

Mutational analysis of the group A streptococcal operon encoding streptolysin S and its virulence role in invasive infection

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Summary

The pathogen group A *Streptococcus* (GAS) produces a wide spectrum of infections including necrotizing fasciitis (NF). Streptolysin S (SLS) produces the hallmark β -haemolytic phenotype produced by GAS. The nine-gene GAS locus (*sagA–sagI*) resembling a bacteriocin biosynthetic operon is necessary and sufficient for SLS production. Using precise, in-frame allelic exchange mutagenesis and single-gene complementation, we show *sagA*, *sagB*, *sagC*, *sagD*, *sagE*, *sagF* and *sagG* are each individually required for SLS production, and that *sagE* may further serve an immunity function. Limited site-directed mutagenesis of specific amino acids in the *SagA* pre-peptide supports the designation of SLS as a bacteriocin-like toxin. No significant pleiotrophic effects of *sagA* deletion were observed on M protein, capsule or cysteine protease production. In a murine model of NF, the SLS-negative M1T1 GAS mutant was markedly diminished in its ability to produce necrotic skin ulcers and spread to the systemic circulation. The SLS toxin impaired phagocytic clearance and promoted epithelial cell cytotoxicity, the latter phenotype being enhanced by the effects of M protein and streptolysin O. We conclude that all genetic components of the *sag* operon are required for expression of functional SLS, an important virulence factor in the pathogenesis of invasive M1T1 GAS infection.

Introduction

Group A *Streptococcus* (GAS) is a leading human pathogen causing common infections such as pharyngitis ('strep throat') and impetigo (Cunningham, 2000; Bisno *et al.*, 2003). During the last three decades, a resurgence of severe invasive GAS infection has been documented worldwide (Efstratiou, 2000). Prominent among invasive GAS syndromes are the destructive soft tissue infection necrotizing fasciitis (NF) and the multisystem disorder of streptococcal toxic shock syndrome (STSS), each carrying significant risk of morbidity and mortality even with aggressive medical therapy (Stevens, 1999; Sharkawy *et al.*, 2002). While GAS strains of many M protein (*emm*) genotypes are capable of producing significant disease, strains representing one globally disseminated clonal M1T1 GAS strain have persisted for over 20 years as the single most prevalent isolate from invasive GAS infections (Cockerill *et al.*, 1997; Cleary *et al.*, 1998; Muroso *et al.*, 1999; Chatellier *et al.*, 2000), including all nine surveillance centres of the United States Centers for Disease Control Emerging Infections Program Network in 2002 (<http://www.cdc.gov/ncidod/dbmd/abcs>).

A hallmark phenotypic feature of GAS is the distinct zone of β -haemolysis surrounding colonies grown on blood agar media. This phenomenon reflects complete lysis of red blood cells produced by the potent oxygen-stable cytolysin known as streptolysin S (SLS). SLS exists primarily in cell-bound form (Ginsburg, 1999) and is delivered most effectively to target cells by direct contact with GAS (Ofek *et al.*, 1990). The cytolytic spectrum of SLS is broad including lymphocytes, neutrophils, platelets, cancer cell lines and subcellular organelles (Keiser *et al.*, 1964; Taketo and Taketo, 1966; Ginsburg, 1972; Hryniewicz and Pryjma, 1977). Insertion of SLS into the cell membrane results in the formation of transmembrane pores and osmotic cell lysis, similar to that observed with complement-mediated cytotoxicity (Ginsburg, 1999; Carr *et al.*, 2001).

