Roles of the plasminogen activator streptokinase and the plasminogen-associated M protein in an experimental model for streptococcal impetigo

Mikael D. Svensson,¹ Ulf Sjöbring,¹ Feng Luo² and Debra E. Bessen²

Author for correspondence: Debra E. Bessen. Tel: +1 203 432 7890. Fax: +1 203 432 6533.
e-mail: debra.bessen@yale.edu

¹ Department of Laboratory Medicine, Lund University, Lund, Sweden

² Department of Ecology and Evolutionary Biology, Yale University, 165 Prospect Street, New Haven, CT, USA

Primary infection by group A streptococci (GAS) takes place at either the throat or skin of the human host, often leading to pharyngitis or impetigo, respectively. Many GAS strains differ in their preference for throat and skin tissue sites. Previous epidemiological findings show that many of the strains displaying strong tropism for the skin have a high-affinity binding site for plasminogen, located within M protein (PAM), a prominent surface fibril. Plasminogen bound by PAM interacts with streptokinase, a plasminogen activator secreted by GAS, to yield bacterial-bound plasmin activity. In this study, PAM and streptokinase were tested for their roles in infection using an experimental model that closely mimics human impetigo. Inactivation of genes encoding either PAM or streptokinase led to a partial, but significant, loss of virulence *in vivo*, as measured by net growth of the bacteria and pathological alterations. The relative loss in virulence *in vivo* was greater for the streptokinase mutant than for the PAM mutant. However, the PAM mutant, but not the streptokinase mutant, displayed a partial loss in resistance to phagocytosis *in vitro*. The combined experimental and epidemiological data provide evidence that PAM and streptokinase play a key role in mediating skin-specific infection by GAS. In addition, secreted cysteine proteinase activity due to SpeB leads to degradation of streptokinase in stationary phase broth cultures. Since SpeB is also a determinant of tissue-specific GAS infection at the skin, direct interactions between these two proteolytic pathways may constitute an important pathogenic mechanism. An integrated model for superficial infection at the skin is presented.

Keywords: streptococci, proteolysis, plasmin, inflammation

INTRODUCTION

Group A streptococci (GAS) are a highly prevalent group of bacterial pathogens that afflict humans. They are well-adapted to superficial epithelial tissue (oropharynx or epidermis), where they can be highly adept at generating progeny that are transmitted to new human hosts. As a consequence of their successful evolutionary strategy, GAS often cause mild disease at

the throat or skin, in the form of pharyngitis or impetigo, respectively.

Decades of epidemiological findings support the existence of GAS strains that have strong preferences for either the throat or skin, as well as other strains whose tissue site preference is less clear cut (Anthony *et al.*, 1976; Bisno & Stevens, 2000; Maxted, 1980; Wannamaker, 1970). Thus, there should exist tissue-specific genotypes among GAS strains. The *emm* pattern is a genetic marker that distinguishes many throat- and skin-tropic strains of GAS (Bessen *et al.*, 1996, 2000; Dicuonzo *et al.*, 2001); this pattern is defined by the chromosomal arrangement of *emm* subfamily genes. The *emm* genes, in turn, encode M surface proteins,

Abbreviations: GAS, group A streptococci; HA, hyaluronic acid; Plg, plasminogen; PMN, polymorphonuclear leukocyte; URT, upper respiratory tract.

which display extensive heterogeneity in their structure and function (Cunningham, 2000; Fischetti, 2000). Plasminogen (Plg)-binding group A streptococcal M protein (PAM) is uniquely associated with *emm* pattern D strains (Svensson *et al.*, 1999), which are usually recovered from impetigo lesions and rarely from cases of pharyngitis. Despite their niche separation, there is an ample flow of neutral housekeeping genes between throat and skin strains (Kalia *et al.*, 2002), and high rates of genetic recombination within GAS as a whole (Feil *et al.*, 2001). In instances where neutral housekeeping alleles are randomly distributed with respect to ecologically distinct populations (Kalia *et al.*, 2002), genetic variation that is strongly associated with the different populations may be directly responsible for adaptation to the ecological niche, i.e. *emm* gene products (or closely linked genes) may have a direct role in tissue tropism. Thus, based on the population genetics findings, a reasonable argument can be made for PAM as a strong candidate for having a tissue-specific role in the development of streptococcal impetigo.

Human Plg can be bound by at least three distinct GAS surface proteins, whereupon it can be converted to its active form (plasmin), through the action of streptokinase, a secreted product of GAS that functions as a Plg activator (Lottenberg *et al.*, 1992; Pancholi & Fischetti, 1992, 1998). Plasmin acts as a broad-spectrum proteinase. It has been proposed that the acquisition of unregulated surface-associated plasmin by GAS and other bacterial pathogens promotes its spread into deep host tissue (Boyle & Lottenberg, 1997; Coleman *et al.*, 1997; Rasmussen & Bjorck, 2002; Sodeinde *et al.*, 1992). However, PAM is distinguished from other GAS Plg-binding proteins in that it has a high affinity for Plg (affinity constant, $8 \times 10^{-8} \text{ M}^{-1}$) (Berge & Sjöbring, 1993). The concentration of human Plg in tissue is highest in the circulation and interstitial fluids ($1\text{--}2 \times 10^{-6} \text{ M}$), suggesting that PAM is adapted for functioning in a micro-environment where the Plg concentration is low. This is consistent with the idea that the interaction of PAM with streptokinase promotes adaptation at a superficial tissue site (i.e. the epidermis).

To test the contribution of specific GAS products to skin infection, an *in vivo* model was developed that closely mimics impetigo in humans, based on histopathology (Scaramuzzino *et al.*, 2000). Human neonatal foreskins are grafted onto the backs of SCID mice, which fail to reject the xenotransplants. In an effort to mimic natural infection in humans, skin grafts are slightly damaged and GAS are topically applied. The hu-skin-SCID mouse model for impetigo exhibits a high degree of sensitivity, as evidenced by low inoculating doses for virulent strains, and high specificity, whereby *emm* pattern D strains display greater overall levels of virulence than typical throat strains (*emm* pattern A–C). Using the hu-skin-SCID mouse model in conjunction with isogenic mutants of GAS, it can be shown that the secreted cysteine proteinase SpeB plays a key role in virulence (Svensson *et al.*, 2000). Furthermore, levels of secreted cysteine proteinase activity are highest for *emm* pattern

D strains and lowest for *emm* pattern A–C strains. Thus, experimental findings combined with epidemiological data can provide compelling evidence for the role of a streptococcal product as a determinant of tissue tropism.

