DNase Sda1 Provides Selection Pressure for a Genetic and Phenotypic Switch Promoting Invasive Group A Streptococcal Infection

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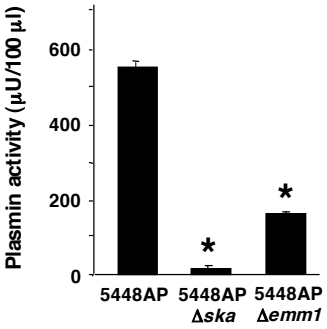
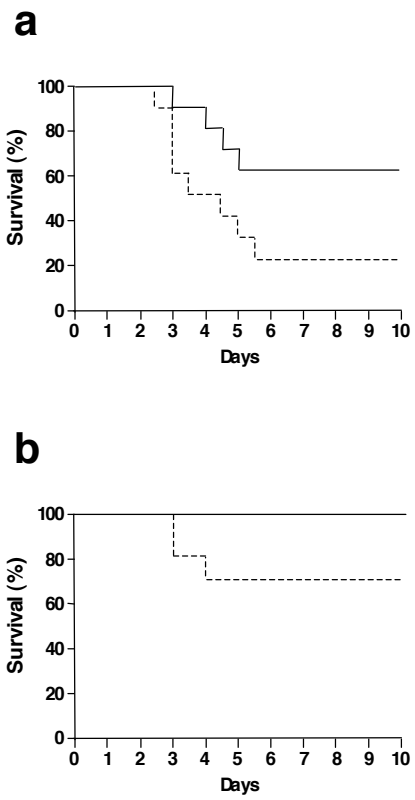
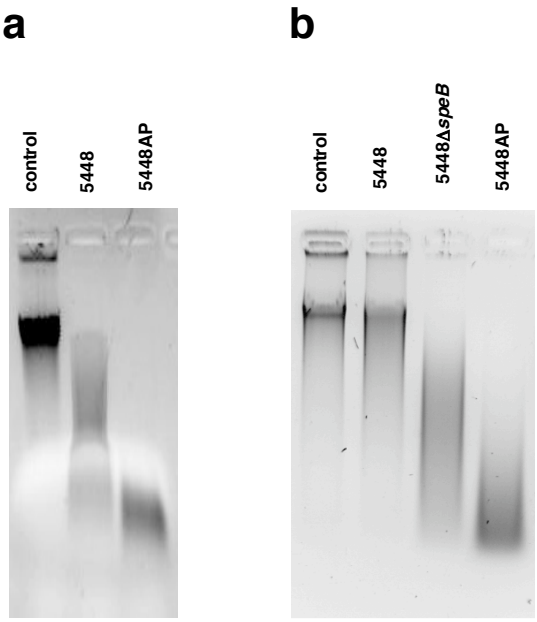


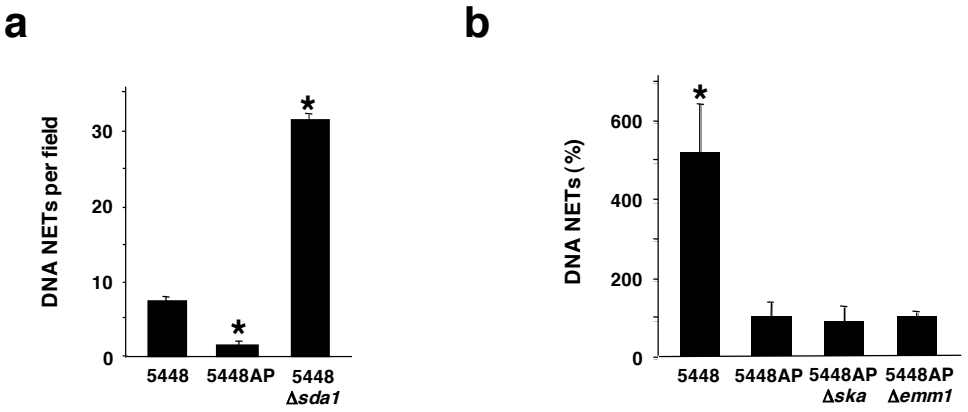
Supplementary Fig. 1. Surface plasmin activity following overnight growth at 37 °C in human plasma. 5448AP accumulates significantly higher levels of surface plasmin activity ($n = 3$; mean ± SD) compared to 5448APΔ*ska* and 5448APΔ*emm1*. Asterisk indicates statistically significant difference from 5448AP, where $P < 0.05$.



