## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

### Genetic Manipulation of GAS

Precise in-frame allelic replacement and complementation of the GAS *gacI* gene was performed as follows. First, 971 bp of sequence immediately upstream of *gacI* was amplified with the primers *gacI*upF, 5′-gcgctcgagggccaaacctcatacgattagtg-3′ + *gacI*upR+*cat*, 5′-ggtggtatatccagtgatttttttctccatgaaaacttctcctattcatttcaatta-3′, and 902 bp immediately downstream of *gacI* amplified with the primers *gacI*downF+*cat*, 5′-tactgcgatgagtggcagggcggggcgtaaaatgacggtaacagccagtttat-3′ + *gacI*downR, 5′-gcgaagcttcgaatgaccaatgtcataatacattat-3′. The *gacI*upR+*cat* and *gacI*downF+*cat* primers were constructed with 25-bp 5’ extensions corresponding to the 5’ and 3’ ends of the *cat* gene, respectively. The upstream and downstream PCR products were then combined with the 660-bp amplicon of the complete *cat* gene as templates in a second round of PCR using primers *gacI*upF and *gacI*downR. The resultant PCR amplicon, containing an in-frame substitution of *gacI* with *cat*, was subcloned into temperature-sensitive vector pHY304, and allelic exchange mutagenesis in GAS 5448 was performed as described previously to generate the stable mutant 5448ΔGacI. Precise in-frame allelic replacement of *gacI* with *cat* in the 5448ΔGacI chromosomal was confirmed by PCR, restriction enzyme digestion, and DNA sequence analysis. Genetic complementation of ΔGacI with the *gacI* gene on multicopy plasmid vector pDCerm resulted in incomplete complementation suggesting perturbation of gene regulatory networks or improper stoichiometry of the enzymes involved GAC biosynthesis. Therefore, we performed genomic complementation with a ‘watermarked’ copy of *gacI* to allow discrimination from authentic WT cultures. Briefly, the *gacI* gene plus flanking regions (as above) was amplified using primers *gacI*upF and *gacI*downR and proofreading enzyme PfuUltra II and cloned into pCR2.1-TOPO (Invitrogen). Mutagenesis of bp 324 (A to T; silent mutation valine) was performed using primers *gacI**Fwd 5′-gtattagttcaatcttcagttaatatggtaattggatcac-3′ + *gacI**Rev 5′-gtgatccaattaccatattaactgaagattgaactaatac-3′ according to the manufacturer’s instructions (Quickchange lightning kit; Strategene), yielding plasmid pCR2.1-TOPO_*gacI**. After confirmation of

the intended mutation by sequence analysis, the insert was subcloned into temperature sensitive pHY304 and transformed into electrocompetent 5448ΔGacI bacteria. Single crossover chromosomal insertions and double crossover events were selected by temperature shifting in the absence of antibiotic selection. The confirmed complemented point mutant knockin was designated GacI*.

### Bacterial Growth Conditions and Growth Curve Analysis

GAS strains were propagated in Todd-Hewitt broth (THB, Hardy Diagnostics) under static conditions or on Todd-Hewitt agar (THA) at 37°C. Unless indicated otherwise, logarithmic growth phase cultures of optical density at 600 nm ($OD_{600}$) = 0.4 or ~1 × $10^8$ colony-forming units (CFU)/ml were used for all experiments. Erythromycin selection was used at 5 μg/ml for streptococci and 500 μg/ml for *Escherichia coli*. For growth curve analysis vernight cultures of WT, ΔGacI mutant, or GacI* GAS were inoculated in fresh THB to $OD_{600}$ = 0.1. Replicate tubes were incubated at 37°C under static conditions, with hourly $OD_{600}$ measurements to monitor growth kinetics.

### Latex Agglutination Assay

Latex agglutination tests for GAS (Remel pathoDx) were performed according to the manufacturer's instructions using overnight cultures.

### Genome Sequencing, Analysis, and Functional Prediction

All published GAS genome sequences in the SEED database (URL: pubseed.theseed.org) were searched for a conserved chromosomal cluster of genes predicted to encode rhamnose polysaccharide-related functions. We employed protein similarity, chromosomal region comparison and function prediction tools offered through the SEED servers (Aziz et al., 2012) to reannotate the *gacA-L* gene cluster. Whole genome sequencing of SDSE 2005-0193 and 2006-0098 was performed on the Illumina Genome Analyzer II platform. Illumina sequence reads were deposited in the European Nucleotide Archive with

the accession numbers ERS017851 and ERS017852 respectively. Draft genomes were generated by *de novo* assembly of raw Illumina data using Velvet (Zerbino and Birney, 2008) with Abacas (Assefa et al., 2009) used to order contigs to the GGS_124 reference, accession number AP010935 (McMillan et al., 2010). A genome map of 2005-0193 and 2006-0098 in context of the GGS_124 reference genome was determined by BLAST comparisons using BRIG (Alikhan et al., 2011). Genome architecture of the carbohydrate loci was determined by Clustal alignments of regions between *dnaG* and *infC* and by tBLASTx analysis of assembled draft genomes.