In this study, mutants of *emm* pattern D strain ALAB49 were constructed, whereby the *pam* gene or the *ska* gene underwent allelic replacement. The isogenic mutants of ALAB49 were compared to the parent strain for virulence *in vivo*, using the hu-skin-SCID mouse model for impetigo.

METHODS

Bacterial culture. The ALAB49 parent strain (ALAB49 wt) was recovered from an impetigo lesion from a patient in Alabama in 1986; it is *emm53* and *emm* pattern D (Enright *et al.*, 2001; Svensson *et al.*, 2000). A streptomycin-resistant variant was selected as described previously (Scaramuzzino *et al.*, 2000; Svensson *et al.*, 2000). GAS were grown in Todd–Hewitt broth with 1% yeast extract (THY), or on THY/blood agar plates, at 37 °C for the times indicated. Included in the growth medium were 70 µg streptomycin ml⁻¹ plus 200 µg kanamycin ml⁻¹ for the mutants (Scaramuzzino *et al.*, 2000; Svensson *et al.*, 2000).

Mutant construction. Directed mutagenesis, via allelic replacement and insertion of the Ω Km-2 interposon, using the temperature-sensitive shuttle vector pJRS233 harbouring an erythromycin-resistance (*Em*^R) gene, was used to generate Δ *pam*, Δ *ska* and Δ *speB* mutants of ALAB49 (Caparon, 2000; Kotarsky *et al.*, 2000; Perez-Casal *et al.*, 1993; Ringdahl *et al.*, 1998; Svensson *et al.*, 2000). Plasmid vectors used for GAS gene inactivation were constructed in *Escherichia coli*. Following electroporation of GAS with purified plasmid DNA, *Em*^R transformants were selected by growth on medium containing 15 µg erythromycin ml⁻¹. Colony picks of *Em*^R transformants were grown to high density in THY broth at 30 °C and shifted to the non-permissive temperature (in the presence of 200 µg kanamycin ml⁻¹), to select for chromosomal integration. Transformants arising from double crossovers were identified by screening for erythromycin sensitivity by replica plating. Construction of the ALAB49 Δ *speB* mutant has been described in detail already (Svensson *et al.*, 2000). For inactivation of the central *emm* gene of ALAB49, encoding PAM (*emm53* or *pam*), two PCR-amplified fragments were prepared, corresponding to the 3' portion of *mrp53* (upstream) through the 5' portion of *emm53* and to the 3' portion of *emm53* through the 5' portion of *enn53* (downstream). The two PCR fragments were ligated into pJRS233, with Ω Km-2 introduced between the two PCR fragments, and directed mutagenesis was performed as indicated above. The ALAB49 Δ *ska* mutant was constructed using the replacement vector described for strain AP53 (Ringdahl *et al.*, 1998). This vector was constructed from pJRS233 via ligation of two PCR-amplified fragments corresponding to the 5' and 3' portions of the *ska* gene, which were flanked by the Ω Km-2 interposon. All mutations were confirmed by PCR-based mapping using the general approach described by Svensson *et al.* (2000).

***In vivo* model for impetigo.** The hu-skin-SCID mouse model for streptococcal impetigo was used to measure the virulence of ALAB49 wt and its isogenic mutants (Scaramuzzino *et al.*, 2000; Svensson *et al.*, 2000). Briefly, human neonatal foreskins were grafted onto SCID mice. Healed skin grafts were

subsequently damaged by a series of gentle, superficial cuts made with a scalpel blade and topically inoculated with 50 µl THY broth containing the appropriate concentration of bacteria that had been freshly grown for 24 h at 37 °C in THY broth. A wide range of inoculation doses was tested, based on the dilution of the broth cultures. To determine the actual dose administered, duplicate 10-fold dilutions were plated onto agar; duplicate c.f.u. counts were highly concordant with each other and with inoculums used in other experiments. The ratios of c.f.u. to optical density (OD) were consistent for ALAB49 wt and isogenic mutants, suggesting low variation in the mean chain length. At all steps, broth cultures were vigorously vortexed, to break up any aggregates.

Infected human skin grafts were occluded with a bandage. At 7 days post-inoculation, the bandages were removed and the human skin grafts were scored for gross pathology and the animals killed. Following biopsy, each graft was weighed and then split in (approximately) half through the centre with a blade. Half of the graft was used for histopathology and the other half of the graft was used to determine the c.f.u. count. The portion of the graft used for determining the c.f.u. count was reweighed, and bacteria were released from the tissue by vigorous vortexing; final calculations were made by taking a measured portion of the supernatant containing released bacteria, determining the c.f.u. counts following 10-fold serial dilutions (averaged duplicates) on THY/blood agar plates (containing kanamycin for mutant strains) and multiplying the c.f.u. mg⁻¹ by the weight of total tissue biopsied prior to splitting. Spleens were recovered and cultured for the growth of GAS; all mice infected with ALAB49 wt, ALAB49Δ*pam* and ALAB49Δ*ska* were negative for septicaemia, with the exception of one spleen which yielded a single colony of GAS. Haematoxylin- and eosin-stained tissue sections were scored, in a blinded fashion, for inflammation and epidermal destruction on a number scale of 0–3, reflecting the severity of the alterations (3 being the most severe). Although gross and microscopic pathology scoring is subjective, it is highly reproducible, even when done in a blinded fashion (Scaramuzzino *et al.*, 2000).

***In vitro* assays**

Mutants were compared to the ALAB49 wild-type strain for several phenotypic traits that are of relevance to the directed mutagenesis and/or other known virulence properties of GAS.

(i) **Bactericidal assay.** The Lancefield basic bactericidal test was used to measure antiphagocytic activity, but with bacterial inoculating doses ranging from 150 to 780 c.f.u. ml⁻¹, which more closely reflects the Minneapolis method (Johnson *et al.*, 1996). The ability of GAS to survive and multiply in heparinized human blood following a 3 h rotation at 37 °C was assessed by c.f.u. counts. Since this assay measures exponential growth, variance can be large; therefore, to minimize the effects of data skewing, bacteria were tested in four separate experiments, rather than tested as replicates in a single experiment. GAS yielding a 32-fold increase in c.f.u. values (5 population doublings) are regarded as having a completely intact antiphagocytic capacity (Johnson *et al.*, 1996).

