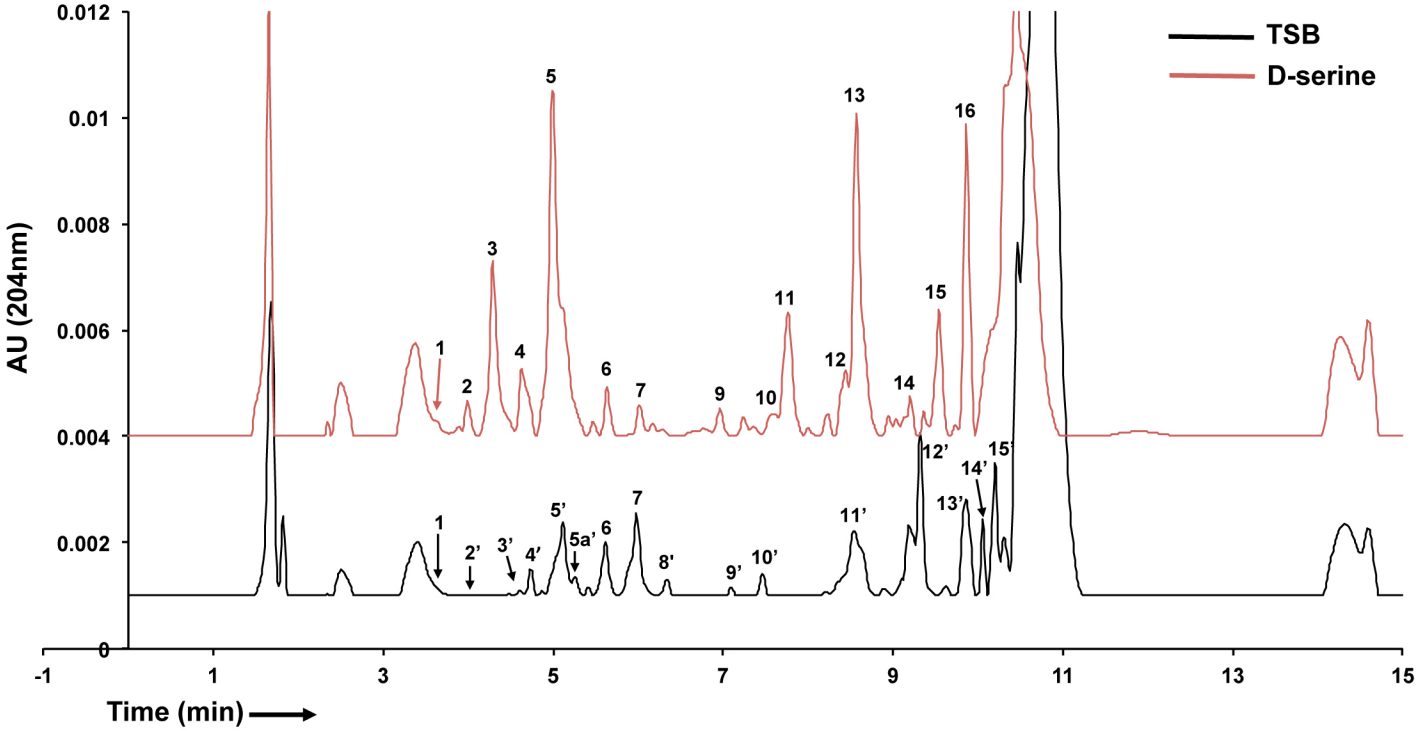
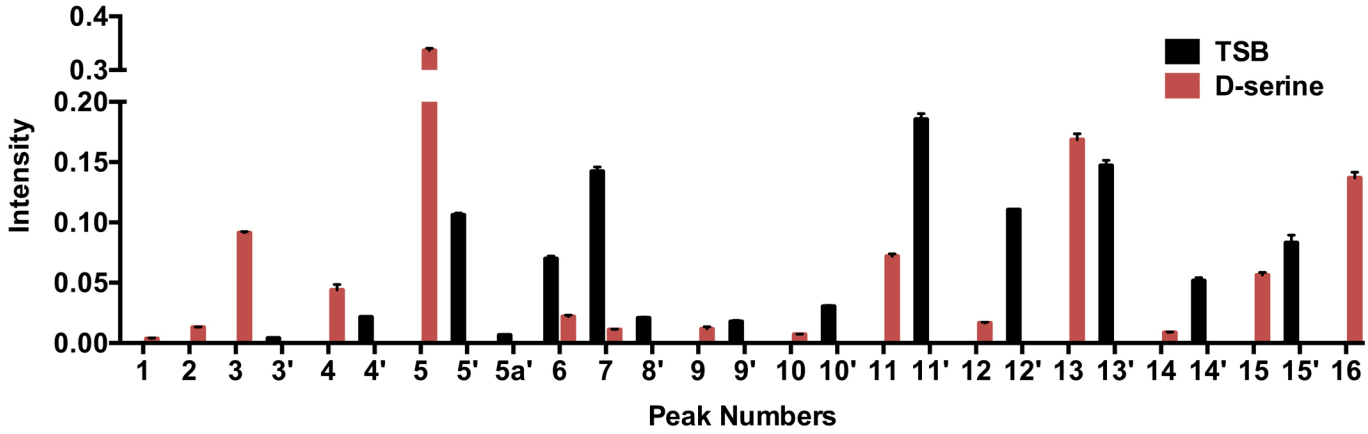

A.
Associated
Supernatant
E.fs
VRE
E.fm
E.fs
VRE
E.fm
α-WGA
α-His
His-P1
His-sP1
B.
Associated
Supernatant
UB
E.fs
VRE
E.fm
E.fs
VRE
E.fm
Streptavidin
α-WGA
b-P1
Streptavidin
α-WGA
b-sP1
C.
PGN
b-P1
b-P1 + PGN
b-sP1
b-sP1 + PGN
α-WGA
Streptavidin
D.
Associated
Supernatant
UB
P1
sP1
PBS
P1
sP1
PBS
Streptavidin
α-His
D-alanine
Streptavidin
α-His
D-serine
E.
Associated
Supernatant
(+)
1
2
3
4
5
50 kDa
α-IAFGP-GST
25 kDa
α-GST
F.
Associated
Supernatant
E.fs
E.fm
E.fs
E.fm
α-WGA
α-His
His-P1
His-sP1
G.
Associated
Supernatant
WT
ΔtagO
+TagO
WT
ΔtagO
+TagO
α-WGA
α-His
His-P1
His-sP1
H.
UB
Associated
UB-LTA
Streptavidin
α-IAFGP-GST
α-LTA
b-IAFGP
Streptavidin
α-GST
α-LTA
b-GST

Figure S4.

A.



B.



C.

