SUPPLEMENTAL INFORMATION

A role of epithelial cells and virulence factors in biofilm formation by *Streptococcus pyogenes in vitro*

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SUPPLEMENTARY FIGURES

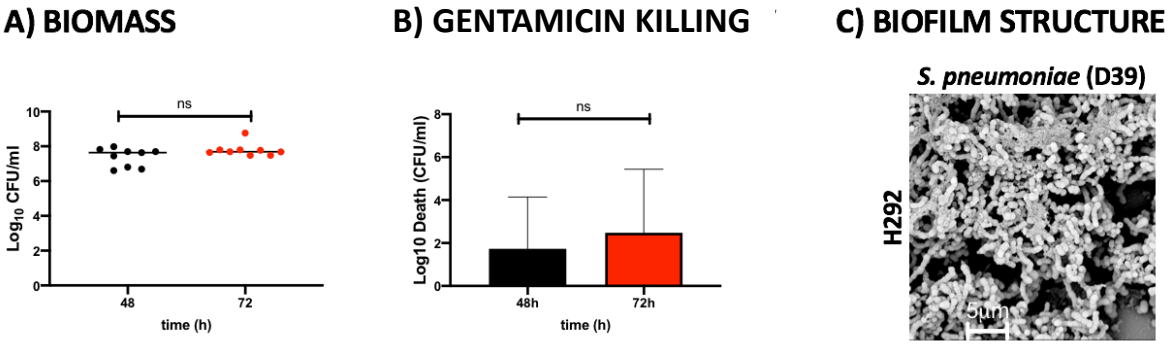

Figure S1. Biofilm growth, antibiotic resistance, and structure of *Streptococcus pneumoniae* on pre-fixed epithelial cells. (A and B). Biofilms developed over 48 (black color) and 72 h (red color) at 34°C in CDM were assessed for biomass and gentamicin killing. (A) Biomass formation on pre-fixed epithelial H292 cells for *Streptococcus pneumoniae* (Spn-D39) was evaluated by assessing the Log_{10} colony forming units [CFU] per ml. The results represent data from 3 separate experiments of 3 individual biofilms each (n = 9) and data was compared using the Mann-Whitney U test. (B) Gentamicin sensitivity in Spn-D39 biofilms was determined by calculating the Log_{10} death in CFU/ml (i.e., the total biomass CFU/ml – biofilm biomass CFU/ml after treatment with 500 µg/ml gentamicin for 3 h). The results represent the mean data from three separate experiments of three individual biofilms each (± SD; n = 3), and data was compared using Student's t-test. For all statistical analyses: ns, non-significant difference. (C) The structure of Spn-D39 biofilms formed on pre-fixed epithelial cells for 72 h was visualized using SEM (scale bar = 5 µm).