(ii) **Binding of Plg to bacteria.** The binding of radiolabelled Plg, from both human and mouse sources, to whole bacteria was measured, using a standard assay (Ringdahl *et al.*, 1998; Svensson *et al.*, 1999). Heat-killed ALAB49 wt and isogenic mutants were tested over a range of concentrations, generated by serial fivefold dilutions of bacteria. Prior to heat-killing,

GAS were grown to an OD₆₂₀ value of 0.8 in THY broth, which is equivalent to the late-exponential phase.

(iii) **Plasmin acquisition in plasma.** Plasmin acquisition by viable ALAB49 wt and the isogenic mutants (10¹⁰ c.f.u.), which were grown overnight and washed with PBS, was assessed by measuring bacterial-bound plasmin following their inoculation into fresh THY broth containing 30 % freshly prepared human plasma, for the times indicated, as described by Ringdahl *et al.* (1998). Bacterial-bound Plg was eluted with glycine (pH 2.0) and monitored by Western immunoblot using a rabbit antibody specific for human Plg (Dako A/S).

(iv) **Streptokinase production.** ALAB49 wt and isogenic mutants were grown in THY broth overnight, washed in fresh THY broth and then grown to several OD values reflecting different stages of growth. Bacteria were pelleted by centrifugation and 1 ml of the supernatant was precipitated in 10 % trichloroacetic acid (Svensson *et al.*, 2000). The suspension was incubated on ice for 1 h and the precipitated proteins were collected by centrifugation. The final pellet was resuspended in SDS sample buffer and analysed by SDS-PAGE and Western blot. The immunoblots were incubated with polyclonal rabbit antibodies raised against purified streptokinase derived from the group C streptococcal strain H46A, generously provided by Dr Horst Malke (Friedrich Schiller University, Jena, Germany). Streptase (highly purified streptokinase from group C streptococci) was purchased from Hoechst Marion Roussel AB.

(v) **Capsule content.** The ALAB49 wild-type strain and isogenic mutants were grown to exponential phase, and their hyaluronic acid (HA) capsule contents were measured using Stains-All (Sigma) as described by Ashbaugh & Wessels (2001). Overnight THY broth cultures of bacteria were diluted 1:100 in fresh THY broth and grown to an OD₆₀₀ value of between 0.2 and 0.3 (mid-exponential phase), prior to HA extraction. C.f.u. counts were determined in duplicate and data were expressed as c.f.u. (fg HA)⁻¹, based on a standard curve generated with known quantities of bovine HA.

Statistical analyses. Statistical tests were performed using STATISTICA 5.5 (StatSoft). Tests included the *t*-test for independent samples (unpaired, two-tailed), the non-parametric Mann–Whitney *U*-test, Fisher's exact test for independence (two-tailed) and Pearson's correlation coefficient.

RESULTS

***In vitro* effects of *ska* and *pam* inactivation**

Allelic replacement of the *ska* and *pam* genes of strain ALAB49 with the ΩKm-2 cassette resulted in several phenotypic changes, as measured *in vitro*. The heat-killed mutant and wild-type organisms were tested over a wide range of concentrations for binding of radio-labelled human Plg (Fig. 1). At a high, but subsaturating, concentration of bacteria, ALAB49 wt and the ALAB49Δ*ska* mutant bound nearly equivalent amounts of human Plg (~80 %); titration of bacteria showed similar binding patterns for the wild-type strain and the Δ*ska* mutant over a range of concentrations. In contrast, the ALAB49Δ*pam* mutant displayed a near-complete loss in binding, even at the highest bacterial concentration tested (~5 % bound) (Fig. 1a). The low-level binding of Plg by the Δ*pam* mutant is probably due to low-affinity Plg-binding proteins, such as α-enolase or

Fig. 1. Binding of human- and mouse-derived Plg by whole bacteria. (a, b) Heat-killed bacteria were tested over a wide range of concentrations for adsorption of ¹²⁵I-labelled Plg derived from human (a) and mouse (b) plasma. Shown are strains ALAB49 wt (●), ALAB49Δska (▲) and ALAB49Δpam (■). (c) The binding of ¹²⁵I-labelled human (○) and mouse (Δ) Plg to ALAB49 wt is compared directly.

Fig. 2. Immunoblot of streptokinase in bacterial culture supernatants. Late-exponential (lanes 1–3) and stationary (lanes 4–9) phase cultures of ALAB49 wt (lanes 1, 4, 6), ALAB49Δpam (lanes 2, 5, 7), ALAB49Δska (lanes 3 and 9) and ALAB49ΔspeB (lane 8) are shown. The cysteine proteinase inhibitor E64 was added to some bacterial cultures during their growth (lanes 6 and 7). Late-exponential cultures were grown to an OD₆₂₀ value of 0.8 in THY broth, whereas stationary phase cultures were grown overnight (14–16 h). Purified recombinant streptokinase derived from group C streptococci (Streptase) was included in lane 10.

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Lottenberg *et al.*, 1992; Pancholi & Fischetti, 1992, 1998). When live bacteria were used in place of heat-killed bacteria, ALAB49 wt bound a high proportion of total Plg, whereas the Δpam mutant bound Plg at a very low level (data not shown).

Under similar experimental conditions, with sub-saturating concentrations of bacteria, ALAB49 wt and the Δska mutant bound ~62% of mouse Plg, whereas ALAB49Δpam bound only 8% (Fig. 1b). A direct comparison of binding of human versus mouse Plg by ALAB49 wt is shown in Fig. 1(c). Over a range of bacterial concentrations, mouse Plg is bound about 10- to 20-fold less effectively than human Plg. The fact ALAB49 wt can bind mouse Plg reasonably well may be important for the hu-skin-SCID mouse model, whereby mouse Plg is present in extravasated plasma that is dragged by polymorphonuclear leukocytes (PMNs) as they migrate from the circulation to the site of infection. Nonetheless, the presence of human Plg within the human skin graft, or its synthesis *in situ* upon stimulation by damage or bacteria, cannot be ruled out. PAM binds human Plg with an affinity constant of $8 \times 10^{-8} \text{ M}^{-1}$ (Berge & Sjöbring, 1993), and the concentration of Plg in the circulation and interstitial fluids is $1-2 \times 10^{-6} \text{ M}$.