Supplementary Fig. 2. Survival curves following GAS infection of humanized plasminogen transgenic mice. (**a**) Survival curves following subcutaneous infection of humanized plasminogen transgenic mice ($n = 10$) with GAS strain 5448 (6.6×10^7 CFU/dose; solid line) and 5448AP (2.9×10^7 CFU/dose; dashed line). (**b**) Survival curves following subcutaneous infection of humanized plasminogen transgenic mice ($n = 10$) with GAS strain 5448 (5.8×10^6 CFU/dose; solid line) and 5448AP (1.3×10^7 CFU/dose; dashed line).



Supplementary Fig. 3. DNase expression by GAS strains. (**a**) DNase expression in tissue cage fluid 24 h following inoculation with 5448 or 5448AP. DNase activity of uninoculated tissue cage fluid was also determined (control). (**b**) DNase expression in stationary-phase culture supernatants as assessed by degradation of calf thymus DNA (control).



Supplementary Fig. 4. NETs clearance by GAS strains. (**a**) Quantification of the clearance of NETs. NETs were visualized using Sytox Orange staining and enumerated for each treatment by counting three fields of view from three independent wells ($n = 9$; mean ± SD). Asterisk indicates statistically significant difference from 5448, where $P < 0.05$. (**b**) NETs clearance by GAS strain 5448 in comparison to 5448AP, 5448APΔ*ska* and 5448APΔ*emm1*. Asterisk indicates statistically significant difference from 5448AP (100%), where $P < 0.05$.

Supplementary Table 1. *CovRS* DNA sequence analysis of selected GAS M1T1 strain 5448 SpeB-negative derivatives isolated 3 d following subcutaneous infection of C57BL/J6 mice.

GAS strain	Mouse ID	Tissue[a]	Mutation[b]	Consequence[c]
5448-APD1	OS41B	Blood	C to T nt 838 *covS*	H to Y aa 280 *covS*
5448-APD2	OS41B	Lesion	C to T nt 838 *covS*	H to Y aa 280 *covS*
5448-APD3	OS41B	Lesion	C to T nt 838 *covS*	H to Y aa 280 *covS*
5448-APD4	OS45R	Blood	C to T nt 838 *covS*	H to Y aa 280 *covS*
5448-APD5	OS45R	Blood	C to T nt 838 *covS*	H to Y aa 280 *covS*
5448-APD6	OS46L	Lesion	Δ nt 83 *covS*	Truncation in CovS
5448-APD7	OS46L	Lesion	Δ nt 83 *covS*	Truncation in CovS
5448-APD8	OS46L	Lesion	G to A nt 331 *covR*	A to T aa 111 *covR*
5448-APD9	OS322B	Lesion	Δ nt 406-1503 *covS*	Truncation in CovS
5448-APD10	OS492L	Lesion	C to T nt 838 *covS*	H to Y aa 280 *covS*

[a]Mouse tissue from which GAS strain was isolated 3 d post-subcutaneous infection.

[b]Mutation positions are based upon nucleotide (nt) position in the *covR* or *covS* genes, relative to each ATG start codon.

[c]Substitutions in CovR and CovS are based upon amino acid (aa) position in each open reading frame, relative to each start codon.

Supplementary Table 2. Oligonucleotide primers used in this study for construction of recombinant GAS strains.

Primer	Direction	bp	Sequence
RCSdapHY304Fwd	Forward	29	GGGCTGCAGCTTAAACGTTGGTATTTTTA
RCSdapHY304Rev	Reverse	28	GGGGAATTCGGATAGCTTACAACTAGTG
SdaF2	Forward	21	ATGTCTAAACATTGGAGACAT
SdaR4	Reverse	20	ATCAGATGATAAAGCAGACA
SmeZ-UpF	Forward	20	TGGCTGACAACTGTCAGGAA
SmeZ-UpR	Reverse	54	GGTGGTATATCCAGTGATTTTTTTCTCCATAAATAGCCT CTTTTCAGGAGTTAT
SmeZ-DownF	Forward	54	TACTGCGATGAGTGGCAGGGCGGGGCGTAATTCAATTT TTCAATATAACTTTTA
SmeZ-DownR	Reverse	20	AACGACACCTCTTTCAGCGA
Emm1-dis-F-BamHI	Forward	30	GCGGATCCTAGTCCTGACTCGCTTGGTCTA
Emm1-dis-R-XbaI	Reverse	30	GCATCTAGACTTGCAGCAAACAATCCCGCA
M1ska-dis-F-BamHI	Forward	30	GCGGATCCAACATCACAACCTGCTCACGGA
M1ska-dis-R-XbaI	Reverse		GCGTCTAGACGCGCACATGTCCCTTTAACAA
T7-For	Forward	21	GTAATACGACTCACTATAGGG
Emm1out-R	Reverse	24	GAGGTTAAGGCTAACGGTGATGGT
M1skaout-R	Reverse	24	TTGAGCCCTGGTCTGAAATCGTCA

Supplementary Table 3. Oligonucleotide primers used in this study for *covRS* PCR and sequence analysis.