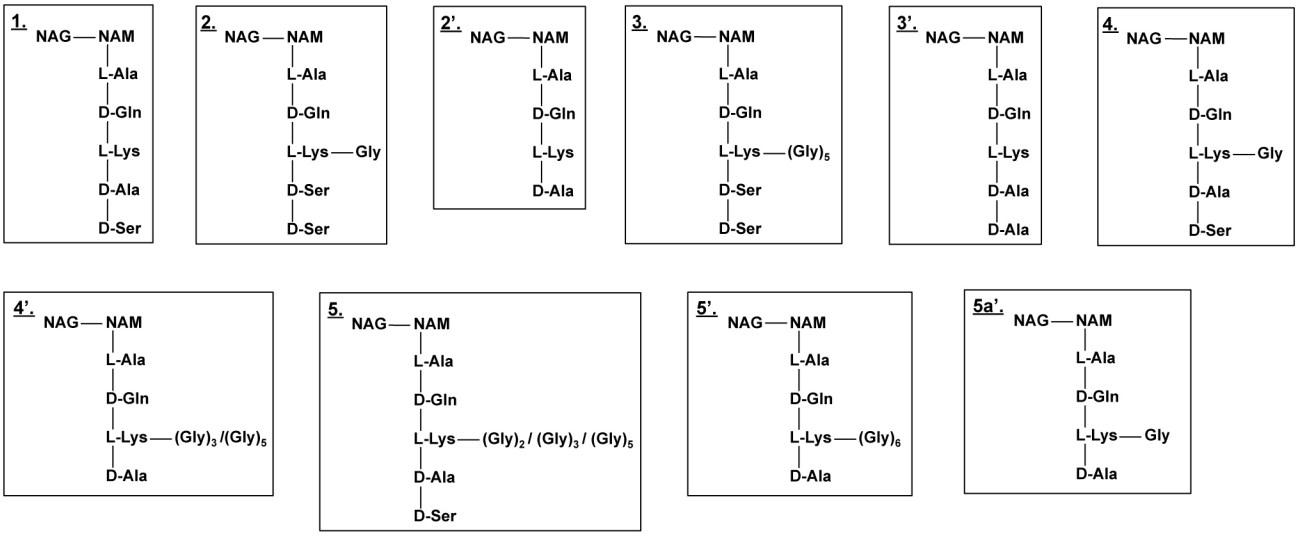
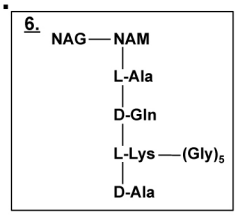
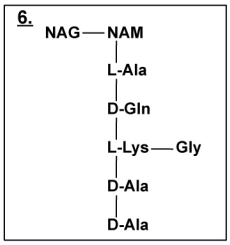
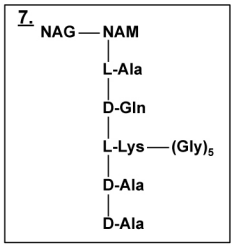


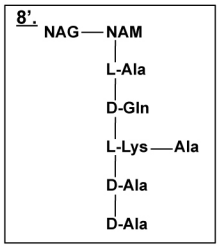
Figure S4 (contd.)

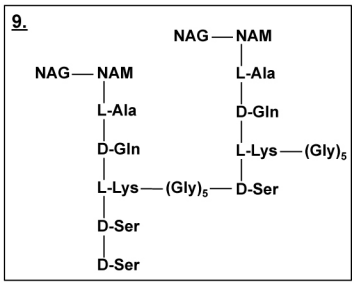
C.