The GAS chromosomal locus for SLS production was first identified by J. DeAzavedo and colleagues through generation and analysis of SLS-deficient transposon

mutants (Borgia *et al.*, 1997; Betschel *et al.*, 1998). Subsequent chromosome walking studies performed by B. Beall, and informed by the first GAS genome project (Ferretti *et al.*, 2001), recognized the existence of a nine-gene cluster (*sagA–I* for streptolysin-associated genes) in the region of transposon insertions. The functional boundaries of the *sag* locus were then defined by plasmid integrational mutagenesis and its organization as an operon confirmed by RNA analysis (Nizet *et al.*, 2000). Heterologous expression of the entire GAS *sag* locus in a non-haemolytic strain of *Lactococcus lactis* yielded robust β -haemolytic transformants, confirming that the operon is both necessary and sufficient for SLS production (Nizet *et al.*, 2000). Individual gene homologies and structural features of the operon suggested that SLS is a bacteriocin-like peptide toxin, with structural gene *sagA* encoding a 53-amino-acid prepropeptide precursor of the mature toxin (Nizet *et al.*, 2000). This hypothesis was corroborated by the research group of B. Kreikemeyer (Carr *et al.*, 2001), and later others (Dale *et al.*, 2002), who demonstrated that antibodies generated against synthetic peptides corresponding to the predicted SagA propeptide neutralized SLS activity. Highly homologous *sag* operons producing SLS toxins are responsible for the β -haemolytic phenotypes of human isolates of groups C and G streptococci (GCS and GGS) and the zoonotic pathogen *Streptococcus iniae* (Fuller *et al.*, 2002; Humar *et al.*, 2002).

Streptolysin S contributes significantly to GAS virulence potential in animal models of infection (Betschel *et al.*, 1998; Humar *et al.*, 2002; Fontaine *et al.*, 2003; Sierig *et al.*, 2003; Engleberg *et al.*, 2004). Like the genetically distinct β -haemolysin of the human pathogen group B *Streptococcus* (GBS), SLS could theoretically enhance GBS pathogenicity by causing direct tissue injury, promoting cellular invasion, impairing phagocytic clearance or additional yet to be defined mechanisms (Nizet, 2002). However, the analysis of SLS-associated virulence phenotypes has been complicated by reports that various mutations in the region of the *sagA* gene could be associated with alterations in expression of other GAS virulence genes including M protein, cysteine protease and streptokinase (Li *et al.*, 1999; Biswas *et al.*, 2001; Mangold *et al.*, 2004).

In the present study, we employ precise in-frame allelic replacement, single-gene complementation and limited site-directed mutagenesis to test the specific requirement of individual GAS *sag* locus genes for the production of SLS. Using a precise allelic replacement Δ *sagA* mutant created in the background of a serotype M1T1 invasive human disease isolate, we then probe the contribution of SLS to cellular injury, phagocytic resistance and virulence in a murine model of GAS necrotizing soft tissue infection.

Results

Requirement of individual sag locus genes in SLS production

Earlier studies establishing the role of the *sag* locus in SLS production employed transposon mutants (Betschel *et al.*, 1998; Li *et al.*, 1999) or insertional inactivation mutants (Nizet *et al.*, 2000; Biswas *et al.*, 2001) likely to exert polar effects on transcription of downstream genes in the operon. To assess the specific requirement of individual *sag* genes in SLS production, we combined precise in-frame allelic exchange mutagenesis with single-gene complementation analysis. Bacterial strains used in this study are listed in Table 1. GAS strain 5448 is a serotype M1T1 isolate from a patient with necrotizing fasciitis and streptococcal toxic shock syndrome expressing SLS and streptolysin O (SLO), cysteine protease, superantigens SpeA, SpeF, SpeG and SmeZ and is genetically representative of the globally disseminated M1T1 clone that is the leading cause of invasive GAS infections (Kansal *et al.*, 2000). GAS strain NZ131, a serotype M49T14 skin isolate from a patient with glomerulonephritis, expresses SLS and SLO, and is frequently used in genetic studies because of its increased transformability (Simon and Ferretti, 1991). Precise in-frame allelic replacement of the *sagA*, *sagB*, *sagC*, *sagD*, *sagE*, *sagF* or *sagG* genes in M49 GAS strain NZ131 produced a completely non-haemolytic phenotype (Fig. 1). Allelic replacement of *sagA* in the M1 GAS strain 5448 also eliminated SLS production. Complementation experiments were performed to reintroduce the corresponding single gene to each allelic exchange mutant. Full complementation was noted for *sagA*, *sagC* and *sagF*, while partial complementation was seen with *sagB*, *sagD* and *sagG* (Fig. 1). These experiments demonstrate a unique requirement for each of these six *sag* operon genes in production of functional SLS toxin. We speculate that partial complementation in the case of the individual genes mentioned could reflect toxicity of overexpression (note SagB and SagD are predicted cytoplasmic proteins) or upsetting the stoichiometry of complex assembly (SagG is predicted to participate with SagH and SagI in forming an ABC-type transporter).