Primer	Direction	bp	Sequence
p1	Forward	19	GCTATTCCGGTACAGGTCT
p2	Forward	19	GTCAATGGTCGTGAAGGGT
p3	Forward	22	GATGTCTATATTCGTTATCTCC
p4	Forward	22	GATGATTTTTACCACAGATAAC
p5	Forward	20	GCATATTGGTCTCTTACAAC
p6	Forward	21	GCAAATTGTAGATGGGTATCA
p7	Reverse	20	GCGGAAAATAGCACGAATAC
p8	Reverse	20	AGGCAATCAGTGTAAAGGCA
p9	Reverse	21	CTTGTGCCAAATAACTCAACA
p10	Reverse	21	ATCAAAAGCCTGCTCAAATGA
p11	Reverse	21	CTTTCATGTCATCCATCATTG
p12	Reverse	19	TTGCTCTCGTGTGCCATCT

Supplementary Materials and Methods

Culture of group A streptococci. *S. pyogenes* strains were routinely propagated at 37 °C on horse blood agar (BioMérieux) or in static liquid cultures of Todd-Hewitt broth (Difco) supplemented with 1% (w/v) yeast extract (THBY). Invasive GAS isolate 5448 (M1T1) and the isogenic animal-passaged SpeB-negative variant 5448AP have been described previously[11]. The isogenic mutants 5448Δ*sda1*[5], 5448Δ*speB*[11] and GAS strain SF370[19], have also been described previously.

Construction of recombinant GAS strains. Allelic exchange was used to precisely replace the deleted *sda1* chromosomal locus in 5448Δ*sda1* with the WT *sda1* gene to construct strain 5448RC*sda1*$^+$. The technology employed to construct 5448RC*sda1*$^+$ was similar to that used in the construction of 5448Δ*sda1*[5]. The PCR primers RCSdapHY304Fwd and RCSdapHY304Rev were employed for amplification of flanking DNA upstream and downstream of *sda1* in the 5448 chromosome (**Supplementary Table 2**). Following amplification, the *sda1* gene was cloned by *Pst*I/*Eco*RI digestion and T4 ligation into the temperature-sensitive plasmid pHY304[5]. The resulting plasmid (pHY*sda1*) was transformed into 5448Δ*sda1* by electroporation. Integration of pHY*sda1* into the chromosome via single-crossover was achieved by culture at the permissive temperature for plasmid replication (30 ˚C). Following subculture at 37 ˚C, single-crossover chromosomal insertions were selected using chloramphenicol (Δ*sda1*) and erythromycin (pHY304). Double-crossover was achieved by serial passage at 30 ˚C, and double-crossover reverse-complemented mutants were identified following removal of antibiotic selection. The reverse-complemented strain 5448RC*sda1*$^+$ was characterized as sensitive to both

chloramphenicol and erythromycin; confirmed as *sda1* PCR-positive using the forward primer SdaF2 and reverse primer SdaR4 (**Supplementary Table 2**); and able to express Sda1 upon assaying for DNase activity (as described below).

The isogenic 5448Δ*smez* mutant was constructed in a manner identical to 5448Δ*sda1*, as previously described[5]. A precise, in-frame allelic exchange replacement of the *smeZ* gene in GAS strain 5448 with a chloramphenicol acetyltransferase (*cat*) antibiotic resistance cassette was generated. The specific primer sets used for amplification of the flanking DNA upstream and downstream of *smeZ* in the 5448 chromosome are given (**Supplementary Table 2**). The primers SmeZ-UpR and SmeZ-DownF contain a 25 bp 5' extension corresponding to the 5' and 3' ends of the *cat* gene, respectively.

Integrational mutagenesis of *ska* and *emm1* was performed essentially as previously described[20]. Internal fragments of the genes *ska* and *emm1* were PCR amplified from GAS strain 5448 using specific primer pairs (**Supplementary Table 2**) and cloned by *Bam*HI/*Xba*I digestion and T4 ligation into the temperature-sensitive plasmid pVE6007[21]. The resultant plasmids were transformed into 5448AP by electroporation and chloramphenicol resistant transformants were grown at the permissive temperature for plasmid replication (30 ˚C). Single-crossover Campbell-type chromosomal insertions were selected by shifting to the non-permissive temperature (37 ˚C), while maintaining chloramphenicol selection. Integrational knockouts were confirmed by PCR using the forward primer T7-For and reverse primer emm1out-R or M1skaout-R (**Supplementary Table 2**). Confirmed integrational knockouts were designated 5448APΔ*emm1* and 5448APΔ*ska*.

