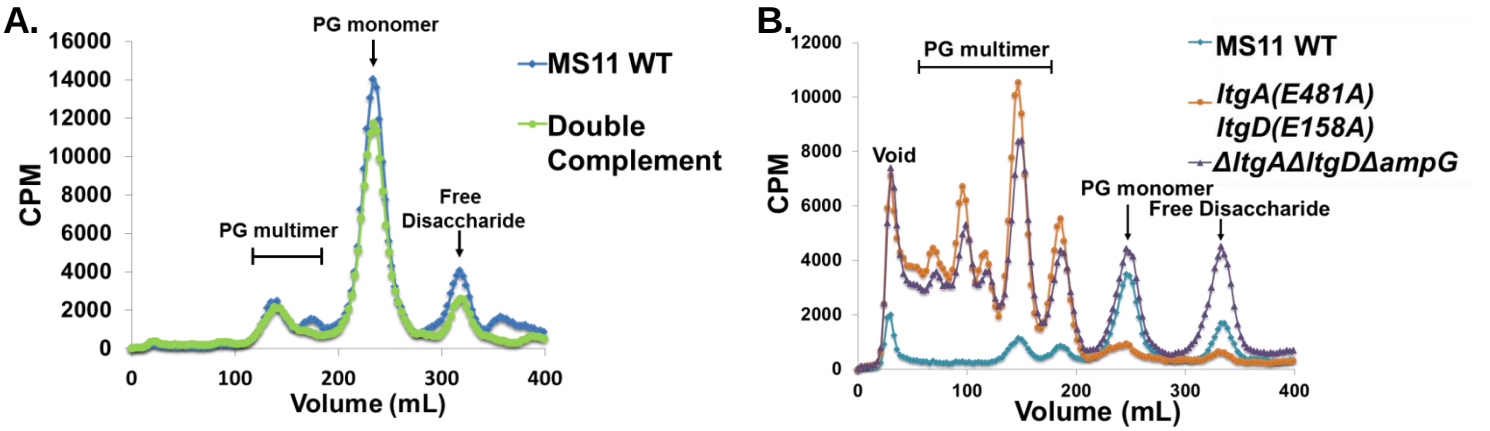
Supplemental Table 1. Bacterial Strains and plasmids used in this study.