The level of streptokinase production by the mutant and wild-type organisms was monitored by Western immunoblot, using a polyclonal antiserum directed against streptokinase (Fig. 2). Following growth to late-exponential phase, culture broth supernatants derived from both ALAB49 wt and the ALAB49Δpam mutant revealed a strongly immunoreactive band at 44 kDa (Fig. 2, lanes 1 and 2), which corresponded to the expected size for streptokinase. In contrast, the ALAB49Δska mutant lacked a 44 kDa band, but instead displayed a strongly immunoreactive band of 27 kDa (Fig. 2, lane 3), consistent with the expected size for the truncated gene product.

Fig. 3. Immunoblot showing acquisition of Plg and plasmin by wild-type and mutant bacteria. Bacterial-bound proteins eluted from ALAB49 wt (lanes 1–3), ALAB49 Δ *ska* (lanes 4–6) and ALAB49 Δ *pam* (lanes 7–9) following addition of human plasma and subsequent incubation at 37 °C for 0 (lanes 1, 4, 7), 30 (lanes 2, 5, 8) and 60 (lanes 3, 6, 9) min are shown. Two micrograms of purified human Plg was included in lane 10.

Acquisition of bacterial-bound plasmin was also measured for ALAB49 wt and the mutant organisms (Fig. 3). Both ALAB49 wt and the ALAB49 Δ *ska* mutant bound human Plg. The wild-type strain, but not the Δ *ska* mutant, displayed conversion of Plg to plasmin following 60 min incubation in 30% human plasma in THY broth, resulting in an immunoreactive band of a lower molecular mass (Fig. 3, lane 3), following elution from the bacteria and analysis by Western immunoblot using an antibody directed against plasmin(ogen). The ALAB49 Δ *pam* mutant failed to bind detectable levels of Plg (Fig. 3, lanes 7–9). Data on Plg binding by live ALAB49 wt and mutant strains (Fig. 3) are consistent with findings on heat-killed bacteria (Fig. 1). The plasmin acquisition assay using mouse plasma as a source was not conducted due to the extreme difficulty in obtaining mouse plasma devoid of even a small degree of activation of the coagulation-fibrinolytic pathways, without the addition of protease inhibitors.

It has long been recognized that the *emm* gene products of GAS can contribute to the ability of these organisms to resist opsonophagocytosis in the absence of immune sera (Cunningham, 2000; Scott *et al.*, 1986). Following rotation in whole human blood, the ALAB49 Δ *ska* mutant is equally resistant to phagocytosis when compared to ALAB49 wt (Table 1; *t*=non-significant, *t*-test, unpaired two-tailed). Both the ALAB49 Δ *ska* mutant and ALAB49 wt show mean net increases in their c.f.u. values that are greater than 200-fold (> 7 population doublings), exceeding the 32-fold threshold (5 popu-

lation doublings) which defines survival due to an anti-phagocytic effect (Johnson *et al.*, 1996). In contrast, the ALAB49 Δ *pam* mutant displays a significant loss in survival relative to the wild-type (*t*=0.038). However, the Δ *pam* mutant shows a mean net increase in its c.f.u. counts of 21-fold (2.9 population doublings), indicative of partial and incomplete growth attenuation in human blood; overall, the ALAB49 Δ *pam* mutant exceeded the five-population doubling threshold in only one of the four experiments. The finding of partial attenuation for the ALAB49 Δ *pam* mutant is not surprising since, for strains having multiple *emm* loci, each *emm* gene product contributes partially to the antiphagocytic effect (Husmann *et al.*, 1995; Podbielski *et al.*, 1996). Although the range of inoculum doses is slightly lower for ALAB49 Δ *pam*, differences in inoculums for ALAB49 and each mutant are not statistically significant.

In addition to M protein, the HA capsule of GAS can promote resistance to phagocytosis by PMNs, as well as affect GAS adherence to keratinocytes (Darmstadt *et al.*, 2000; Schrager *et al.*, 1998). To better ensure that unintended mutations were not selected for following directed mutagenesis, ALAB49 wt was compared to the Δ *ska* and Δ *pam* mutants for HA content (Ashbaugh & Wessels, 2001). Following growth to mid-exponential phase, differences in HA content [c.f.u. (fg HA) $^{-1}$] between wild-type and mutant organisms were very slight [20.5, 19.5 and 19.3 c.f.u. (fg HA) $^{-1}$ for wild-type, Δ *ska* and Δ *pam*, respectively, measured in duplicate and averaged]. The data suggest that the mutagenesis procedure did not lead to the selection of mutants with alterations in their capsule contents. Additional calculations show that the number of c.f.u. per millilitre of THY broth culture at OD₆₀₀ was 3.5, 3.8 and 3.1 $\times 10^9$, respectively, for the wild-type, Δ *ska* and Δ *pam* strains. Since optical density measures turbidity, which is independent of variations in chain length, there is strong evidence that the mutations do not affect streptococcal chain length.

Growth curves in THY broth, measured at OD₆₂₀, were nearly identical for ALAB49 wt and the Δ *ska* and Δ *pam* mutants (data not shown). In addition, the wild-type and the mutant constructs produced equivalent amounts

Table 1. Bacterial growth in whole human blood and the ability to resist phagocytosis

Strain	Range of inoculums (c.f.u. ml $^{-1}$)	Mean inoculum (c.f.u. ml $^{-1}$)	<i>t</i> -Test (versus wild-type), for inoculums	Growth following 3 h rotation in blood (log two-fold increase)*					<i>t</i> -Test (versus wild-type), for growth
				Expt 1	Expt 2	Expt 3	Expt 4	Mean	
ALAB49 wt	460–780	600	NA	8.325	8.616	6.370	6.677	7.497	NA
<i>ska</i> mutant	350–740	505	NS	7.800	7.827	7.078	7.657	7.591	NS
<i>pam</i> mutant	150–570	375	NS	4.700	5.810	1.202	0.000	2.928	0.038

NA, Not applicable; NS, non-significant.

* Experiments 1–4 utilized a total of three different blood donors.

Table 2. Gain or loss in bacterial counts following inoculation of hu-skin-SCID mice

ALAB49 wt		<i>pam</i> mutant		<i>ska</i> mutant	
Inoculum ($\times 10^6$)	Log two-fold increase in c.f.u. count	Inoculum ($\times 10^6$)	Log two-fold increase in c.f.u. count	Inoculum ($\times 10^6$)	Log two-fold increase in c.f.u. count
0.006	0.80	0.005	−9.97	0.038	1.27
0.018	10.92	0.024	10.63	0.076	−13.29
0.023	11.99	0.035	7.23	0.280	−5.62
0.089	8.51	0.165	−6.41	0.343	−16.61
0.320	5.20	0.240	−12.23	0.409	0.33
0.405	1.79	0.305	5.15	0.908	−1.08
1.550	0.80	0.348	−10.04	1.400	0.19
2.700	2.10	3.050	−8.04	5.363	0.10
		9.050	2.3		
<i>t</i> -Test (two-tailed) versus ALAB49 wt		0.4514 NS	0.0393*	0.5324 NS	0.0067†

ns, Non-significant.