Molecular and phenotypic analysis of GAS. To screen GAS strains for mutations in the *covRS* locus, we designed 12 primers for PCR and DNA sequence analysis (**Supplementary Table 3** and **Fig. 1a**). Firstly, primers p1 and p12 were used to PCR amplify the intact *covRS* locus from genomic DNA which was extracted by phenol-chloroform. Then, an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) was used to directly sequence the amplified PCR product with the 12 primers and the sequence assembled by the use of Sequencher version 4.5 (Gene Codes Corporation). Using BLASTN analysis, the assembled sequences were aligned against GAS genomes and a single adenine base insertion mutation was identified at position 877 in the 5448AP *covS* gene, using numbering relative to the ATG start codon of 5448 *covS*. Other *in vivo*-derived, SpeB-negative GAS strain 5448 derivatives were analyzed for *covRS* mutations in an identical manner.

SpeB-positive and SpeB-negative isolates were routinely identified by the Columbia skim milk agar assay[22]. Quantitative SpeB assays were undertaken as previously described[23]. Bacterial surface plasmin acquisition from human plasma assays and western blot identification of α-enolase, GAPDH, streptokinase and M1 protein, were conducted essentially as previously described[4], with exception that cross-specific rabbit M53 protein-specific serum was used to identify M1 protein. GAS strain NS1133[24] was used as an internal control for bacterial surface plasmin acquisition assays[4] undertaken by incubating bacteria overnight in human plasma. Plasminogen-binding assays were conducted as previously described[24].

Virulence of GAS in a humanized plasminogen transgenic mouse model. Transgenic humanized plasminogen *AlbPLG1* mice heterozygous for the human plasminogen transgene[16] were backcrossed greater than $n = 6$ with C57BL/J6 mice (Animal Resources Centre, Perth, Australia). GAS strains 5448 and 5448AP were harvested at logarithmic phase (OD_{600} approx. 0.4), washed twice with sterile 0.7% saline and diluted to the required dose. The number of viable bacteria was determined by counting colony forming units (CFU) after plating a dilution series onto blood agar. The SpeB expression status of 5448 and 5448AP was also determined as described above ($n = 50$). The 5448AP inoculum was found to be 100% SpeB-negative, while the 5448 inoculum was 100% SpeB-positive. Groups of *AlbPLG1* mice ($n = 10$) were subcutaneously infected with GAS and mortality was monitored for 10 d. Alternatively, groups of *AlbPLG1* mice ($n = 5$) were subcutaneously infected with either 5448 or 5448AP for 48 h and the lesion (site of infection), blood, spleen and liver harvested. Lesion, spleen and liver samples were homogenized in 2 ml of sterile 0.7% saline. The number of viable bacteria was determined by counting CFU after plating a dilution series onto blood agar.

Isolation of mRNA and real-time PCR analysis. In order to isolate *in vivo*-derived RNA, we utilized the subcutaneous Teflon chamber model[25]. Teflon chambers were inserted surgically under the skin of 6-week-old female BALB/c mice. Three weeks after surgery, tissue chamber fluid (TCF) was collected and tested for sterility. Mice that had contaminated TCF, or those that had open surgical wounds, were excluded from further experimentation. To prepare inocula, bacteria were grown overnight in THBY, checked for SpeB phenotype as detailed above, then subcultured for 18 h in THBY. Bacterial pellets were washed twice in sterile phosphate buffered saline (PBS)

and resuspended in sterile PBS to 1 × 10^9 CFU/ml. We injected 100 μl of this bacterial suspension into the subcutaneous chambers using sterile 25-gauge needles. At 24 h post-injection, sterile 25-gauge needles were used to collect the TCF to analyze bacterial content and SpeB status, and to extract RNA from recovered bacteria[11, 25]. This 24 h time point was chosen as WT 5448 GAS recovered from mouse infection chambers were > 95% SpeB-positive (data not shown), whereas after 3 d *in vivo* the WT subcutaneous bacterial population contain a significant proportion (up to 74%) of SpeB-negative phenotype[4]. 5448AP cells recovered from 24 h infection chambers were uniformly SpeB-negative. RNA was extracted from bacterial pellets using RNeasy kits (Qiagen), treated with DNase (Ambion) for 1 h to remove contaminating genomic DNA, and then recovered using RNeasy columns (Qiagen). The absence of genomic DNA in the RNA samples was confirmed by PCR using primers specific for the *speB* gene[11] (data not shown). The intactness and purity of isolated RNA was assessed using an Agilent Technologies Bioanalyzer (data not shown). Superscript II (Invitrogen) was used to reverse transcribe RNA into cDNA, following the manufacturer's protocol; cDNA was immediately diluted with four volumes of sterile water then aliquoted for real-time PCR reactions. We performed all Sybr-Green real-time quantitative PCR reactions using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) and calculated relative expression amounts using the delta-delta CT method[11]. All real-time PCR reactions were performed using biological triplicates; product specificity was confirmed by the presence of a single peak in dissociation curves. Fluorescence in all real-time quantitative PCR reactions was measured at 75 ˚C, a temperature at which any potential primer dimers would melt, ensuring that the PCR product measured was the