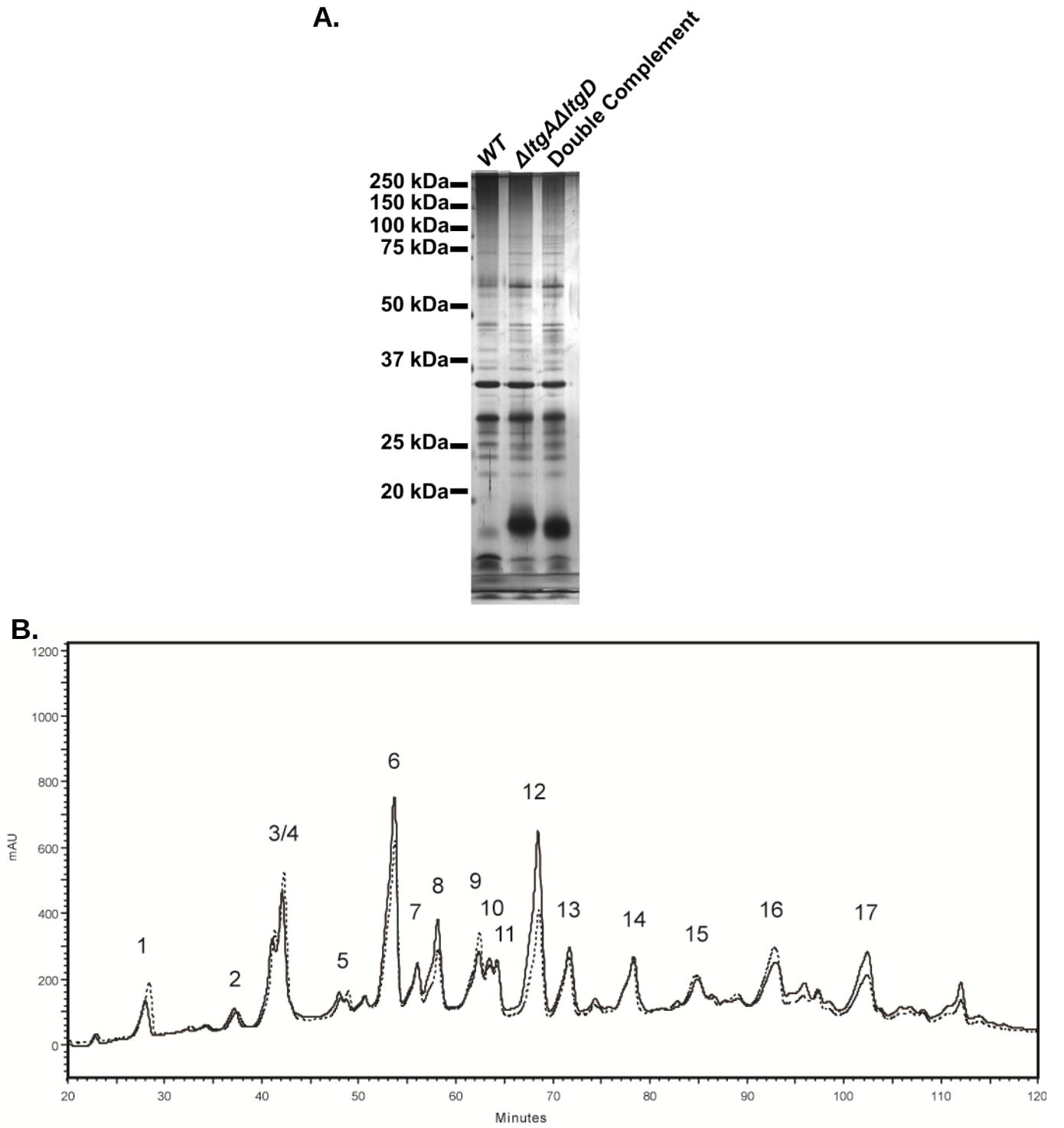
Plasmid or strain	Properties	Source or Reference
pKH37	Complementation vector, Cm^R	Kohler, P.L., *et al.*
pMR68	Complementation vector, Erm^R	Ramsey, M.E., *et al.* (2012)
pKH96	*ltgA* complementation in pKH37, Cm^R	This work
pRS62	*ltgD* complementation in pMR68, Erm^R	This work
pRS91	LtgA active site mutation construct, Erm^R	Schaub, et al. under review
pRS92	LtgD active site mutation construct, Erm^R	Schaub, et al. under review
pDG132	*ampG* deletion vector, Erm^R	Garcia, D.L., and Dillard, J.P.
pKH40	827-bp NruI-AgeI *pacA* fragment of RD_5 in EcoRV-XmaI pIDN1	Dillard, J.P., and Hackett, K.T.
pKH38	*pacA* in SpeI-KpnI-digested pKH35, *pacA* complementation, Cm^R	Dillard, J.P., and Hackett, K.T.
WT, MS11 VD300 $P+_{nv}$	Wild-type RecA + MS11 *N. gonorrhoeae* with nonvariable VD300 pilin	This work
ΔltgAΔltgD	KH560, *ltgA ltgD* double mutation in MS11	Cloud-Hansen, K.A., *et al.*
ltgA+ *ltgD*+ Double Complement	RS512, *ltgA* and *ltgD* complementation in KH560 with pKH96 and pRS65	This work
ltgA(E481A)ltgD(E158A)	RS559, *ltgA(E481A) ltgD(E158A)* double point mutation in MS11	This work
ΔltgAΔltgDΔampG	RS562, *ampG* mutation by pDG132 in KH560	This work
ΔpacA	*pacA* mutation in VD300 MS11 with pKH40	This work
ΔltgAΔltgDΔpacA	*pacA* mutation in *ΔltgAΔltgD* KH560 with pKH40	This work
pacA+ in *ΔltgAΔltgDΔpacA*	*pacA* complementation in *ΔltgAΔltgDΔpacA* with pKH38	This work
ΔpacAΔmsbB	KH624, *pacA msbB*::aph-3 in MS11 background	This work