### Effect of Tunicamycin on GAS Growth and GAC Expression

Bacteria were grown overnight in the presence of 0.25, 0.1, or 0.025 µg/ml tunicamycin and growth was assessed by monitoring $OD_{600}$. Microscopic morphology was acquired using the bright field channel. Bacteria harvested after overnight culture were resuspended at $OD_{600}$ of 1.0 in PBS, and 100 µl incubated with 100 U/ml mutanolysin and decrease in $OD_{600}$ recorded using Bioscreen C MBR machine. GAC from WT GAS cultured in the presence of 0.1 or 0.025 µg/ml tunicamycin was extracted and analyzed as described below.

### Rabbit Polyclonal Antiserum

GAC purified from WT GAS (WT GAC) and the ΔGacI mutant (ΔGAC) were coupled to recombinant pneumococcal protein SP_0435 by streptavidin-biotin affinity interactions and complexes purified by gel filtration chromatography to > 95% purity (Zhang et al., 2013). The GAS homologue of SP_0435 (elongation factor) is absent from the published GAS surface proteomes (Rodriguez-Ortega et al., 2006; Severin et al., 2007), is not labeled by biotinylation like other GAS surface proteins (Cole et al., 2005), and is not immunoreactive to pooled hyperimmune sera from an Australian Aboriginal population in which GAS is highly endemic (Cole et al., 2005). Polyclonal rabbit antibodies were raised against MAPS-conjugated WT GAC and ΔGAC through Cocalico Biologicals (Reamstown, PA). After initial

immunization with 20 µg GAC conjugate (100 µg protein), 4 boosts with 10 µg GAC conjugate (50 µg protein) were performed on days 14, 21, 49 and 70, with test bleeds performed on days 0, 35, and 56 to monitor antibody titers by ELISA. Rabbits were exsanguinated under anesthesia by terminal cardiac puncture 10 days after the final immunization. ELISA was performed using purified WT GAC or ΔGAC to determine specific IgG titers. Titer of the anti-WT GAC serum was 1:51,800 against purified GAC.

### GAS Virulence Determinants and Traits

Two independent assays quantified hyaluronic acid capsule expression as previously described (Cole et al., 2012). SpeB proteolytic activity was assessed in stationary phase GAS culture supernatants (Cole et al., 2010). For fibrinogen (Fg) binding, 96-well plates were coated with human Fg, washed, blocked, and incubated with $2 \times 10^7$ CFU bacteria. Adherent bacteria were released by 0.25% trypsin/1 mM EDTA and CFU enumerated. For cell surface plasmin accumulation, bacteria were grown to exponential phase in the presence of 1 U/ml human plasminogen + 7 µM human Fg (Wang et al., 1995) or THB alone, and incubated with substrate S-2251 for 1 h at 37°C. Cell surface plasmin activity was calculated as absorbance units ($405_{nm}$)/CFU.

### Neutrophil Phagocytosis

Neutrophil phagocytosis was quantified using FITC-labeled bacteria under shaking conditions after 15 min incubation at 37°C and analyzed by flow cytometry (Rooijakkers et al., 2005), as well as by by confocal microscopy after addition of the lipophilic styryl dye FM5-95 (10 µg/ml) to label neutrophil membranes.

### Preparation of Platelet Releasates

Whole blood from consenting, healthy, drug-free donors was anticoagulated with sodium citrate, and washed platelets were prepared in Walsh buffer and suspended to $5 \times 10^8$ platelets/ml (Leng et al., 1998).

Platelets were then stimulated with thrombin, centrifuged at 2,000 × *g*, and supernatant containing the platelet releasate was used for bactericidal assays at a final concentration of 25%. A role for thrombin itself was excluded by adding exogenous thrombin or by blocking thrombin activity through the addition of hirudin (data not shown).