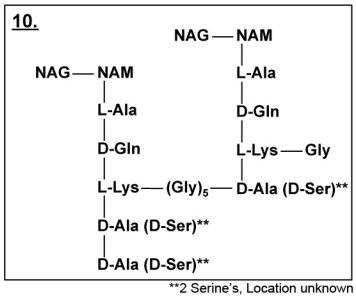




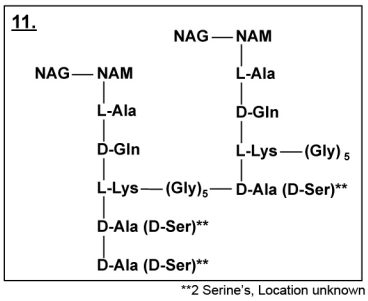




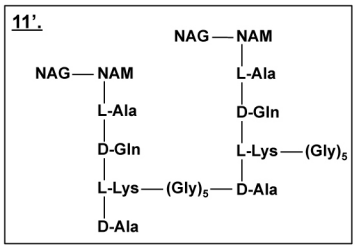


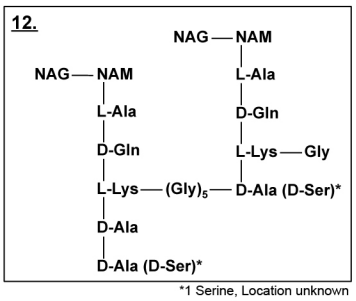


**2 Serine's, Location unknown

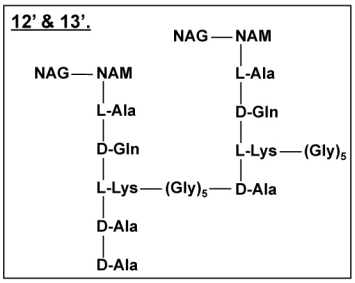


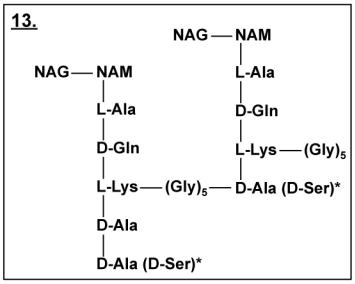
**2 Serine's, Location unknown



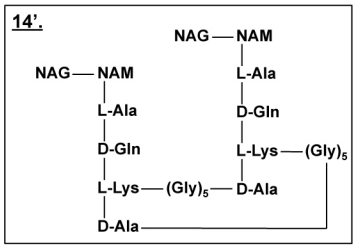


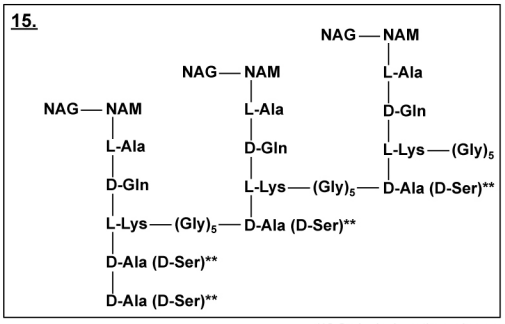
*1 Serine, Location unknown



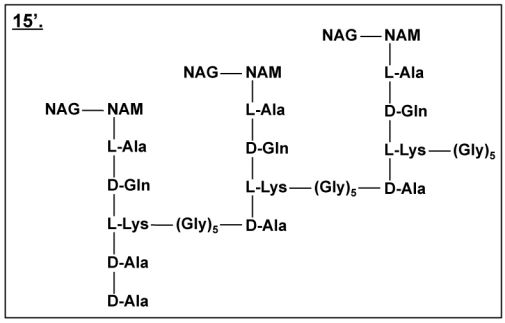


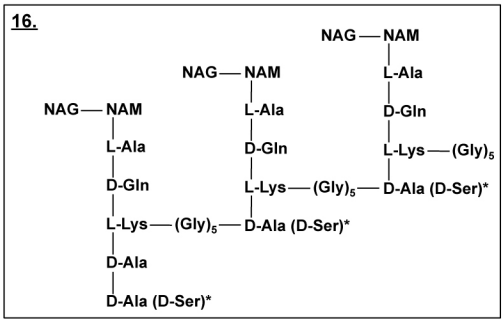
*1 Serine, Location unknown





**2 Serine's, Location unknown

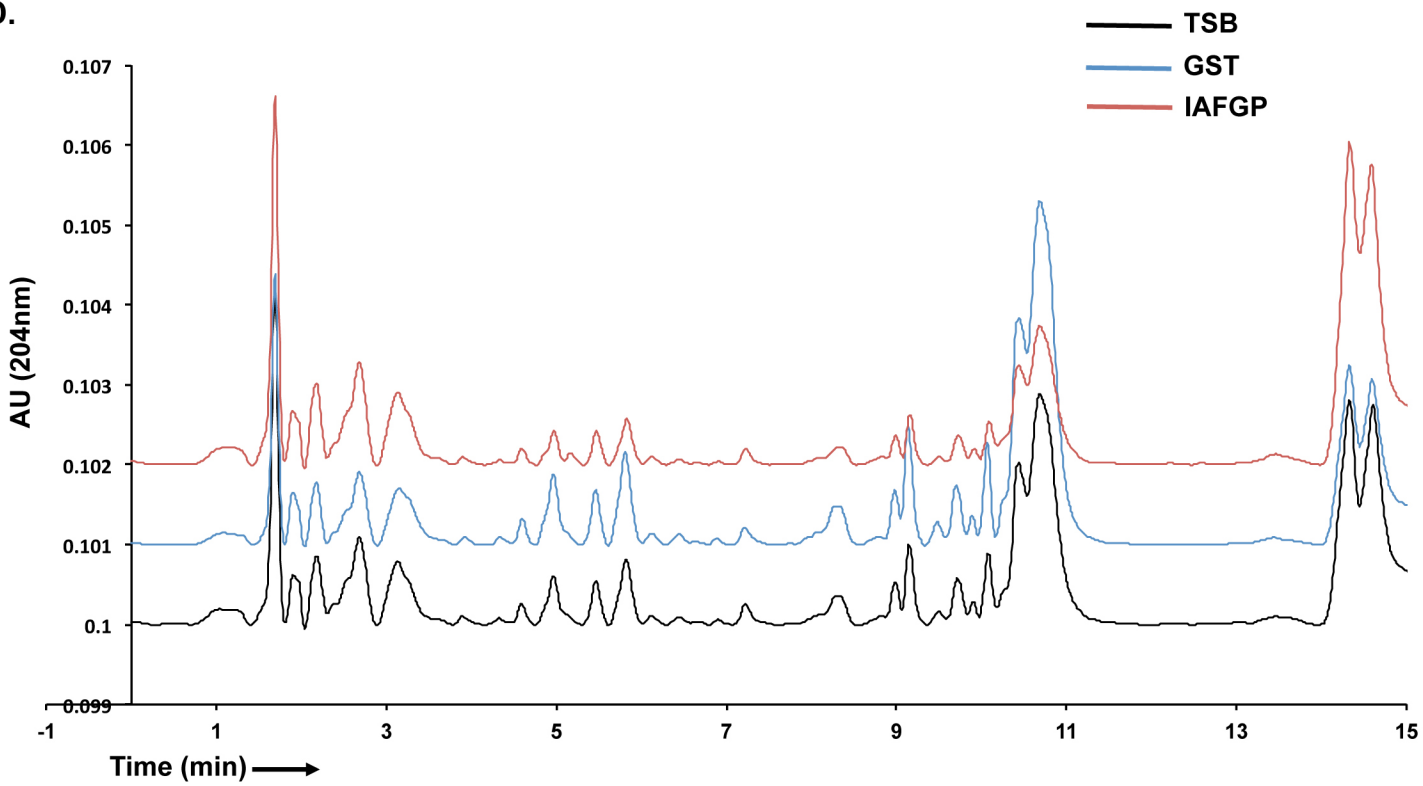




*1 Serine, Location unknown

Figure S4 (contd.)

D.



E.

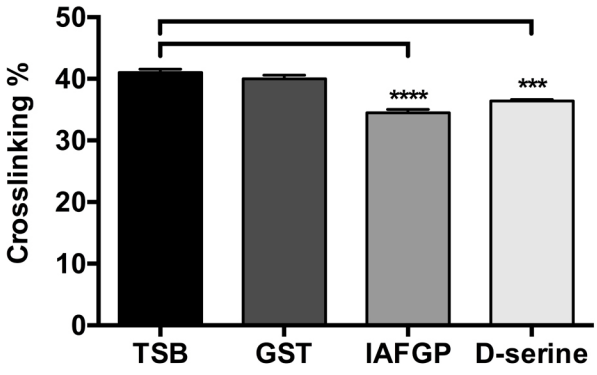


Figure S5.