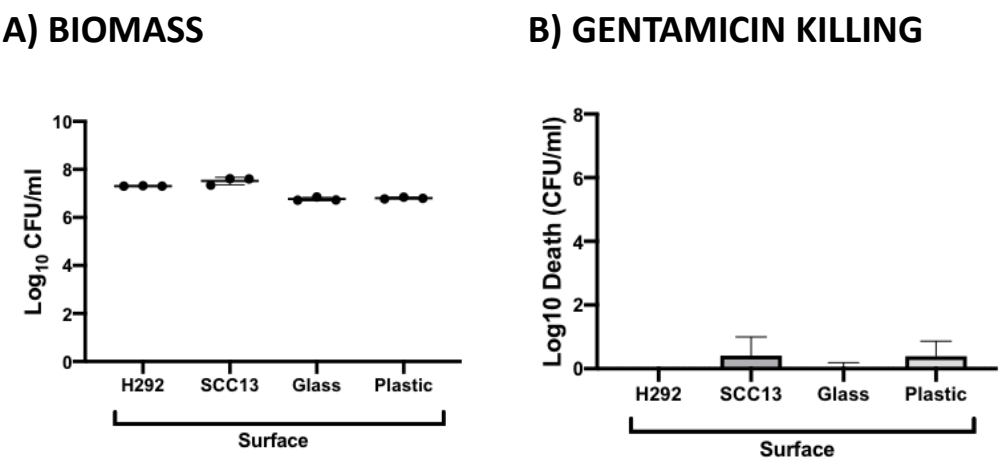

Figure S2. Biofilm growth and antibiotic resistance of GAS-771 formed on biological vs abiotic surfaces. Biofilms developed over 72 h at 34°C on biological (pre-fixed respiratory H292 cells or SCC13 keratinocytes) or abiotic (Glass or Plastic) surfaces were assessed for biomass and gentamicin sensitivity. (A) Biomass formation of biofilms formed on biological (pre-fixed H292 cells or SCC13 keratinocytes) or abiotic (Glass or Plastic) surfaces for the GAS-771 strain was evaluated by measuring the Log_{10} colony forming units [CFU] per ml. The results represent data from one experiment with 3 individual biofilms (± SD; n = 3). (B) Determination of gentamicin killing in GAS-771 biofilms was performed by calculating the Log_{10} death in CFU/ml (i.e., the total biomass CFU/ml – biofilm biomass CFU/ml after treatment with 500 μg/ml gentamicin for 3 h). The results represent the mean data from one experiment of three individual biofilms (± SD; n = 3). Student's t-test was used for comparison of biomass (A) or gentamicin killing (B) between groups of data points.