* Significant.

† Highly significant.

of the mature 28 kDa form of SpeB in late-stationary phase culture supernatants, and displayed complete conversion of SpeB from the 40 kDa zymogen form (data not shown). Despite the strongly polar nature of Ω Km-2, evidence for decreased expression of downstream genes in our mutants is lacking. The *ska* transcript is monocistronic (Malke, 2000; Malke *et al.*, 2000) and, based on the SF370 (M1) genome (Ferretti *et al.*, 2001), the nearest downstream gene is 4.5 kb away (SPy1984, encoding a putative ribonucleotide reductase, NrdI); intervening ORFs are transcribed on the opposite strand, and *rel* shares a bidirectional transcriptional terminator with *ska* (Malke, 2000; Malke *et al.*, 2000). Using gene-specific primers, the relative chromosomal arrangements of *ska* (SPy1979), *rel* (SPy1981) and *nrdI* (SPy1984) were found to be similar for ALAB49 and SF370 (data not shown), unlike the gene order observed for some strains of *Streptococcus dysgalactiae* subsp. *equisimilis* (Geyer & Schmidt, 2000). *emm* genes are also transcribed monocistronically in several GAS strains that have multiple *emm* genes (Bessen & Fischetti, 1992; Podbielski *et al.*, 1995; Yung & Hollingshead, 1996).

In summary, phenotypic analysis of the Δ *ska* and Δ *pam* mutants constructed in ALAB49 revealed that each mutant exhibits the changes that were expected, but neither mutant has measurable alterations in several unrelated properties that may be key for virulence. As expected, the ALAB49 Δ *ska* mutant is unchanged in its Plg binding, resistance to non-immune phagocytosis, HA capsule and SpeB contents and growth in THY broth; however, it fails to secrete streptokinase and fails to convert surface-bound Plg to plasmin. The ALAB49 Δ *pam* mutant is unchanged in its streptokinase and SpeB production, HA capsule content and growth curve but, as expected, shows loss in binding Plg and loss in the antiphagocytic effect, properties that are both attributed to the M53 protein (i.e. PAM).

***In vivo* effects of *ska* and *pam* inactivation**

The ALAB49 Δ *ska* and Δ *pam* mutants were tested for virulence in the hu-skin-SCID mouse model for impetigo, over a wide range of inoculating doses. In this model, virulence can be defined as a function of the increase in c.f.u. counts following topical inoculation of GAS onto slightly damaged (scratched) human skin grafts. The 7 day time point chosen for skin biopsy is based on empirical evidence showing a large net growth of virulent strains and histopathological alterations that closely mimic a late stage of the human disease (Scaramuzzino *et al.*, 2000; Svensson *et al.*, 2000). The hu-skin-SCID mouse model is technically challenging, and there is a limit to the number of graft biopsies that can be processed at any one time. Furthermore, since the inoculum doses, expressed as c.f.u. values, are only known in retrospect, it is not possible to test two different constructs at precisely the same dose using the highly accurate c.f.u. measure. Given these constraints, we chose an experimental design whereby each graft

receives a different inoculating dose, spanning a wide range of doses, rather than multiple grafts receiving only one or two identical doses. This approach further ensures against overlooking a critical threshold dose, which may differ for the wild-type and each mutant. In natural infections, inoculating doses are likely to fall within a wide range and be dependent on the degree of direct contact and various risk factors, such as skin integrity.

For skin graft biopsies taken at 7 days post-inoculation (Table 2), each mutant showed a partial attenuation in virulence, relative to ALAB49 wt. There were no significant differences for the ranges of inoculating doses tested for ALAB49 wt versus each mutant (Table 2). The difference between ALAB49 wt and each of the mutants in terms of the two-log change in c.f.u. counts (i.e. doubling or halving of the bacterial population) was statistically significant ($t < 0.05$; unpaired, two-tailed). Although the Δ *ska* mutant showed greater attenuation than the Δ *pam* mutant ($t = 0.0067$ and 0.0393 , respectively), the Δ *ska* versus Δ *pam* mutants failed to show significant differences relative to one another. Using a non-parametric statistical test (Mann-Whitney *U*-test), loss of virulence for the Δ *ska* mutant was highly significant ($P < 0.001$), whereas the Δ *pam* mutant showed no significant difference relative to ALAB49 wt. Thus, it appears that the Δ *ska* mutant is highly attenuated for virulence, whereas the Δ *pam* mutant is attenuated to a lesser degree. However, the Δ *pam* mutant exhibited net reproductive growth for only 44 % of the inoculated skin grafts, in contrast to 100 % of the grafts infected with ALAB49 wt ($P = 0.029$, Fisher's exact test, two-tailed). If only half of the infections caused by PAM-deficient strains are productive, there may be a significant impact on transmission dynamics (Anderson, 1998). Although statistical analysis strongly supports partial attenuation in virulence resulting from the *ska* and *pam* mutations, the loss in virulence does not follow a strict dose-response for either mutant (Table 2); this may be partly due to microscopic graft-to-graft variation in the depth of the skin surface scratches, a procedure that is difficult to control with precision and which can on occasion extend beyond the epidermis into the dermal layer (Scaramuzzino *et al.*, 2000).

Gross pathology was scored for grafts infected with ALAB49 wt and each of the mutants, using a relative scale measuring severity of infection – a high score of 3 indicates a purulent exudate (pus) (Scaramuzzino *et al.*, 2000; Svensson *et al.*, 2000). The gross pathology score for the Δ *ska* and Δ *pam* mutants was significantly different when compared to ALAB49 wt (Table 3; all *P* values < 0.02). Virulence, as defined by a net gain or loss in c.f.u. values, is generally consistent with the severity of infection, as defined by grossly visible alterations in the skin graft.

Previous histopathological analysis shows that topical inoculation of skin grafts with ALAB49 wt and other virulent GAS strains typically leads to loss of the stratum

Table 3. Gross and microscopic (blinded review) pathological changes in human skin grafts at 7 days post-inoculation with GAS

Scores for tissue alterations: 0, no change; 1, mild changes; 2, moderate changes; 3, severe changes (Scaramuzzino *et al.*, 2000).