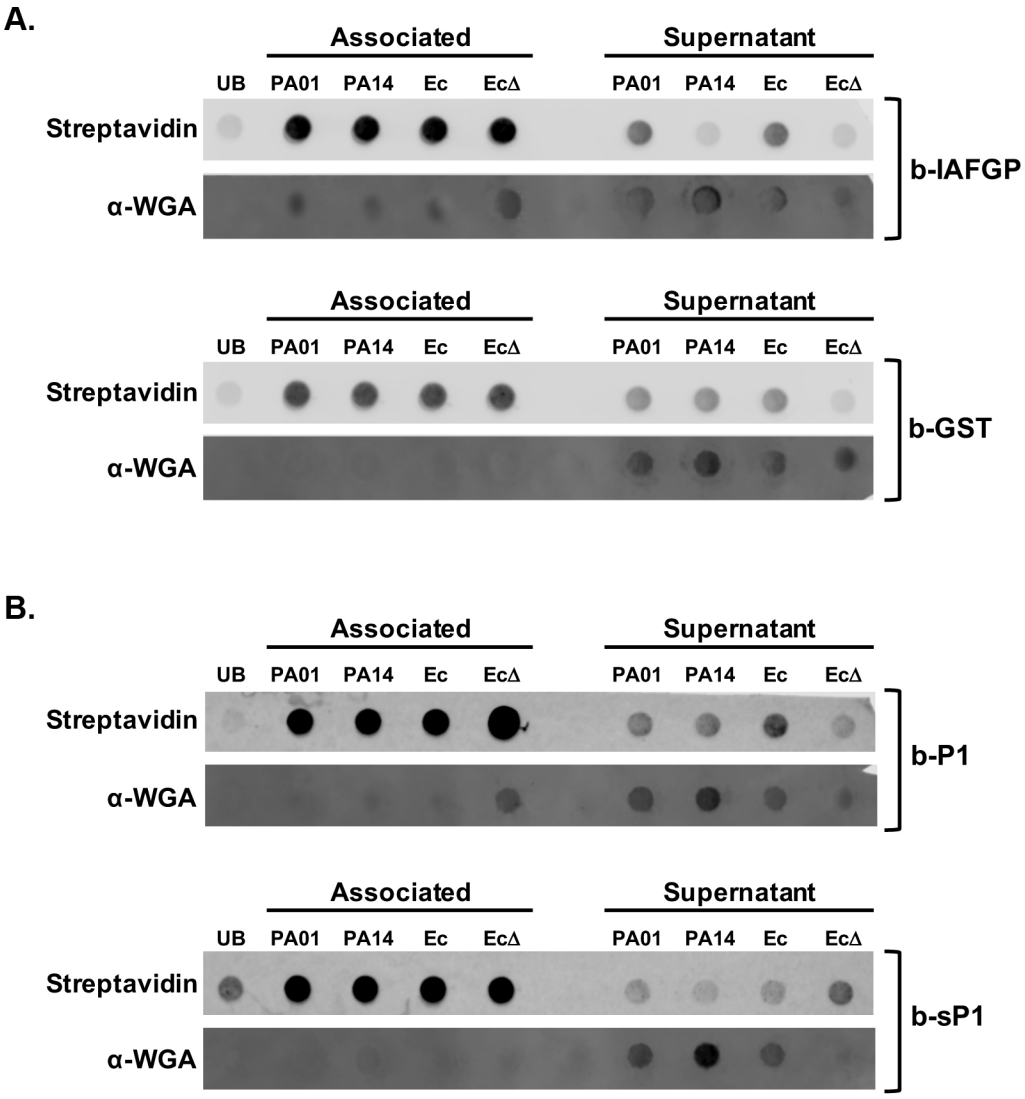

A.
Associated
Supernatant
UB
PA01
PA14
Ec
EcΔ
PA01
PA14
Ec
EcΔ
Streptavidin
α-WGA
b-IAFGP
Associated
Supernatant
UB
PA01
PA14
Ec
EcΔ
PA01
PA14
Ec
EcΔ
Streptavidin
α-WGA
b-GST
B.
Associated
Supernatant
UB
PA01
PA14
Ec
EcΔ
PA01
PA14
Ec
EcΔ
Streptavidin
α-WGA
b-P1
Associated
Supernatant
UB
PA01
PA14
Ec
EcΔ
PA01
PA14
Ec
EcΔ
Streptavidin
α-WGA
b-sP1

Figure S5 (contd).

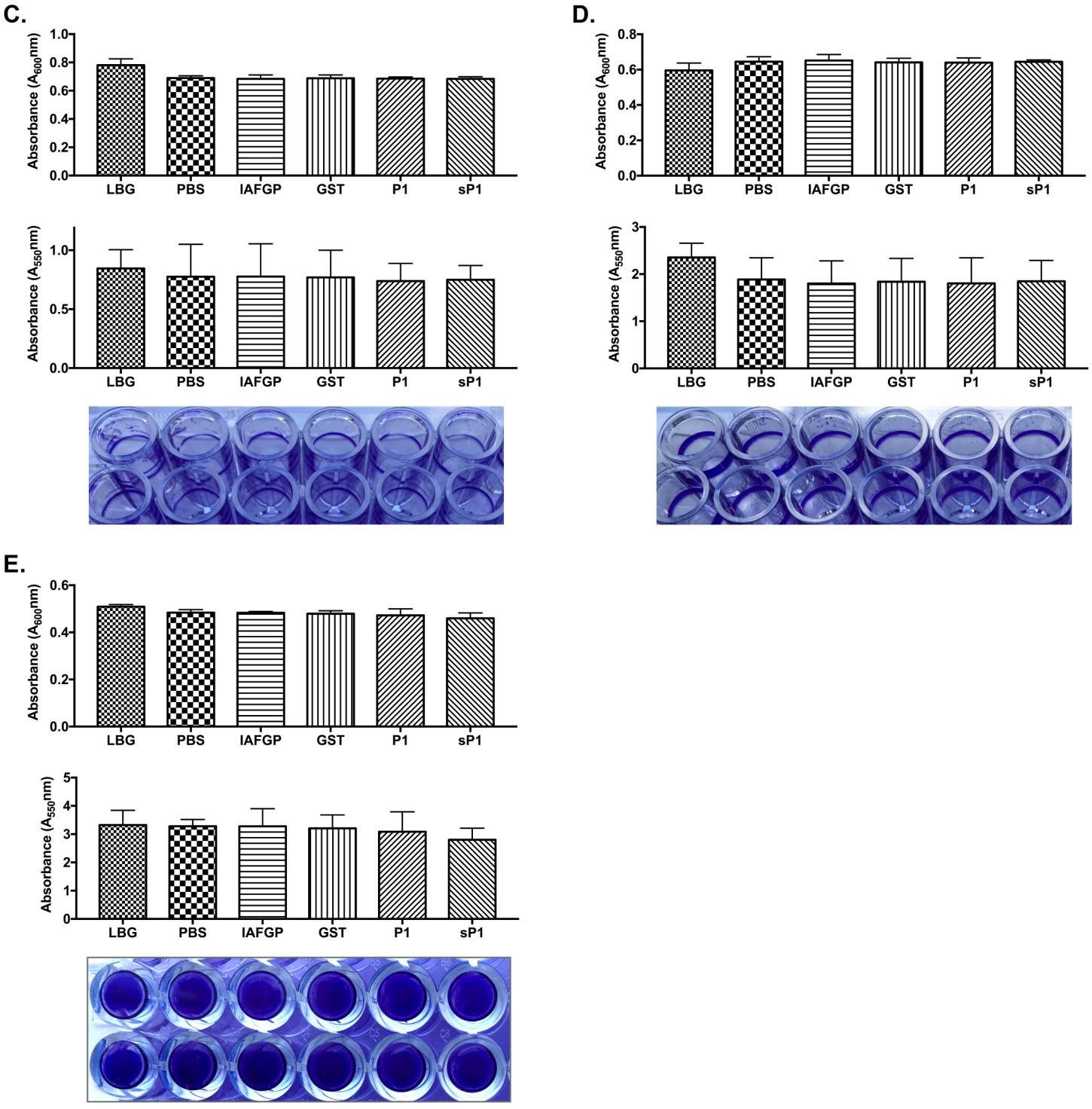


Figure S6.