The sagE gene appears to encode an immunity function

Multiple initial attempts to complement the *sagE* allelic exchange mutant were unsuccessful. Because the predicted gene product SagE shares homology to a bacteriocin immunity protein of *Lactobacillus planterum*, we hypothesized that elimination of *sagE* gene in the face of continued SLS biosynthesis may be lethal to the bacterium. We further hypothesized that this may force a compensatory mutation elsewhere in the operon to eliminate SLS production allow bacterial survival; hence return of

Table 1. Bacterial strains and plasmids.

	Description	Reference
Bacterial strain		
Group A <i>Streptococcus</i> clinical isolates		
NZ131	M49T14, OF+, <i>emm49</i> glomerulonephritis isolate	Simon and Ferretti (1991)
5448	M1T1, OF-, <i>emm1.0</i> necrotizing fasciitis + toxic shock isolate	Kansal <i>et al.</i> (2000)
Group A <i>Streptococcus</i> mutants		
NZ131: <i>sagA</i> Δ <i>cat</i>	M49 GAS with in-frame allelic replacement of <i>sagA</i> by <i>cat</i> (Cm ^R)	This study
NZ131: <i>sagB</i> Δ <i>cat</i>	M49 GAS with in-frame allelic replacement of <i>sagB</i> by <i>cat</i> (Cm ^R)	This study
NZ131: <i>sagC</i> Δ <i>cat</i>	M49 GAS with in-frame allelic replacement of <i>sagC</i> by <i>cat</i> (Cm ^R)	This study
NZ131: <i>sagD</i> Δ <i>cat</i>	M49 GAS with in-frame allelic replacement of <i>sagD</i> by <i>cat</i> (Cm ^R)	This study
NZ131: <i>sagE</i> Δ <i>cat</i>	M49 GAS with in-frame allelic replacement of <i>sagE</i> by <i>cat</i> (Cm ^R)	This study
NZ131: <i>sagF</i> Δ <i>cat</i>	M49 GAS with in-frame allelic replacement of <i>sagF</i> by <i>cat</i> (Cm ^R)	This study
NZ131: <i>sagG</i> Δ <i>cat</i>	M49 GAS with in-frame allelic replacement of <i>sagG</i> by <i>cat</i> (Cm ^R)	This study
5448: <i>sagA</i> Δ <i>cat</i>	M1 GAS with in-frame allelic replacement of <i>sagA</i> by <i>cat</i> (Cm ^R)	This study
NZ131:M-Pr.KO	NZ131 <i>emm49</i> KO by plasmid integration (Cm ^R)	This study
NZ131:SLO KO	NZ131 <i>slo</i> KO by plasmid integration (Cm ^R)	This study
NZ131: <i>sagA</i> :Erm	NZ131 with allelic replacement of <i>sagA</i> by <i>Erm</i> (Em ^R)	Nizet <i>et al.</i> (2000)
NZ131: <i>sagA</i> :M-Pr.KO	SLS + M protein double KO (Cm ^R + Em ^R)	This study
NZ131: <i>sagA</i> :SLO.KO	SLS + SLO double KO (Cm ^R + Em ^R)	This study