Gross and pathological parameters	Strain		
	ALAB49 wt	<i>pam</i> mutant	<i>ska</i> mutant
Mean score for			
Gross pathology	2.750	1.389	0.500
Inflammation	1.875	1.111	0.375
Epidermal destruction	1.625	0.667	0.063
<i>t</i> -Value (unpaired, two-tailed <i>t</i> -test) versus wild-type, for			
Gross pathology	NA	0.0148*	0.0005†
Inflammation	NA	NS	0.0265*
Epidermal destruction	NA	NS	0.0176*
<i>P</i> -value (Mann–Whitney <i>U</i> -test) versus wild-type, for			
Gross pathology	NA	0.0105*	0.0006†
Inflammation	NA	NS	0.0250*
Epidermal destruction	NA	NS	0.0250*

NA, Not applicable; NS, non-significant.

* Significant.

† Highly significant.

corneum (keratinized layer) over large sections of skin, infiltration of numerous PMNs throughout the dermis, epidermis and external surface of the skin, and epidermal destruction over large areas of skin. These events have been described in extensive detail, along with accompanying histopathology photographs (Scaramuzzino *et al.*, 2000; Svensson *et al.*, 2000). Similar patterns of histopathological alterations were observed in this study. Six of the seven grafts infected with ALAB49 wt and having a grossly visible purulent exudate on the skin surface at 7 days post-inoculation also scored positive for inflammatory cells present within the tissue, on the external surface and/or in distal foci (Scaramuzzino *et al.*, 2000), according to a microscopic histopathological analysis performed in a blinded fashion (Table 3). The degree of inflammation was lower for grafts inoculated with either the Δ *pam* or the Δ *ska* mutant, with statistically significant differences when Δ *ska* is compared to ALAB49 wt (Table 3). A similar trend was noted for destruction of the epidermal layer (Scaramuzzino *et al.*, 2000), whereby the Δ *pam* and Δ *ska* mutants showed less epidermal destruction than the wild-type, and the difference between the Δ *ska* mutant and the wild-type was statistically significant. The gross and histopathological findings support the conclusion that the Δ *ska* and Δ *pam* mutants are partially attenuated in virulence, relative to ALAB49 wt, with the Δ *ska* mutant showing a greater loss in virulence than the Δ *pam* mutant.

When considering all grafts inoculated with GAS in this study ($n=25$), the correlation between gross pathology score and the sum of the scores for inflammation and

epidermal destruction is statistically significant ($r=0.801$; Pearson's correlation coefficient). Grafts showing a net increase in c.f.u. counts at 7 days post-inoculation (Table 2), as compared to grafts showing a net decrease in c.f.u. counts, reveal differences in gross pathology scores that are highly significant ($t=0.00027$). Grafts showing a net increase or decrease in c.f.u. counts at 7 days post-inoculation also showed a strong correspondence with histopathological scores for epidermal destruction and inflammation ($t=0.0036$ and 0.0008 , respectively). The latter finding is consistent with the idea that bacterial reproduction in tissue depends on a source of nutrients provided by extravasated inflammatory cells and that the inflammatory response and/or replicating bacteria contribute to destruction of the epidermal layer.

The hu-skin-SCID mouse model measures the c.f.u. values for bacteria released from the skin graft surface. However, in severely infected tissue, dermal ulcers packed with bacteria can arise; this is characteristic of ecthyma observed in human disease (Scaramuzzino *et al.*, 2000). Therefore, the number of c.f.u. recovered from heavily infected tissue in the hu-skin-SCID mouse model may be an underestimate of the total bacteria associated with the skin graft. Since the wild-type strain yields the most extensive tissue damage (Table 3), it is possible that the net increase in bacterial growth within the total skin graft is higher than reported for vortex-released bacteria (Table 2). Nonetheless, in natural infections in humans, bacteria present on the superficial skin surface are the most likely to undergo transmission to a new host.

Interactions of streptokinase and PAM with SpeB

In a previous study, it was shown that the secreted cysteine proteinase SpeB is critical for GAS infection in the hu-skin-SCID mouse model for impetigo (Svensson *et al.*, 2000). Other investigators have reported that the M1 protein facilitates the conversion of the zymogen form of SpeB to the mature form (Collin & Olsen, 2000). In contrast, the M53 protein (i.e. PAM) of the ALAB49 strain has no noticeable effect on SpeB conversion. Using culture broth conditions used to prepare inoculums for hu-skin-SCID mice (24 h growth in THY broth), there was complete conversion of SpeB to the mature form, and near-equivalent levels of SpeB for the Δ pam mutant and ALAB49 wt (data not shown). Also, there were no obvious differences in SpeB production by the ALAB49 Δ ska mutant.

Cysteine proteinase activity due to SpeB can result in the degradation of M proteins (Berge & Björck, 1995; Raeder *et al.*, 1998). Studies show that SpeB activity is maximally expressed during the stationary phase of growth (Collin & Olsen, 2000; Lyon *et al.*, 1998), whereas Mga-regulated *emm* transcription peaks during the exponential phase (McIver & Scott, 1997). Control experiments show that the ALAB49 Δ speB mutant (Svensson *et al.*, 2000) binds human Plg at levels equivalent to those measured for ALAB49 wt and ALAB49 Δ ska, following growth in THY broth to late-exponential phase (Fig. 1a; data not shown). The ALAB49 Δ speB mutant shows no change in anti-phagocytic activity in the bactericidal assay (Svensson *et al.*, 2000), consistent with the presence of functionally intact PAM in ALAB49 wt. Thus, under the set of *in vitro* conditions tested, there is no evidence for modulation of PAM by SpeB in ALAB49 wt nor is there any indication that the loss of PAM influences SpeB activity. Nonetheless, we cannot rule out the possibility that SpeB has an effect on PAM under other experimental conditions (such as stationary phase) or that SpeB modulates the low-affinity Plg-binding surface proteins of ALAB49.