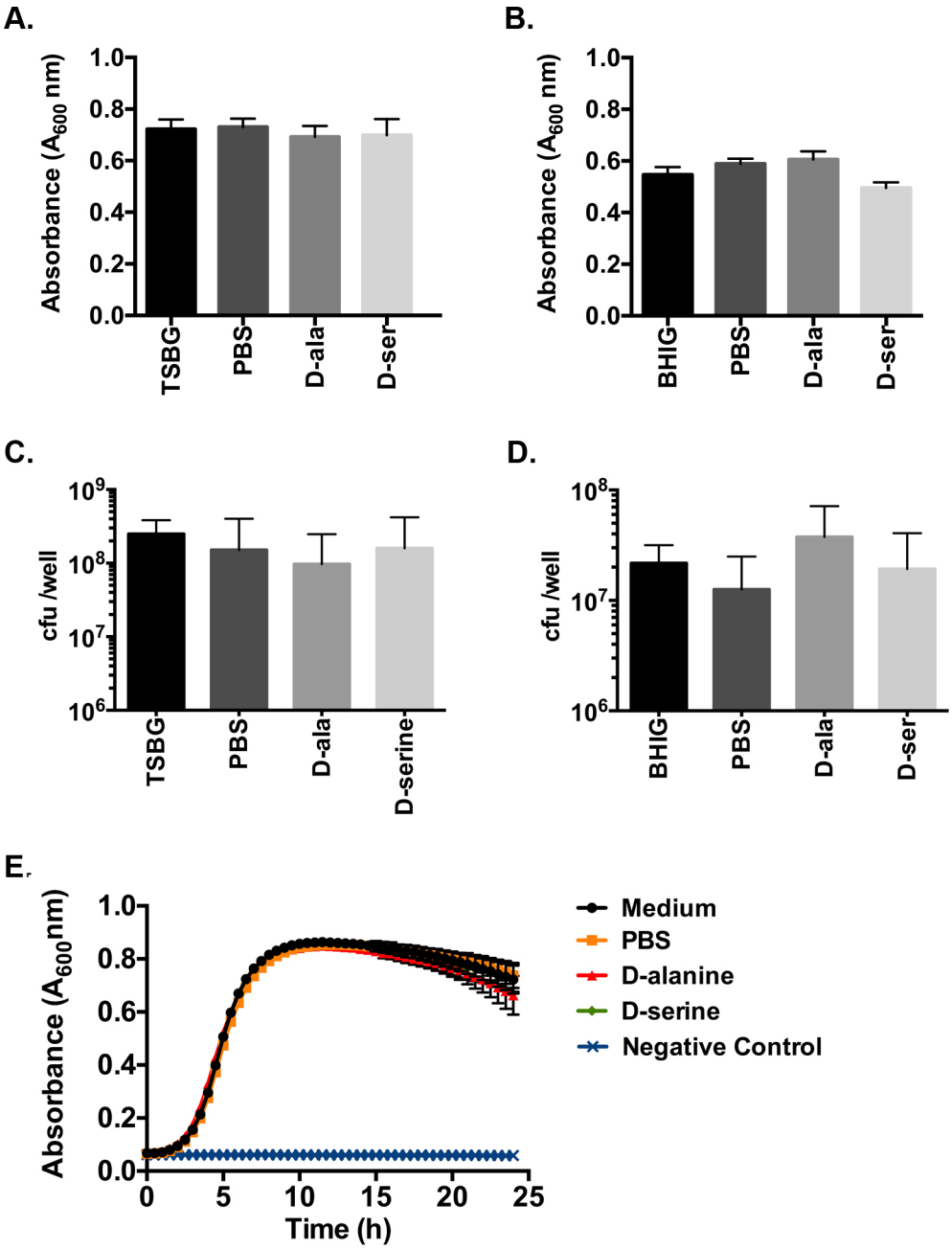

A.
Absorbance (A_{600} nm)
1.0
0.8
0.6
0.4
0.2
0.0
TSBG
PBS
D-ala
D-ser
B.
Absorbance (A_{600} nm)
1.0
0.8
0.6
0.4
0.2
0.0
BHIG
PBS
D-ala
D-ser
C.
cfu /well
10^9
10^8
10^7
10^6
TSBG
PBS
D-ala
D-serine
D.
cfu /well
10^8
10^7
10^6
BHIG
PBS
D-ala
D-ser
E.
Absorbance (A_{600}nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
Time (h)
Medium
PBS
D-alanine
D-serine
Negative Control

Figure S7.

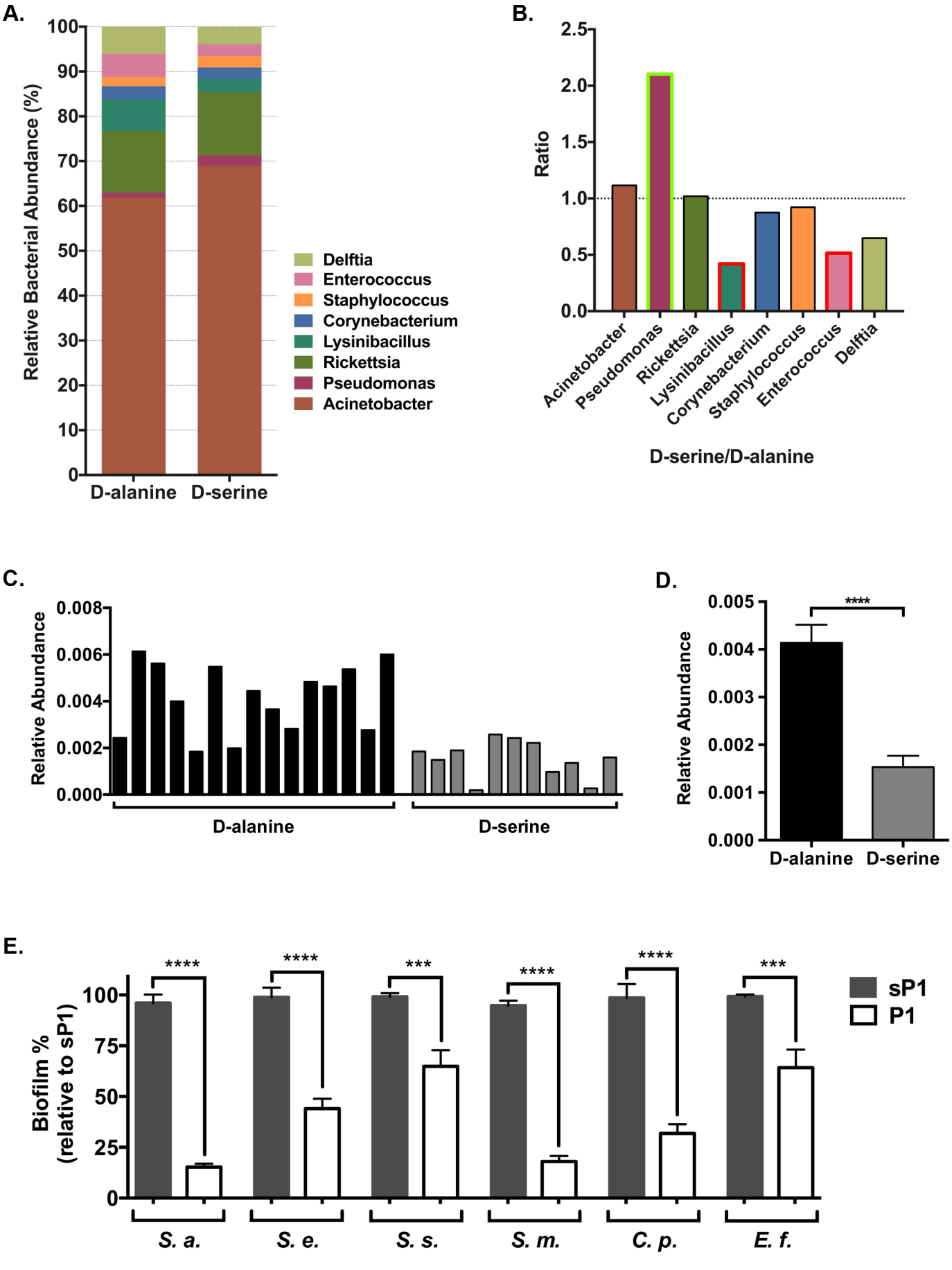

A.
Relative Bacterial Abundance (%)
100
90
80
70
60
50
40
30
20
10
0
D-alanine
D-serine
Delftia
Enterococcus
Staphylococcus
Corynebacterium
Lysinibacillus
Rickettsia
Pseudomonas
Acinetobacter
B.
Ratio
2.5
2.0
1.5
1.0
0.5
0.0
Acinetobacter
Pseudomonas
Rickettsia
Lysinibacillus
Corynebacterium
Staphylococcus
Enterococcus
Delftia
D-serine/D-alanine
C.
Relative Abundance
0.008
0.006
0.004
0.002
0.000
D-alanine
D-serine
D.
Relative Abundance
0.005
0.004
0.003
0.002
0.001
0.000
****
D-alanine
D-serine
E.
Biofilm %
(relative to sP1)
100
75
50
25
0
****
****
***
****
****
***
S. a.
S. e.
S. s.
S. m.
C. p.
E. f.
sP1
P1

Figure S8.