Supplemental Table 2. Peptidoglycan composition of cell wall from WT and *ΔltgAΔltgD* mutant Gc.
Peptidoglycan sacculus isolated using the boiling SDS method was digested with mutanolysin, and soluble peptidoglycan fragments were injected into HPLC. Quantification of fragments was performed by determining the area under the peak (AUP) at A_{206} and is expressed as a percentage of the chromatogram ± standard deviation (SD). Selected peaks were desalted and identified by ESI-MS.
[a]All identified peaks obtained an *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) disaccharide, and the amino acids alanine (Ala), glutamic acid (Glu), and meso-diaminopimelic acid (DAP). Tri, GlcNAc-MurNAc-Ala-Glu-Dap; Tetra, GlcNAc-MurNAc-Ala-Glu-DAP-Ala; OAc, *O*-acetylation on MurNAc; Anh, 1,6-anhydro-muramic acid; ND, peak identity not determined.
P-value was determined using a two-tailed, equal variance *t*-test.

Peak	Peak Identity[a]	Exact Mass	Calculated $[M+H]^+$	Observed *m/z*	WT AUP	WT SD	*ΔltgAΔltgD* AUP	*ΔltgAΔltgD* SD	Ratio of WT:*ΔltgA ΔltgD* AUP	*p*-value
1	Tri	868.35	869.36	869.37	1.81	0.13	2.72	0.27	1:1.50	0.0062
2	Tri	868.35	869.36	869.36	2.08	0.24	1.63	0.55	1:0.78	0.2639
3	Tetra	939.39	940.40	940.39	4.09	0.27	4.15	0.35	1:1.01	0.8253
4	Tri	868.35	869.36	869.37	6.15	0.46	6.43	0.64	1:1.04	0.5705
5	ND				3.71	0.24	3.75	0.07	1:1.01	0.7617
6	Tetra	939.39	940.40	940.39	10.66	0.65	10.30	0.13	1:0.96	0.3984
7	Tetra (OAc)	981.40	982.41	982.41	3.61	0.06	3.81	0.11	1:1.06	0.0457
8	Tri(OAc)	910.37	911.37	911.37	5.73	0.10	4.73	0.06	1:0.83	0.0001
9	ND				5.45	0.31	5.89	0.04	1:1.08	0.0695
10	ND				2.31	0.05	2.26	0.05	1:0.98	0.2856
11	ND				2.15	0.08	2.38	0.03	1:1.11	0.0112
12	Tetra (OAc)	981.40	982.41	982.41	10.46	0.46	6.86	0.32	1:0.66	0.0004
13	ND				4.60	0.04	4.25	0.04	1:0.92	0.0003
14	ND				4.62	0.18	4.52	0.42	1:0.98	0.7350
15	(Anh)Tetra	921.38	922.39	922.37	4.30	0.08	5.32	0.47	1:1.24	0.0206
16	Tetra-Tetra	1860.77	1861.78	1861.77	4.97	0.35	6.60	0.60	1:1.33	0.0152
17	ND				4.62	0.41	3.92	0.49	1:0.85	0.1305