### Quantification of C3b Deposition

Exponential phase bacteria were washed, resuspended in HEPES++0.1% BSA, and incubated in a range of serum concentrations for 20 min at 37°C. After washing, samples were incubated with FITC-conjugated goat (Fab)2 anti-C3 antibody (Protos Immunoresearch) and analyzed by flow cytometry.

### Antimicrobial Susceptibility Assays

Exponential phase bacteria were resuspended in PBS and incubated in assay medium: THB + nafcillin (0.2 μg/ml), THB + vancomycin (4 μg/ml), or DMEM 10% THB + lysozyme (2.5 mg/ml). Bacterial survival at indicated time points was determined by dilution plating and expressed as percentage of initial inoculum.

### Autolysis and Oxidative Stress Sensitivity

Log phase GAS were centrifuged, washed twice with PBS and autolysis induced by washing with cold Milli-Q water. Bacteria were then resuspended in PBS containing 0.05% (v/v) Triton X-100 and $OD_{600}$ measured every 30 min at 30°C for 4 h. Log phase bacteria were incubated with 0.05% $H_2O_2$ in THB and surviving CFU calculated at indicated time points; catalase was added to the first dilution to quench residual $H_2O_2$. For superoxide sensitivity, overnight cultures of GAS WT or ΔGacI bacteria were washed once in THB and inoculated into THB + 10 mM paraquat dichloride x-hydrate PESTANAL (Sigma) to a starting $OD_{600}$ = 0.1. Replicate tubes were incubated at 37°C under static conditions and $OD_{600}$ used to monitor growth kinetics.

## Total GAS Protein Profiling

For total protein profile comparisons, exponential phase GAS WT and ΔGacI mutant cultures were resuspended in Tris buffer containing mutanolysin and lysostaphin and incubated at 37°C. Samples were boiled and bacterial lysates separated on 10% and 15% SDS-PAGE gels and silver stained.

## Surface Plasmon Resonance

SPR binding studies were performed on a Biacore T100 instrument (GE Healthcare). LL-37 peptide (Anaspec) was immobilized on a CM5 sensor chip using an amine coupling kit (GE Healthcare). After activation of the flow cell with the EDC/NHS mixture, 10 µg/ml of LL-37 dissolved in 10 mM sodium acetate buffer pH 4.0 was injected for 420 s at flow rate 10 µl/min, then free reactive sites quenched with ethanolamine (1 M). A reference flow cell was only activated and quenched without immobilization of a ligand. HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 0.005 % Tween, pH 7.4) was used as running buffer. Lyophilized WT and ΔGacI mutant GAC were dissolved in PBS pH 7.4 in four different concentrations (0.4, 0.8, 1.2 and 1.6 mg/ml) and used as analyte for 60 s with a flow rate of 30 µl/min. After a dissociation time of 120 s, the sensor chip was regenerated with 10 mM glycine-HCl pH 1.7 for 30 s. PBS containing 0.005% Tween 20 was used as running buffer. The experiment was performed two times with similar results. For analysis, the response unit [RU] values measured in the reference flow cell were subtracted from the RU values detected in the flow cell with immobilized LL-37. Additionally, the RU values of a blank sample (only running buffer) were subtracted from the binding curve of each analyte. An overlay of the SPR sensorgrams was generated using the Biacore T100 Evaluation Software (GE Healthcare).

## Monoclonal Antibody Binding Assay

EIA/RIA 96-well plates were coated with 10 µg of purified WT or ΔGacI GAC. Plates were washed, blocked, and incubated with neat hybridoma supernatants in triplicate overnight at 4°C followed by

peroxidase-conjugated donkey anti-human IgM (Jackson Immunoresearch) and read at an absorbance of 670 nm.

## Antibody Cross-Reactivity Testing

ELISA on human heart lysate was performed as described previously (Henningham et al., 2012), using rabbit antisera raised against WT GAC or ΔGAC and a control rabbit antiserum against M1 protein. For immunohistochemistry, deparaffinized human heart tissue sections were treated with Power Block (BioGenex, Fremont CA) with 1% normal goat serum overnight at 4°C, and anti-WT GAC or anti-ΔGAC antisera incubated at 1:1,000 dilution for 2 h at room temperature, with NRS or anti-human cardiac myosin as negative and positive controls, respectively. Biotin-conjugated FAB' affinity-purified goat anti-rabbit IgG Ab (1:1,000; Jackson ImmunoResearch Laboratories) was incubated on tissues for 30 min and detected with alkaline phosphatase-conjugated streptavidin and Fast Red substrate (BioGenex) against a Mayer's hematoxylin (BioGenex) counterstain.

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