The production of streptokinase by ALAB49 wt and the ALAB49 Δ pam mutant was monitored by Western immunoblot analysis of culture broth supernatants (Fig. 2). Streptokinase was evident in late-exponential phase cultures as a strong 44 kDa band (Fig. 2, lanes 1 and 2), but was significantly degraded in overnight cultures (Fig. 2, lanes 4 and 5). However, addition of the cysteine proteinase inhibitor E64 led to an accumulation of streptokinase in stationary phase cultures (Fig. 2, lanes 6 and 7). That SpeB degrades streptokinase is further supported by findings with overnight culture supernatants of the ALAB49 Δ speB mutant grown in the absence of E64, which yielded ample quantities of non-degraded streptokinase (Fig. 2, lane 8). The finding of degradation of Ska by SpeB is consistent with an earlier report (Johnston & Zabriskie, 1986).

Mid-exponential phase cultures of ALAB49 wt and ALAB49 Δ pam, grown to OD₆₂₀ values of 0.4, each reveal an immunoreactive band that is somewhat less

intense than that observed for late-exponential cultures (data not shown). Streptokinase production was unaffected by the presence of the E64 inhibitor during growth. This finding is consistent with the extracellular production of streptokinase beginning at or before the mid-exponential phase, and streptokinase accumulating throughout the later stages of exponential growth.

In summary, there is no evidence that the inactivation of *pam* or *ska* results in alterations in the production of the mature form of SpeB. However, the accumulation of SpeB in stationary phase broth cultures leads to the degradation of extracellular streptokinase in the ALAB49 wild-type strain.

DISCUSSION

The primary impetus for this study was the observation that GAS strains containing PAM are exclusively *emm* pattern D; furthermore, pattern D strains are disproportionately associated with impetigo lesions [versus the upper respiratory tract (URT)] in human hosts (Bessen *et al.*, 1996, 2000; Svensson *et al.*, 1999). *In vitro* studies demonstrate an interaction between streptokinase and PAM in the generation of bacterial-bound plasmin activity (Ringdahl *et al.*, 1998); therefore, bacterial-bound plasmin may be a determinant for GAS infection at the skin. The findings of this study provide direct experimental evidence that both streptokinase and PAM contribute to virulence during GAS impetigo. When considered together, the experimental and population (epidemiological) findings provide strong evidence that streptokinase and PAM contribute to the establishment of tissue tropism for the skin. Thus, in addition to secreted cysteine proteinase activity due to SpeB (Svensson *et al.*, 2000), the streptokinase-PAM interaction appears to represent a second extracellular proteolytic pathway that is critical for adaptation of GAS at the epidermis. Both SpeB and plasmin function as broad-spectrum proteases.

In the hu-skin-SCID mouse model for impetigo, ALAB49 wt and its mutants exhibit a strong correlation between gross pathological alterations and histopathological findings on the infiltration of inflammatory cells and epidermal erosion. Furthermore, the findings of this study show that the severity of both gross and microscopic alterations correlates with a net increase in bacterial growth at the skin. Previous studies have shown that at an early stage of infection with virulent GAS (48 h post-inoculation) the stratum corneum is lost over large sections of skin, and single cocci or short chains are observed in close association with the newly exposed apical surface of the viable keratinocytes; furthermore, the number of tissue-associated bacteria is low and few PMNs are present (Scaramuzzino *et al.*, 2000). We propose that at this early stage of infection, the bacteria are in a nutrient-poor environment (Fig. 4). A nutrient-poor environment is consistent with a stationary phase of growth and maximal expression of cysteine protease activity due to SpeB (Lyon *et al.*, 1998; Podbielski *et al.*, 1999; Svensson *et al.*, 2000).

Fig. 4. Integrated model for the pathogenesis of GAS impetigo. It is proposed that SpeB acts at an early stage of infection and is involved in the early amplification of the inflammatory response, whereby PMNs and plasma begin to accumulate in the vicinity of the bacteria adherent to the apical surface of keratinocytes. Loss of SpeB prevents the inflammatory response and bacteria fail to replicate. Host plasma that enters the infection site constitutes an important source of nutrients, leading to bacterial reproduction. Through the combined action of streptokinase and PAM, bacterial-generated plasmin activity leads to fibrinolysis, which, in turn, prevents the skin lesions from drying out and healing. Through fibrinolytic activity, the window of opportunity for a continuing inflammatory infiltrate and ensuing bacterial reproduction is greatly expanded.

Inactivation of *speB* leads to complete attenuation of virulence in the experimental model for impetigo (Svensson *et al.*, 2000), suggesting that SpeB plays a critical role during early stages of infection, prior to extensive bacterial growth.

By 4 days post-inoculation with virulent GAS, the external surface of the epidermis has been transformed into a nutrient-rich environment (Scaramuzzino *et al.*, 2000). Extravasating PMNs drag along small amounts of host plasma in their migration from dermal vessels to the outer epidermal surface, wherein both PMNs and plasma gradually accumulate to form a copious purulent exudate (pus) that is visible by gross pathology. Some bacterial growth has occurred by 4 days, as evidenced by both a net increase in c.f.u. counts and readily visible, large aggregates of cocci at the outer rim of the pus layer (Scaramuzzino *et al.*, 2000). The massive aggregates of cocci appear to be well-positioned for transmission to new hosts and, because of their high density, resistance to phagocytosis may result from steric hindrance.

Extracellular streptokinase is produced during exponential growth (Fig. 2); therefore, streptokinase appears to be essential at some time during the nutrient-rich phase of infection (Fig. 4). Host Plg is (presumably)

present in the local micro-environment (i.e. purulent exudate) and its activation by streptokinase appears to be critical for ensuing bacterial reproduction, although incomplete attenuation of the ALAB49 Δ *ska* mutant suggests that limited bacterial growth (or survival) can occur in the absence of streptokinase. If streptokinase is critical during the earliest stage of the nutrient-rich phase of infection, it may act, directly or indirectly, to further amplify the inflammatory response, leading to the recruitment of additional PMNs and host plasma. Bacterial-generated plasmin activity can lead to fibrinolysis, which, in turn, may prevent the skin lesion from drying out and healing. If streptokinase plays a role during the late stages of the nutrient-rich phase of infection, it may act to promote invasion of GAS into the host tissue. Unlike PAM, streptokinase does not appear to directly contribute to resistance to phagocytosis, based on findings with the *in vitro* bactericidal assay. By 7 days post-inoculation, bacterial reproduction has increased even further and dermal pockets of Gram-positive cocci are readily observed (Scaramuzzino *et al.*, 2000).