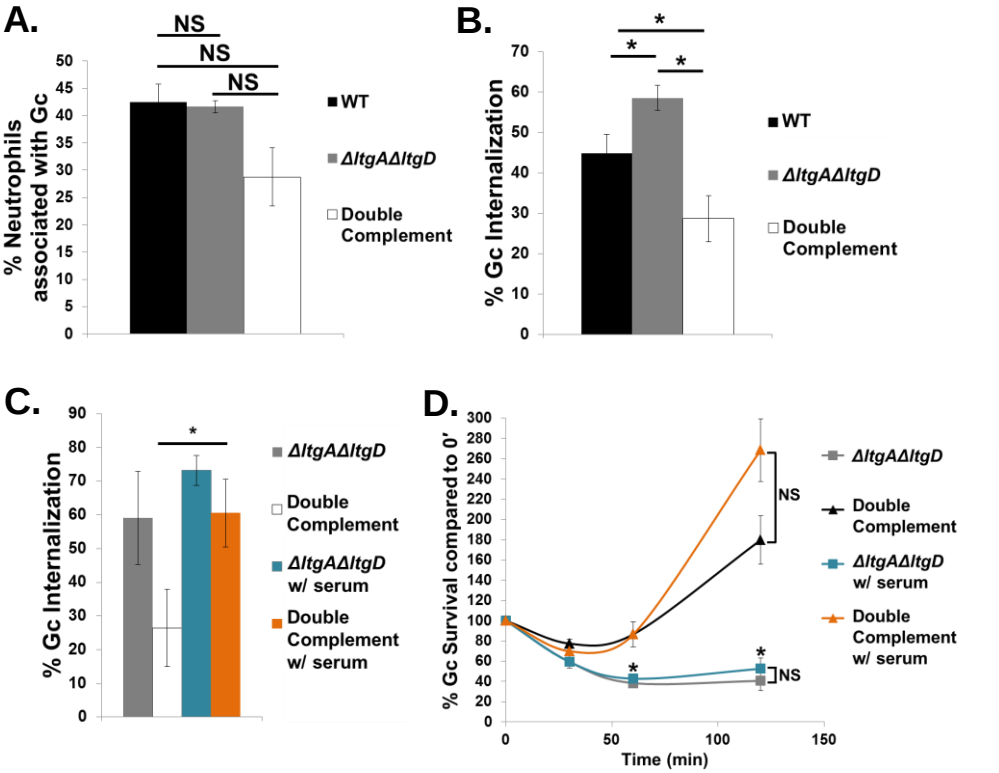
Supplemental Figure 1. **Profiles of PG released extracellularly by strains used in this study.**
(A) Induced *ltgA*+ *ltgD*+ double complement, (B) *ltgA(E481A)ltgD(E158A)* and *ΔltgAΔltgDΔampG* bacteria were labelled with [6-^{3}H]glucosamine, chased for 2.5hr, supernatants harvested and filtered, PG fractionated by gel filtration chromatography, and samples were counted with a liquid scintillation counter.



Supplemental Figure 2. **Protein composition of the outer membrane and cell wall composition of *ΔltgAΔltgD* mutant Gc.**

A. Isolated outer membrane from WT, *ΔltgAΔltgD*, and *ltgA*+ *ltgD*+ double complement Gc (1µg per lane) was separated by SDS-PAGE and subjected to silver staining.

B. PG isolated from intact cell walls of WT (solid line) and *ΔltgAΔltgD* mutant Gc (dotted line) was digested with mutanolysin. A representative HPLC chromatogram of the resulting PG fragments is presented. Fragment quantitation is presented in Table S2.