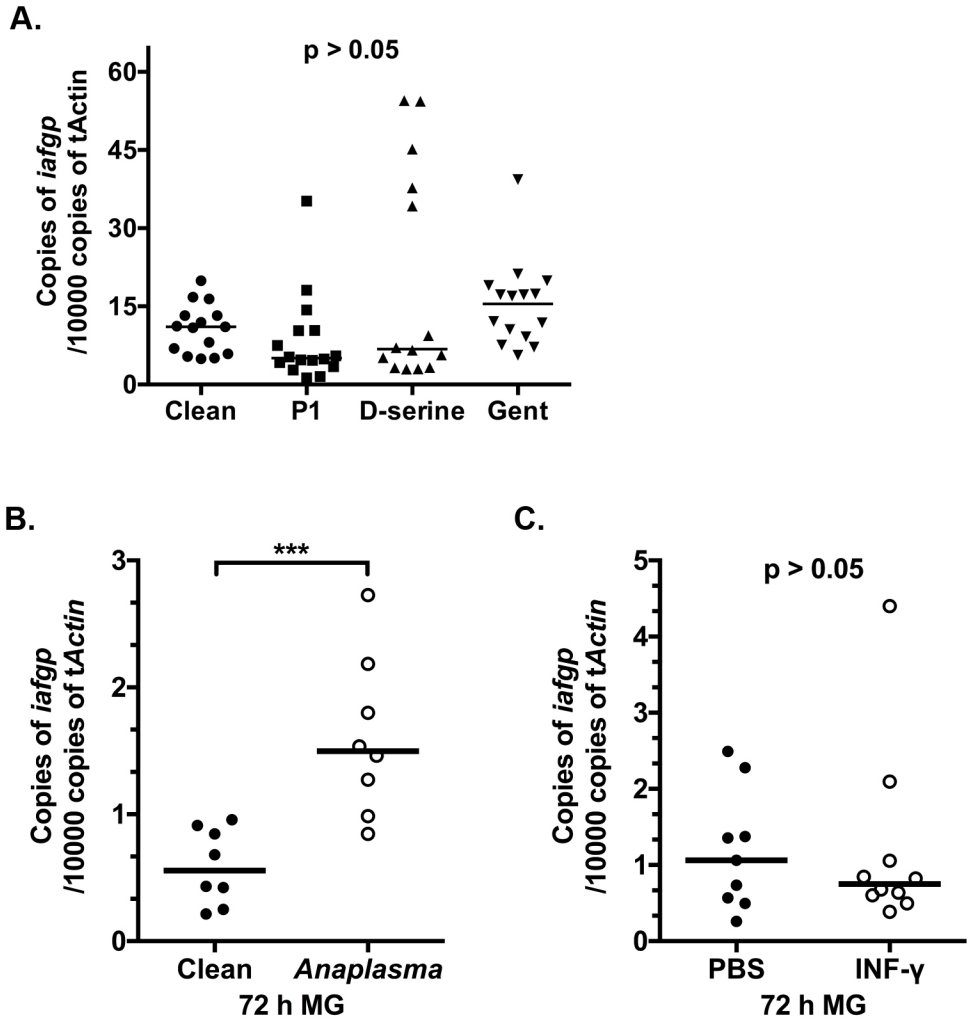

A.
p > 0.05
Copies of iafgp /10000 copies of tActin
60
45
30
15
0
Clean
P1
D-serine
Gent
B.
***
Copies of iafgp /10000 copies of tActin
3
2
1
0
Clean
Anaplasma
72 h MG
C.
p > 0.05
Copies of iafgp /10000 copies of tActin
5
4
3
2
1
0
PBS
INF-γ
72 h MG

Figure S9.

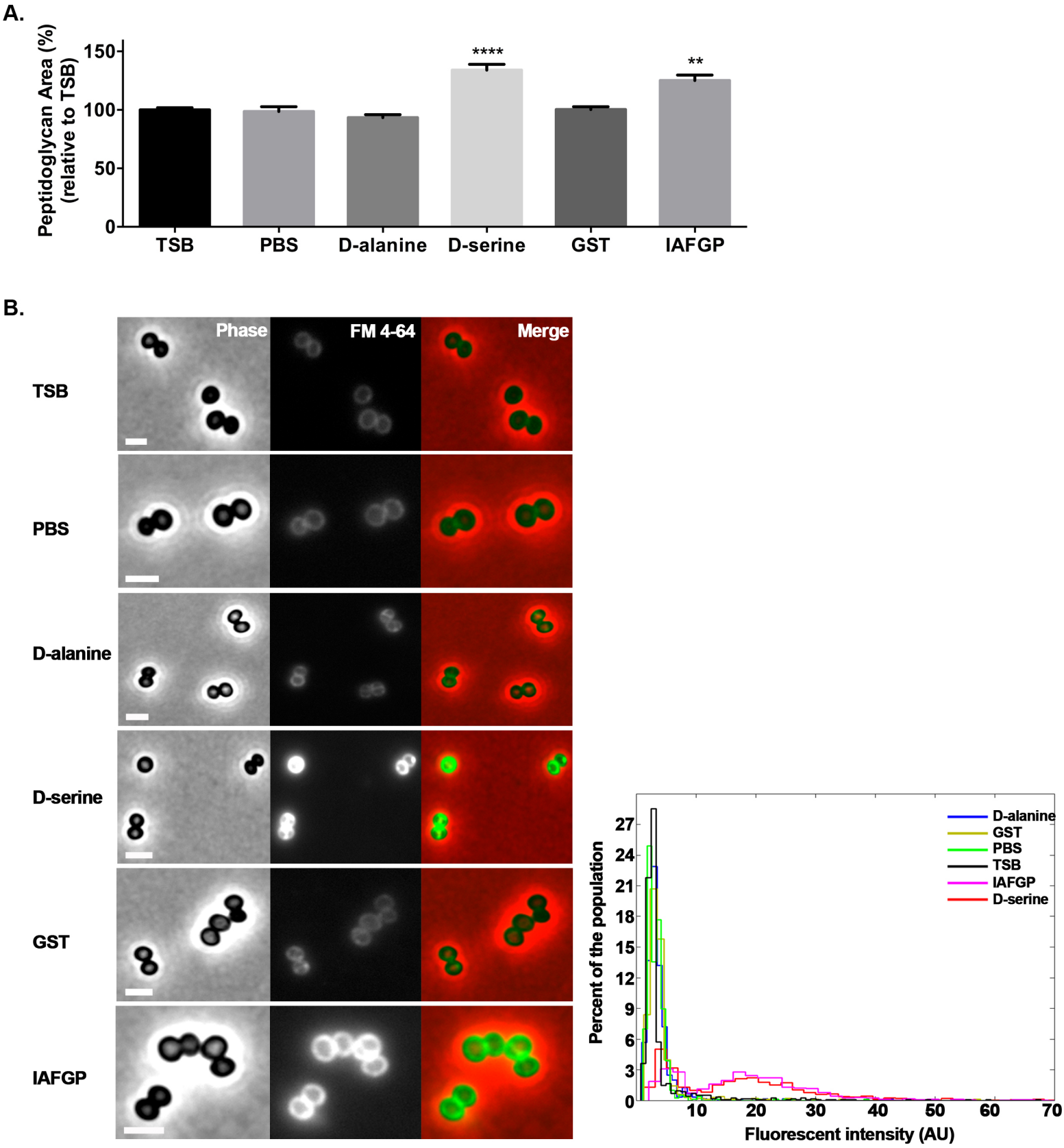

A.
Peptidoglycan Area (%)
(relative to TSB)
150
100
50
0
****
**
TSB
PBS
D-alanine
D-serine
GST
IAFGP
B.
Phase
FM 4-64
Merge
TSB
PBS
D-alanine
D-serine
GST
IAFGP
Percent of the population
27
24
21
18
15
12
9
6
3
0
10
20
30
40
50
60
70
Fluorescent intensity (AU)
D-alanine
GST
PBS
TSB
IAFGP
D-serine

Figure S10.