expected product. Primers used for real-time PCR analysis of *speB* and *sda1* have been previously described[11, 26].

DNase activity assays. Supernatants were collected from mid-logarithmic (OD_{600} = 0.4) or stationary phase cultures of GAS strains grown in THBY. Calf thymus DNA (1.0 μg/μl) was combined with bacterial supernatant (2.5 μl) in final volume of 50 μl buffer (300 mM Tris, 3 mM $CaCl_2$, 3 mM $MgCl_2$) for 15 min at room temperature. To halt DNase activity, 12.5 μl of 0.33 M EDTA was added to the reaction. Visualization of relative DNA degradation was undertaken by side-by-side comparison of DNA using 1% agarose gel electrophoresis.

Live cell imaging for visualization of NETs. NETs were visualized as previously described[5]. Briefly, neutrophils were seeded at 2×10^5 per well in 96-well plates in RPMI without phenol red (Invitrogen). GAS were added to the wells at a multiplicity of infection of 1:100 (GAS:neutrophils) and Sytox Orange (Molecular Probes) added to a final concentration of 0.1 μM. Cells were visualized without fixation or washed using a Zeiss Axiovert 100 inverted microscope with appropriate fluorescent filters, and images captured with a CCD camera. For quantitation, NETs were enumerated for each treatment by counting three fields of view after staining from three independent wells; a NET was defined as a discrete area of bright orange fluorescence larger in size than a neutrophil. Presented data are representative of experiments undertaken on three separate occasions.

Neutrophil killing assays. Neutrophil killing assays were performed as previously described[5]. Briefly, human neutrophils were isolated and purified from venous blood

using the PolyMorphPrep kit (Axis-Shield) as per the manufacturer's instructions and seeded into 96-well plates at 2×10^5 cells/well. Logarithmic-phase bacteria grown in THBY were diluted to the desired concentration in RPMI media containing 2% heat inactivated autologous human plasma, then added to neutrophils at a multiplicity of infection of 1:10 (GAS:neutrophils). Plates were centrifuged at 500 × *g* for 10 min then incubated at 37 °C in 5% CO_2. Following incubation for 1 h, neutrophils were lysed with 0.02% Triton X-100 and the contents of the well serially diluted and plated on Todd-Hewitt agar for overnight incubation and enumeration of CFU. Internal control wells without neutrophils were used to determine baseline bacterial counts at the assay endpoints. Percent survival of GAS was calculated as ([CFU/ml experimental well] / [CFU/ml control well]) × 100%. All assays were performed in triplicate.

Monitoring the *in vivo* phase-shift of GAS strains. Separate cohorts of C57BL/J6 mice (n = 10) were inoculated subcutaneously with a non-lethal dose of GAS to examine the *in vivo* phase-shift of SpeB during infection. The inocula used in these experiments were plated out onto blood agar plates then individual colonies tested for SpeB expression status as described above (n = 50). The 5448, 5448Δ*sda1*, 5448RC*sda1*$^+$ and 5448Δ*smez* inocula were found to be 100% SpeB-positive. On day three post-infection, mice were sacrificed by CO_2 asphyxiation and representative bacteria isolated from skin lesions[4]. The SpeB status of individual colonies (n = 50) was determined as described above.

All animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals (National Health and Medical Research Council,

Australia) and were approved by the University of Wollongong Animal Ethics Committee.

Statistical analyses. Statistical analysis of SpeB expression and status, plasminogen-binding, surface plasmin activity, quantitative real-time PCR, human neutrophil killing assays, and NET quantification were performed using a one way ANOVA with a Dunnett's Multiple Comparison Test. Differences were considered statistically significant at $P < 0.05$. Differences in survival of humanized plasminogen transgenic mice infected with GAS strains 5448, 5448AP and 5448APΔ*ska* were determined by the log-rank test. All statistical tests were performed using GraphPad Prism version 4.02.