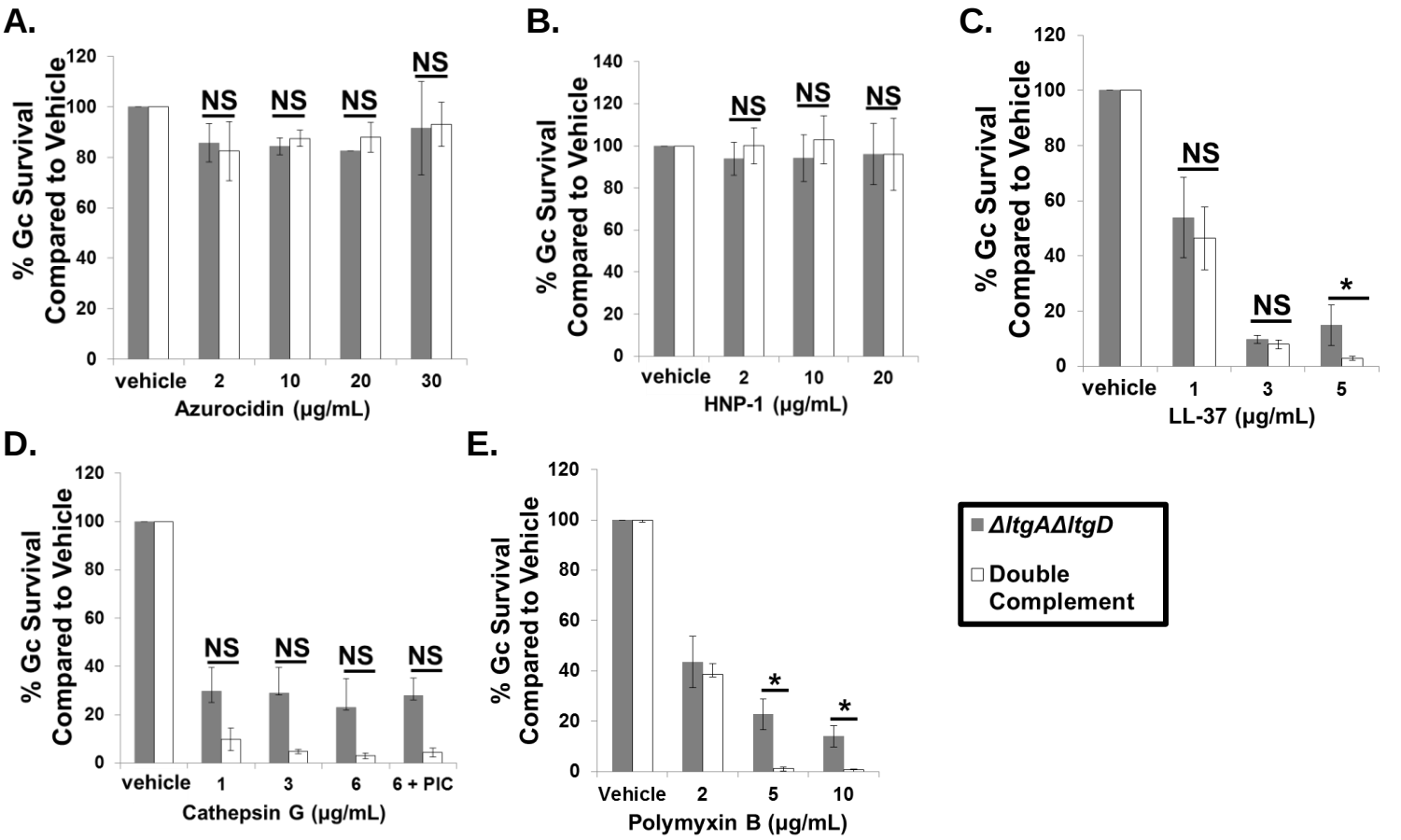
Supplemental Figure 3. **Differences in phagocytosis are not sufficient to explain the *ΔltgAΔltgD* mutant defect.**

A. Adherent, IL-8-treated primary human neutrophils were infected with CFSE-labelled Gc for 1 hr and subsequently analyzed by imaging flow cytometry. Percent neutrophils associated with Gc was enumerated by subtracting the percentage of uninfected neutrophils (total neutrophils not associated with CFSE-labelled Gc divided by the total number of neutrophils) from 100%. n = 4 independent experiments.

B. From Figure 6, extracelluar Gc was discriminated from intracellular Gc by labelling extracellular Gc with Alexa Fluor 647 conjugated soybean lectin. Total impermeant and permeant Gc were stained with Baclight viability dyes Syto9 and propidium iodide, respectively. Percent Gc internalization was determined by dividing total intracellular Gc (sum of total intracellular permeant and impermeant Gc) by total Gc (sum of total permeant and impermeant Gc). **P*<0.05; two-tailed *t*-test, n = 6 independent experiments.