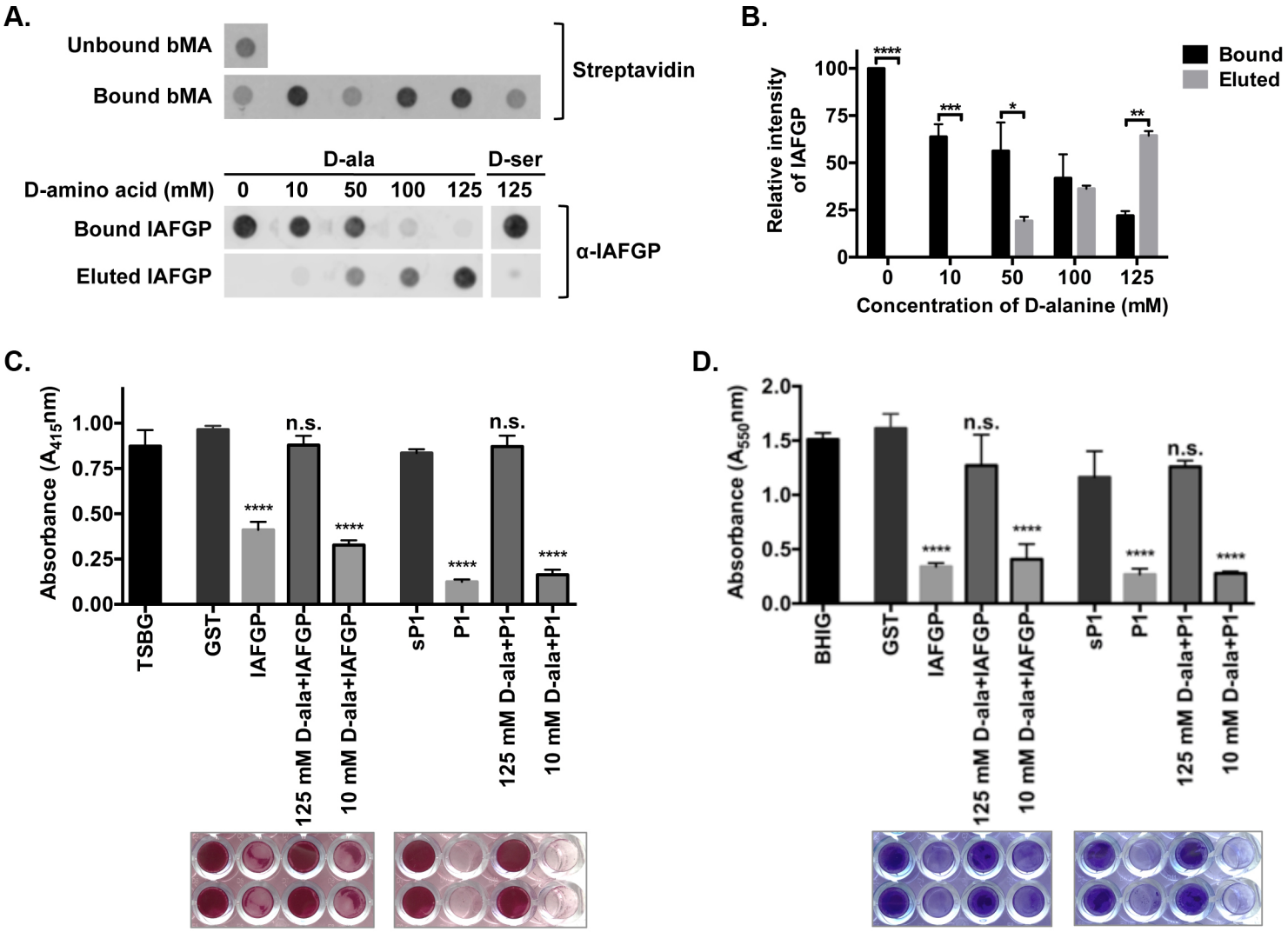

A.
Unbound bMA
Bound bMA
Streptavidin
D-ala
D-ser
D-amino acid (mM)
0
10
50
100
125
125
Bound IAFGP
Eluted IAFGP
α-IAFGP
B.
Bound
Eluted
Relative intensity of IAFGP
Concentration of D-alanine (mM)
C.
Absorbance (A415nm)
TSBG
GST
IAFGP
125 mM D-ala+IAFGP
10 mM D-ala+IAFGP
sP1
P1
125 mM D-ala+P1
10 mM D-ala+P1
n.s.
D.
Absorbance (A550nm)
BHIG
GST
IAFGP
125 mM D-ala+IAFGP
10 mM D-ala+IAFGP
sP1
P1
125 mM D-ala+P1
10 mM D-ala+P1
n.s.

Figure S11.