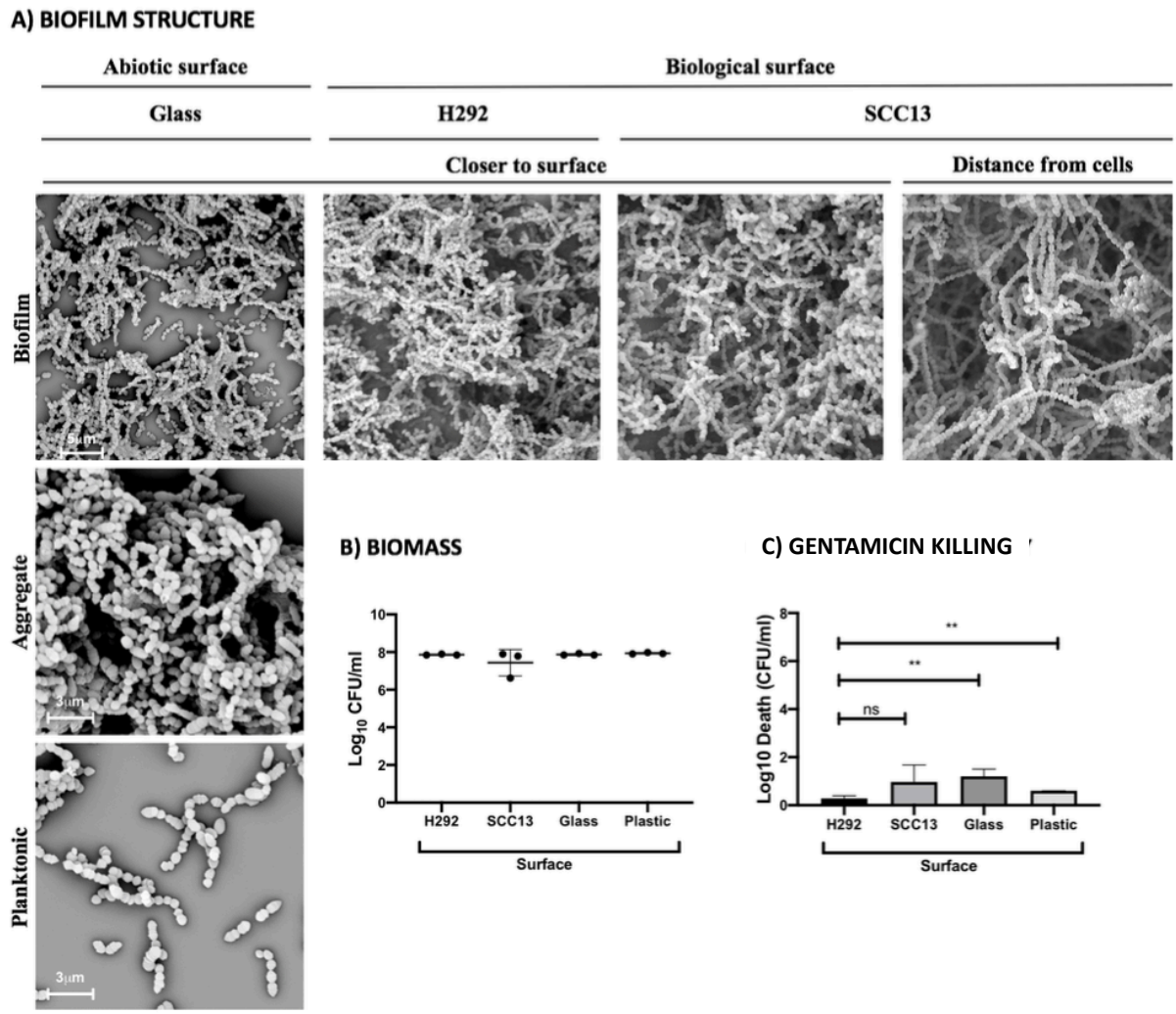

Figure S3. Stages of M5 bacterial growth, antibiotic resistance and structure on abiotic or biological surfaces. (A) M5 (Manfredo) bacteria were used to form biofilms in CDM over 72h at 34 °C on glass or pre-fixed epithelial cells (respiratory H292 cells or SCC13 keratinocytes). Additionally, M5 bacteria were grown planktonically in CDM at 34 °C until reaching the exponential phase and were then seeded onto glass to observe the level of bacterial aggregation. Samples were visualized using SEM for biofilms (scale bar = 5 μm) or for planktonic and aggregated bacteria (scale bar = 3 μm). Although a low level of aggregation occurred in planktonic cultures, visualization of both aggregate and free chains are presented. (B and C). Biofilms formed over 72 h at 34°C on biological (pre-fixed respiratory H292 cells or SCC13 keratinocytes) or abiotic (Glass or Plastic) surfaces were assessed for biomass and gentamicin killing. (B) Biomass was evaluated by measuring the Log_{10} colony forming units [CFU] per ml. The results represent data from one experiment with three individual biofilms each (± SD; n = 3). (C) Determination of gentamicin killing was performed by calculating the Log_{10} death in

CFU/ml (i.e., the total biomass CFU/ml – biofilm biomass CFU/ml after treatment with 500 µg/ml gentamicin for 3 h). The results represent the mean data from one experiment of three individual biofilms each (± SD; n = 3). Student's t-test was used for comparison of biomass (B) or gentamicin sensitivity (C) between groups of data points. **, $P < 0.01$ and ns, non-significant difference.

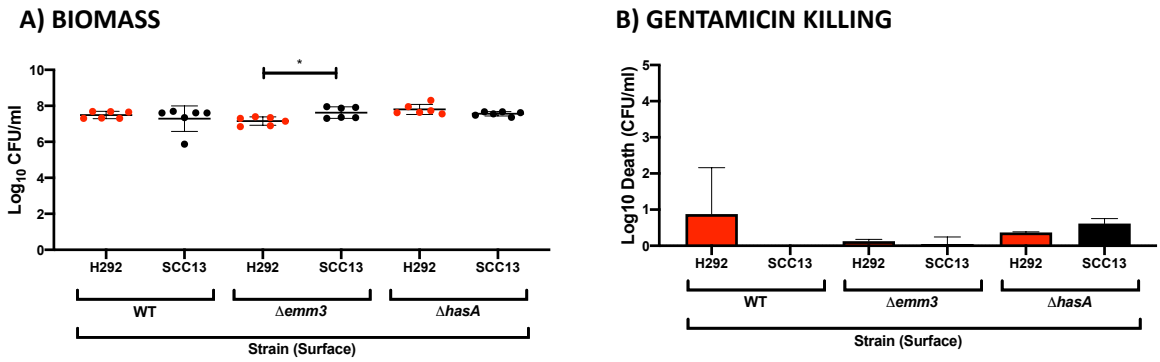

Figure S4. Biofilm growth and antibiotic resistance in the presence or absence of M3 protein or capsule. Biofilms developed over 72 h at 34°C on biological (pre-fixed respiratory H292 cells or SCC13 keratinocytes) were assessed for biomass and gentamicin killing. (A) Biomass formation of biofilms formed on biological (pre-fixed H292 cells or SCC13 keratinocytes) surfaces for the WT (GAS-771) strain and its isogenic mutants lacking M3 protein (Δ*emm3*) or capsule (Δ*hasA*) was evaluated by measuring the Log_{10} colony forming units [CFU] per ml. The results represent data from two experiments with 3 individual biofilms each (± SD; n = 6). (B) Determination of gentamicin sensitivity in WT, Δ*emm3* and Δ*hasA* biofilms was performed by calculating the Log_{10} death in CFU/ml (i.e., the total biomass CFU/ml – biofilm biomass CFU/ml after treatment with 500 µg/ml gentamicin for 3 h). The results represent the mean data from two experiments of six individual biofilms (± SD; n = 3). Mann-Whitney U test was used for comparison of biomass (A) or gentamicin killing (B) between groups of data points.