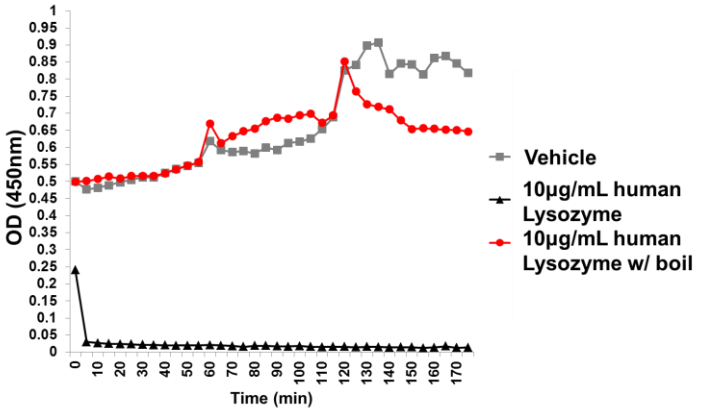
C. Gc was opsonized for 20 min at 37°C with autologous human serum prior to infection of human neutrophils for 1 hr. Extracellular Gc were discriminated with 647-conjugated soybean lectin while total Gc were labelled with the membrane permeant dye Syto9. Percent Gc internalization was determined by dividing intracellular Gc by total Gc. **P*<0.05; paired, two-tailed *t*-test, n = 3 independent experiments.

D. Gc was opsonized with autologous human serum or left non-opsonized as in C, followed by exposure to adherent, IL-8-treated human neutrophils. Percent Gc survival was assessed over time as in Figure 1A. **P*<0.05 for *ΔltgAΔltgD* w/serum compared to double complement w/serum; two-tailed *t*-test, n = 3 independent experiments.

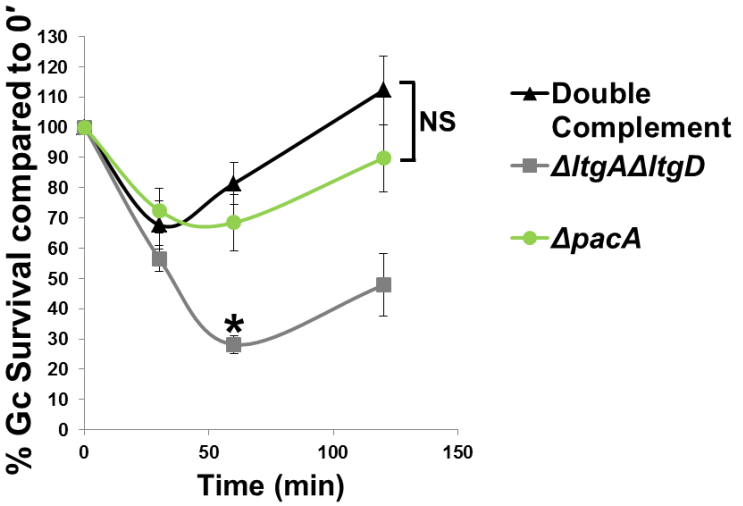


Supplemental Figure 4. LtgA and LtgD are not important for Gc defense against several antimicrobial proteins from neutrophils.

ΔltgAΔltgD (grey bars) and *ltgA+ltgD+* double complement (white bars) Gc were exposed to increasing concentrations of (A) Azurocidin, (B) Human Neutrophil Peptide-1 (HNP-1), (C) LL-37, (D) Cathepsin G with or without protease inhibitor cocktail (PIC), and (E) Polymyxin B. Gc survival at each concentration is expressed in relation to survival in vehicle control, as in Fig. 3A. **P*<0.05 for indicated comparisons; two-tailed *t*-test, n = 3 to 10 biological replicates.



Supplemental Figure 5. *Micrococcus luteus* lysis by human lysozyme is rescued when lysozyme is boiled. Stationary phase *M. luteus* was resuspended in 0.5x GCBL. Human lysozyme diluted in 50mM potassium phosphate buffer, pH 7.0 was boiled for 1 hr. Change in OD_{450}, indicative of bacterial lysis, was measured over time. Shown is one representative of two independent biological replicates.



Supplemental Figure 6. PacA is not important for survival from neutrophils.
ΔltgAΔltgD double mutant, *ΔpacA* single mutant, and *ltgA*+ *ltgD*+ double complement Gc were exposed to adherent, IL-8-treated neutrophils. Percent Gc survival was assessed over time as in Figure 1A. **P*<0.05 for *ΔltgAΔltgD* compared to *ΔpacA*; two-tailed *t*-test, n = 3 independent experiments.