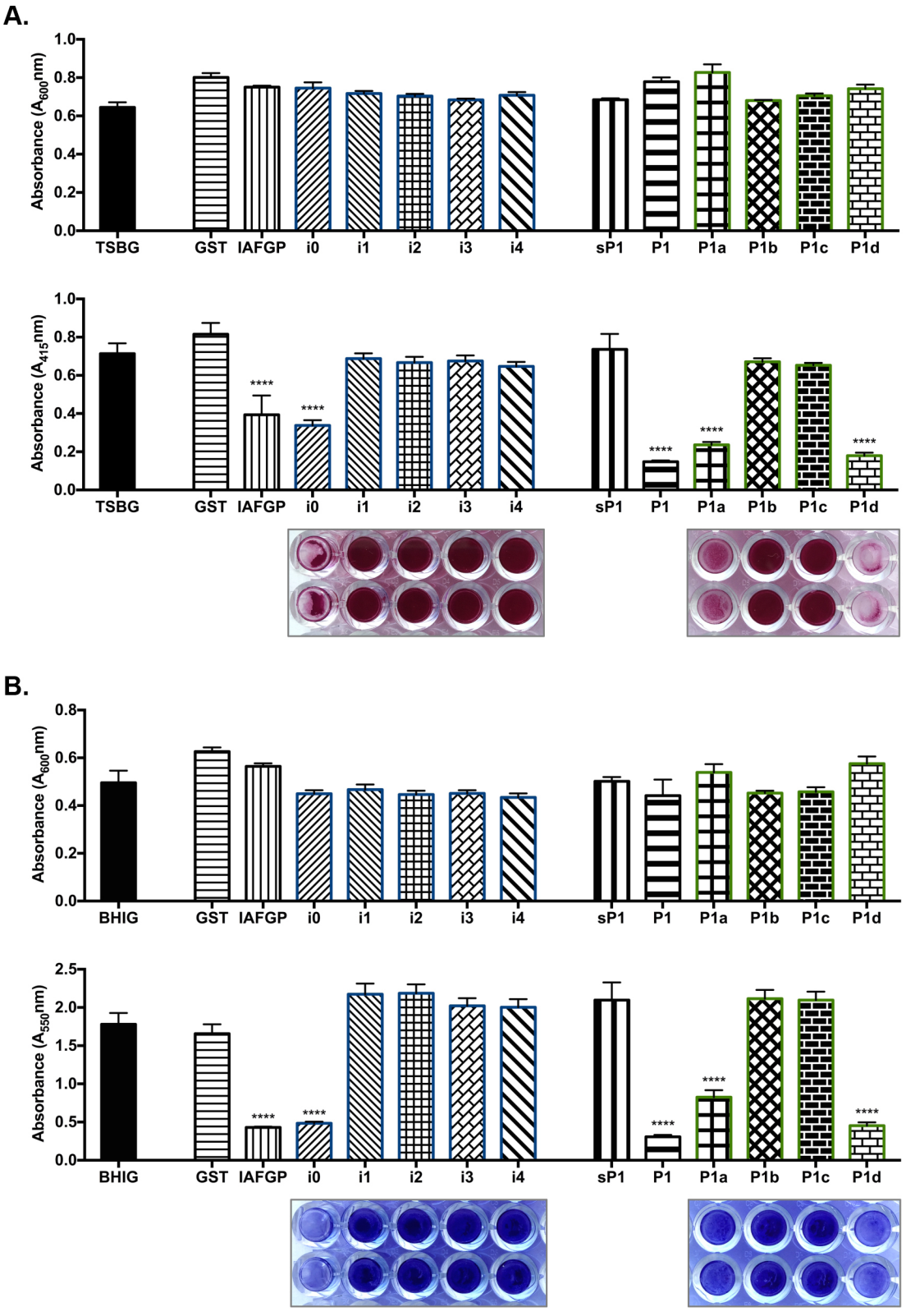
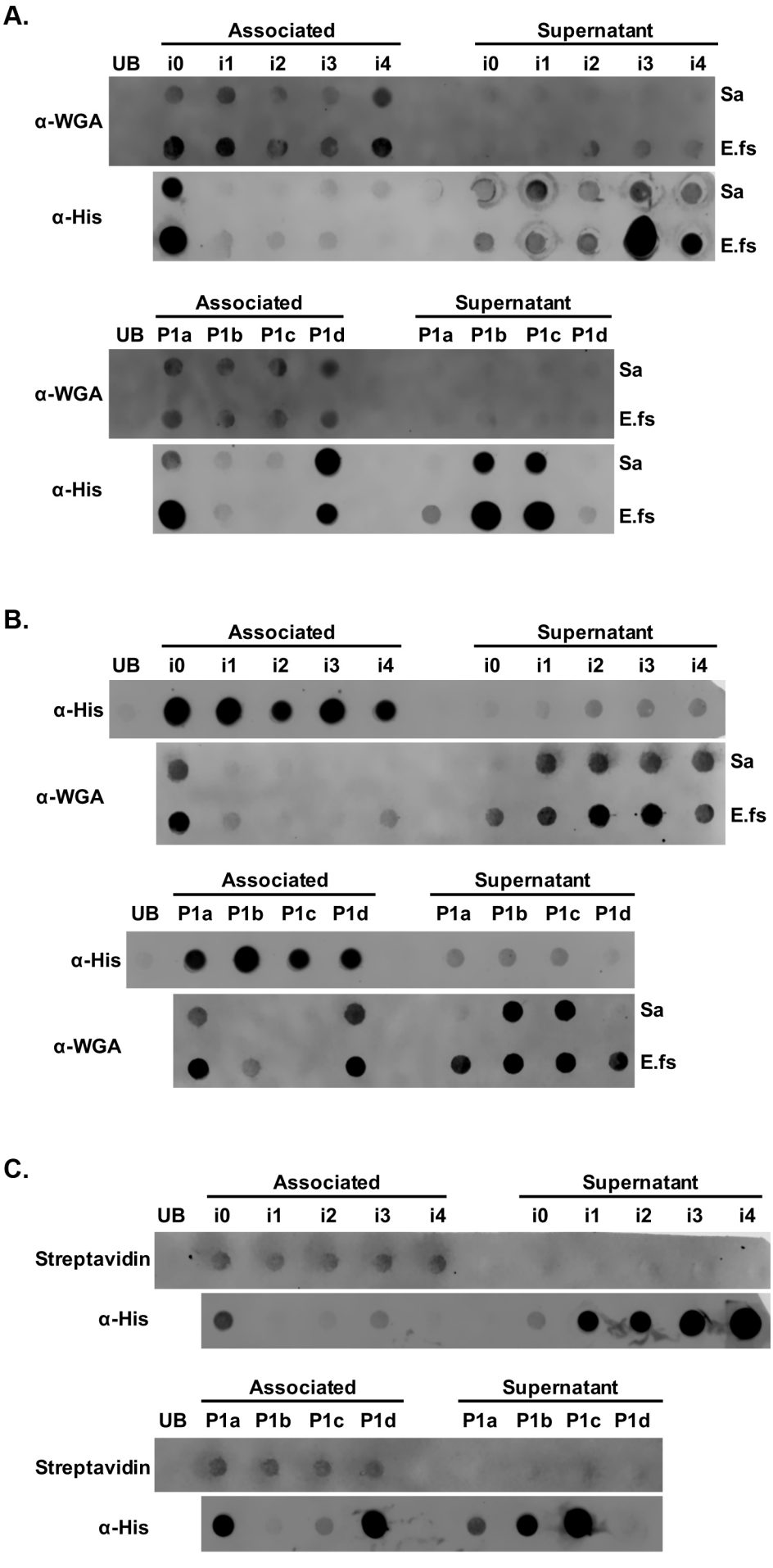

A.
Absorbance (A600nm)
1.0
0.8
0.6
0.4
0.2
0.0
TSBG
GST
IAFGP
i0
i1
i2
i3
i4
sP1
P1
P1a
P1b
P1c
P1d
Absorbance (A415nm)
****
B.
Absorbance (A600nm)
BHIG
Absorbance (A550nm)
2.5
2.0
1.5
1.0
0.5

Figure S12.


A.
Associated
Supernatant
UB i0 i1 i2 i3 i4 i0 i1 i2 i3 i4
α-WGA
Sa
E.fs
α-His
Sa
E.fs
Associated
Supernatant
UB P1a P1b P1c P1d P1a P1b P1c P1d
α-WGA
Sa
E.fs
α-His
Sa
E.fs
B.
Associated
Supernatant
UB i0 i1 i2 i3 i4 i0 i1 i2 i3 i4
α-His
α-WGA
Sa
E.fs
Associated
Supernatant
UB P1a P1b P1c P1d P1a P1b P1c P1d
α-His
α-WGA
Sa
E.fs
C.
Associated
Supernatant
UB i0 i1 i2 i3 i4 i0 i1 i2 i3 i4
Streptavidin
α-His
Associated
Supernatant
UB P1a P1b P1c P1d P1a P1b P1c P1d
Streptavidin
α-His