It is proposed that PAM exerts its virulence properties during the nutrient-rich phase of infection (Fig. 4), since *emm* gene transcription is up-regulated during the exponential phase (McIver & Scott, 1997). It is not known whether the slight attenuation observed for ALAB49 Δ *pam* *in vivo* is related to the partial loss in its antiphagocytic property or to the decreased bacterial-bound plasmin activity *in vitro*. The ALAB49 Δ *ska* mutant exhibits a greater loss in virulence than the ALAB49 Δ *pam* mutant, suggesting that PAM function can be partly compensated by low-affinity Plg-binding proteins present on the bacterial surface (Lottenberg *et al.*, 1992; Pancholi & Fischetti, 1992, 1998) and/or by fluid-phase plasmin activity. Since the affinity of PAM for mouse Plg is 10- to 20-fold lower than its human counterpart, the contribution of PAM to virulence may be delayed until additional plasma is dragged to the epidermal surface by extravasating PMNs. A possible consequence for the hu-skin-SCID mouse model is that the minimal infective dose is somewhat higher than for natural infection in humans.

As ALAB49 re-enters another round of stationary growth, the increased expression of cysteine protease activity due to SpeB results in the degradation of extracellular streptokinase. It is not yet known whether streptokinase is deleterious to GAS transmission and/or the early stages of a newly acquired infection. Conceivably, bacterial-generated plasmin might act to degrade streptococcal adhesins or host keratinocyte receptors that are critical for the initial host-pathogen interaction. Alternatively, SpeB degradation of streptokinase might simply be incidental.

Although the ALAB49 Δ *ska* and Δ *pam* mutants are only partially attenuated in the experimental model for impetigo, it is quite possible that in the natural world comparable mutants would become completely lost. In a human host population, GAS infectious doses are

probably at the lower end of our experimental range. In order for a micro-organism to survive, its basic reproductive rate (R_0) must be greater or equal to one (Anderson & May, 1991; Anderson, 1998), which essentially means that for each infected host that recovers, the organism must be successfully transmitted to at least one new host. If bacterial doses are naturally low and the mutated variant can induce infection in only a fraction of newly exposed hosts, the organism will quickly disappear.

From the standpoint of evolutionary success, changes in c.f.u. counts are highly relevant measures. Fitness is defined in the context of the environmental niche, and the organism with the highest fitness is that which yields the greatest number of progeny in that niche (i.e. it is the most highly adapted). An increase in the number of (transmissible) progeny increases the frequency of adaptive alleles in the gene pool of that subpopulation. The finding that the PAM phenotype and high levels of SpeB activity display statistically significant associations with *emm* pattern D strains and impetigo isolates (Svensson *et al.*, 1999, 2000) is consistent with the idea that they play a direct role in promoting skin infection. In addition to *emm* pattern D strains, *emm* pattern E strains of GAS are often associated with impetigo, although they are prevalent at the URT as well (Bessen *et al.*, 1996, 2000; Dicuonzo *et al.*, 2001). To date, pattern E strains have not been carefully investigated in the hu-skin-SCID mouse model. Since *emm* pattern E strains lack PAM (Svensson *et al.*, 1999), it is possible that they cause impetigo by using an entirely different molecular strategy than pattern D strains.

Following mutagenesis of any gene, unintended polar effects can potentially arise, not only through the introduction of strong transcriptional terminators within the Ω Km-2 element (as is the case for the ALAB49 Δ *ska* and Δ *pam* mutants) but also through indels generated by allelic replacement and ensuing shifts in codon usage and translational activity. In many respects, an ideal control for the ALAB49 Δ *ska* and Δ *pam* mutants is the reintroduction of the wild-type gene by transcomplementation, although there is a downside lying in variable gene dosage effects. Transcomplementation was achieved for the ALAB49 Δ *pam* mutant, and the binding activity of human Plg to whole bacteria, when tested *in vitro*, was fully restored (data not shown). However, when this construct was tested in the hu-skin-SCID mouse model for impetigo, a confusing picture emerged whereby gross and microscopic pathological alterations were evident in the absence of net bacterial growth at the skin. These findings are probably an artefact of plasmid instability resulting from shifts in selective pressures that occur during the course of infection, i.e. uneven diffusion of the selectable antibiotic to the superficial tissue site and changing needs for PAM. Once the plasmid is lost due to a transient lapse in positive selection, the bacteria can be eliminated following subsequent exposure to antibiotics or PMNs. Despite the lack of this particular control, the numerous phenotypic tests performed *in vitro* provide

strong support that the ALAB49 Δ *ska* and Δ *pam* mutants did not undergo unintended alterations in several phenotypes that are known to affect virulence. Although the Ω Km-2 element contains strong transcriptional terminators, both *ska* and *emm* genes are expressed as monocistronic transcripts, and the ORF transcribed on the strand opposite to *ska* shares a bidirectional transcriptional terminator positioned downstream of *ska* (Bessen & Fischetti, 1992; Malke, 2000; Malke *et al.*, 2000; Podbielski *et al.*, 1995; Yung & Hollingshead, 1996); therefore, expression of adjacent genes should be unaffected. In summary, there is no evidence that the ALAB49 Δ *ska* and Δ *pam* mutants have alterations in other genes.

It has been proposed that bacterial-generated plasmin acts to modulate the micro-environment of the bacterium and its movement through host tissue (Boyle & Lottenberg, 1997; Coleman *et al.*, 1997; Rasmussen & Bjorck, 2002; Sodeinde *et al.*, 1992). However, the ability to spread into deep tissue and cause invasive disease does not appear to be a long-term evolutionary strategy for GAS. The URT and epidermis of the skin are the primary tissue reservoirs for GAS. Because infection at the throat and skin is typically mild and superficial, the infected host is fairly mobile and has a high potential for exposing numerous individuals, via respiratory droplets or direct contact. Since GAS undergo expansive reproductive growth at the superficial epithelium and transmission to new hosts is launched from the superficial epithelium as well, it is at the URT and epidermis where the evolution of GAS is most profoundly shaped by the forces of natural selection. Thus, it seems most likely that the primary function of bacterial-generated plasmin activity is to enhance survival at a superficial epithelial site, as opposed to deep tissue.

The epidemiological findings, in combination with the experimental data, make a strong case for a role for both extracellular plasmin (this study) and cysteine proteinase activities (Svensson *et al.*, 2000) as key determinants of tissue tropism at the skin. An interesting aspect of this finding is that one usually envisions determinants of host or tissue range as attachment factors that recognize a specific host-cell receptor, yet, here, extracellular enzymes appear to play that role. By modulating their local micro-environment through proteolytic pathways, GAS have been highly successful in exploiting this ecological niche.

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