Supplemental Information

Generation of pilus-negative mutants in strains MGAS10870 and MGAS10870*liaS*^{R135G}

Briefly, a three-step process was used to generate a PCR fragment in which the entirety of the *tee3* open reading frame (ORF) was removed. Primer pair MSP328 and MSP363 were used to generate a 1,001-bp fragment including the 29-bp immediately upstream of the *tee3* ORF. Likewise, primer pair MSP331 and MSP365 were used to generate a 1,058-bp fragment including the last 24-bp immediately downstream of the *tee3* ORF. Primer pair MSP364 and MSP366 were used to amplify *aad9* with ends homologous to the immediate upstream and downstream flanking regions of the *tee3* ORF. The three PCR fragments were then used to generate a 2,761-bp fragment using primer pair MSP328 and MSP331 in a ligation PCR in which all of the *tee3* gene was replaced, in-frame, by the spectinomycin cassette, *aad9*. The resulting PCR fragment was then digested with *BamH*I and *Xho*I and ligated into the same sites of pJL1055 to generate pMAS5. Strains MGAS10870 and MGAS10870*liaS*^{R135G} were electroporated with pMAS5 and recombinant mutants selected on THY agar supplemented with spectinomycin.

Human neutrophil survival assays

Human neutrophils (polymorphonuclear leukocytes or PMNs) were isolated from heparinized venous blood of healthy volunteer donors (n=14) in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University. All subjects provided written informed consent. Neutrophils were isolated as previously described (1). Purity (less than 1% peripheral blood mononuclear cell [PBMC] contamination) and viability using propidium iodide uptake were assayed via flow cytometry with a FACSCalibur™ (BD Biosciences, San Jose, CA, USA). Neutrophils were suspended in RPMI 1640 supplemented with 5 mM HEPES. To evaluate GAS survival following neutrophil interaction, neutrophils were

combined with GAS opsonized with autologous serum in serum coated 96-well tissue culture plates at a ~1:10 neutrophil: bacteria ratio. Phagocytosis was synchronized by centrifugation at 500 x g for 8 minutes at 4°C and incubated at 37°C for 3 hours. Neutrophils were lysed with 0.1% saponin (15 minutes on ice) and GAS were plated on THY agar. Colonies were enumerated the following day and percent survival was calculated with the equation (CFU_{+PMN} at 3hr/CFU_{+PMN} at 0hr) x 100.

Cultured human epithelial cell adherence and internalization assays

Adherence to cultured human epithelial cells was carried out as previously described (2-4). HaCaT cells (human skin epithelial cells; P. Sumby) were incubated at 38.5°C and HEp-2 cells (HeLa contaminant; ATCC) were incubated at 37°C. The appropriate GAS strains were grown to mid-exponential phase ($OD_{600} \approx 0.5$) in THY, pelleted, washed with sterile PBS and approximately 1 x 10^7 CFU GAS (MOI ≈ 10) were added to 12 replicate wells, rocked briefly, and incubated for 2 hrs at 37°C. Wells were subsequently washed three times with PBS and incubated at 37°C with 1 mL PBS containing 1% saponin (Oxoid). Cells were released from wells by pipetting, serially diluted, and plated on SBA to enumerate GAS. Percent adherence was calculated by dividing recovered CFU by the original inoculum.

GAS internalization to cultured epithelial cells was carried out as previously described (2). Briefly, GAS strains were added to HEp-2 cells as for adhesion assays above, incubated for 2 hrs, and washed three times with 2 mL PBS. Gentamicin was then added to each well at a concentration of 100µg/ml and incubated for 1 hr at 37°C to kill extracellular GAS. Each well was then washed 3x with 1 ml PBS to remove antibiotics. HEp-2 cells were released from the wells using 0.25% trypsin/ 1M EDTA. Cells were lysed using sterile water, serially diluted, and plated on SBA to enumerate GAS. Percentage internalization was calculated by dividing the recovered CFU by the original inoculum.

Table S1. Strains used in this study.

Strain	Characteristics	Reference
MGAS10870	Invasive	(5)
MGAS10870*liaS*^{R135G}	MGAS10870 with *liaS*^{R135G} carrier mutation	(4)
MGAS10870Δ*tee3*	Δ*tee3*, pilus-negative mutant in MGAS10870	This study
MGAS10870*liaS*^{R135G}/Δ*tee3*	*liaS*^{R135G}, Δ*tee3*; pilus-negative mutant with carrier mutation	This study

Table S2. Plasmids and primers used in this study.

Plasmid	Characteristics	Reference
pMAS5	pJL1055/Δ*tee3*::*aad9*]	This study
Primer	**Sequence (5'-3')**	**Use**
MSP328	GTTACTGGATCCATTGCAGATAAG	PCR
MSP331	TGATAAACTCGAGAAGTATATTGC	PCR
MSP363	CTTTATTAATTTGTTCGTATGTATTCAATTTTTCTCTCTCCAAATTTTTTAAATTCC	PCR
MSP364	GGAATTTAAAAAATTTGGAGAGAGAAAAATTGAATACATACGAACAAATTAATAAAG	PCR
MSP365	ATAACAGATTAAAAAAATTATAAGTGATTAGTCAAAGAATGGTGATG	PCR
MSP366	CATCACCATTCTTTGACTAATCACTTATAATTTTTTTAATCTGTTAT	PCR
nra-for	TAACTGCAAATTGGTGGCGC	TaqMan
nra-rev	GAACATCGCCCTGGCTAGAG	TaqMan
nra-p	TCCATGACAAGGCAAGTAACA	TaqMan Probe
cbp-for	AAGCGACGAAAGCCTCAGTT	TaqMan
cbp-rev	GCCCCATCTGTTGTCAAACC	TaqMan
cbp-p	CCGAAAAGATCTTGTCCCACC	TaqMan Probe
tee3-for	AGCGTGACACTCAAGTTCCA	TaqMan
tee3-rev	TCCACCAATAGCCACAATGCT	TaqMan
tee3-p	ACCCTTGCTCCATTTGCAGT	TaqMan Probe
msmR-for	GTGAGGTTCTTTTCCGCCCT	TaqMan
msmR-rev	AGCAAGAAATCAATCGCATCCC	TaqMan
msmR-p	AGAGCAGAAATAGAAGATGAGGTGA	TaqMan Probe
mga-for	CAAGTCAACAGTGGAGAGAACTAAAATTA	TaqMan
mga-rev	ATGGAGATGTTGAGAGCTTTGCT	TaqMan
mga-p	TCATACGTAACAGAAAACGCTGACGCCATT	TaqMan Probe
spxA2-for	AAGGATGCGCGGGTTATCTT	TaqMan

spxA2-rev	ATGCCAAAGCTCTCGATTGC	TaqMan
spxA2-p	TCAAATCAATAACCTCACTAACACTT	TaqMan Probe
tufA-for	CAACTCGTCACTATGCGCACAT	TaqMan
tufA-rev	GAGCGGCACCAGTGATCAT	TaqMan
tufA-p	CTCCAGGACACGCGGACTACGTTAAAAA	TaqMan Probe

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