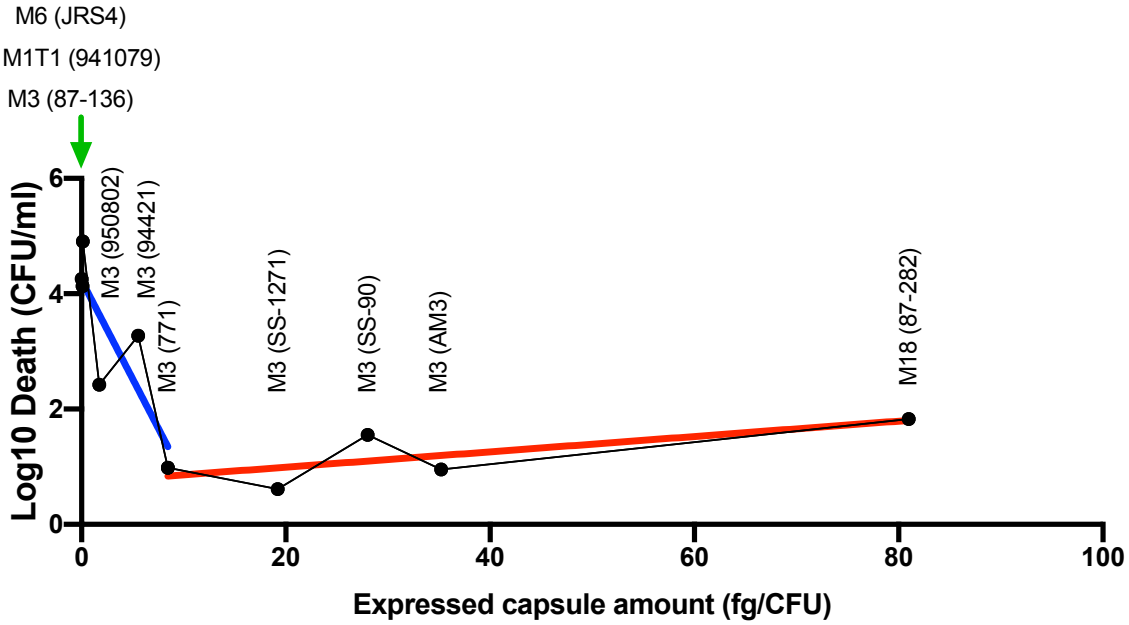

Figure S5. High capsule expression correlates with the ability of GAS to form functional biofilms. The gentamicin sensitivity (in $\log_{10}$ death after gentamicin treatment) of biofilms of the GAS M1T1 (941079), M6 (JRS4), M3 (GAS-771, 87136, 950802, 94421, SS-1271, SS-90 or AM3), or M18 (87-282) strains after 72h at 34°C on pre-fixed H292 cells was plotted against the level of capsule expressed by each strain when grown in media (in fg/CFU). The blue line represents the linear correlation of gentamicin killing and capsule production for strains expressing <8 fg/CFU of hyaluronic acid capsule ($P < 0.05$ by simple linear regression analysis), while the red line represents the lack of a significant correlation ($P > 0.05$ by simple linear regression analysis) for strains expressing >8 fg/CFU of hyaluronic acid. The green arrow indicates the plotted values on the y-axis corresponding to the strains M6 (JRS4), M1T1 (941079), and M3 (87-136) that all expressed very low capsule levels.

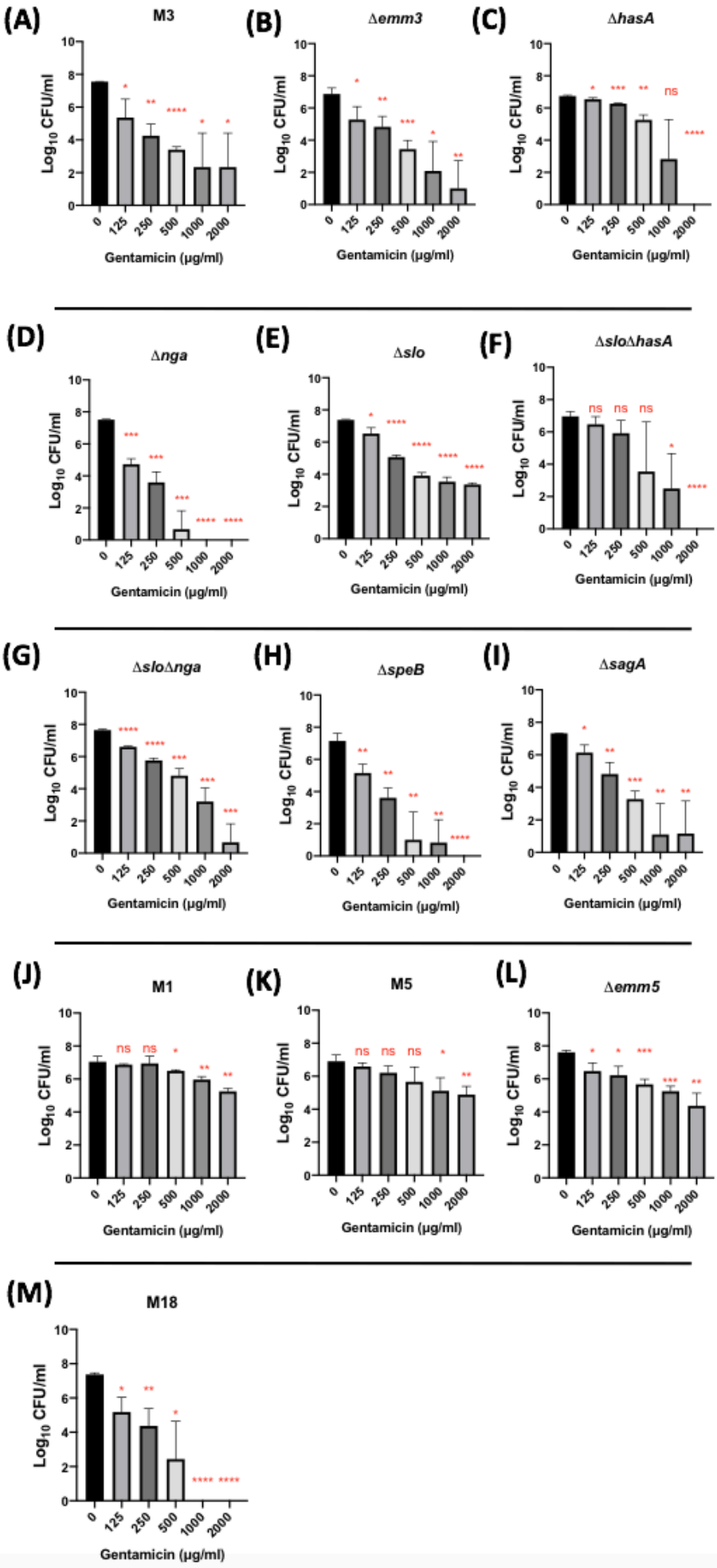
(A)
M3
(B)
Δemm3
(C)
ΔhasA
(D)
Δnga
(E)
Δslo
(F)
ΔsloΔhasA
(G)
ΔsloΔnga
(H)
ΔspeB
(I)
ΔsagA
(J)
M1
(K)
M5
(L)
Δemm5
(M)
M18
Log10 CFU/ml
Gentamicin (μg/ml)
0
125
250
500
1000
2000
ns

Figure S6. Gentamicin sensitivity of planktonic GAS grown in broth. Planktonic GAS bacteria grown in CDM were assessed for gentamicin sensitivity by determining the Log_{10} colony forming units [CFU] per ml after exposure to indicated gentamicin concentrations (in μg/ml) for 3 h at 34 °C. Each assay was performed three times (n=3). Differences in gentamicin induced killing compared to the non-treated control were analyzed using the Student's t-test. Statistical significance is displayed as follows: *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ and ns, non-significant difference.