<i>Escherichia coli</i>		
DH5α	<i>end A1 hsd R17 (r_k-m_k⁺) sup E44 thi-1 rec A1 gyr A (Nal^R) Rel A1 Δ(lac ZYA-arg F) U169, φ80 dLac Δ(lac Z)M15)</i>	Woodcock <i>et al.</i> (1989)
MC1061	F ⁻ <i>ara D139 Δ(ara ABC-leu)7696 Δ(lac) X74 gal U gal K Hsd R2, r_k-m_k⁺ mcr B1 rps L (Str^R)</i>	Wertman <i>et al.</i> (1986)
Top 10	F ⁻ <i>mcr A Δ(mrr-hsd RMS-mcr BC) φ80lac ZΔM15 Δ(lac) X74 deoR rec A1 ara D139 Δ(ara-leu)7697 gal U gal K rps L (Str^R) end A1 nup G</i>	Grant <i>et al.</i> (1990)
<i>Lactococcus lactis</i>		
NZ9000	MG1363 (lacking nisin operon); <i>pepN::nisRK</i>	Kuipers <i>et al.</i> (1998)
Plasmid		
Mutagenesis studies		
pCR2.1-TOPO	ColE <i>ori</i> , Amp ^R , Kn ^R , <i>lacZα</i> , T-A cloning vector	Invitrogen
pHY304	Temp ^S pVE6007Δ derivative, Em ^R + <i>lacZα</i> /MCS of pBluescript	Pritzlaff <i>et al.</i> (2001)
pACYC184	rep(p15A), Cm ^R , Tet ^R	Rose (1988)
pVE6007Δ	Temperature sensitive derivative of p WV01, Cm ^R	Maguin <i>et al.</i> (1992)
pSagAΔ <i>cat</i> -KO	pHY304 containing <i>sagA</i> Δ <i>cat</i> allele + flanking DNA, Em ^R , Cm ^R	This study
pSagBΔ <i>cat</i> -KO	pHY304 containing <i>sagB</i> Δ <i>cat</i> allele + flanking DNA, Em ^R , Cm ^R	This study
pSagCΔ <i>cat</i> -KO	pHY304 containing <i>sagC</i> Δ <i>cat</i> allele + flanking DNA, Em ^R , Cm ^R	This study
pSagDΔ <i>cat</i> -KO	pHY304 containing <i>sagD</i> Δ <i>cat</i> allele + flanking DNA, Em ^R , Cm ^R	This study
pSagEΔ <i>cat</i> -KO	pHY304 containing <i>sagE</i> Δ <i>cat</i> allele + flanking DNA, Em ^R , Cm ^R	This study
pSagFΔ <i>cat</i> -KO	pHY304 containing <i>sagF</i> Δ <i>cat</i> allele + flanking DNA, Em ^R , Cm ^R	This study
pSagGΔ <i>cat</i> -KO	pHY304 containing <i>sagG</i> Δ <i>cat</i> allele + flanking DNA, Em ^R , Cm ^R	This study
pVE <i>emm49</i> -KO	pVE6007Δ containing 900 bp of intragenic fragment of <i>emm49</i>	This study
pVE <i>slo</i> -KO	pVE6007Δ containing 1151 bp of intragenic fragment of <i>slo</i>	This study
Complementation and heterologous expression studies		
pDC125	<i>E. coli</i> /streptococcal promoterless shuttle vector, JS-3 replicon, Em ^R (<i>erm</i>)	Chaffin <i>et al.</i> (1998)
pDC123	<i>E. coli</i> /streptococcal shuttle expression vector, JS-3 replicon, Cm ^R (<i>cat</i>)	Chaffin and Rubens (1998)
pDCerm	pDC123 derivative with Em ^R (<i>erm</i> of Tn916ΔE) replacing <i>cat</i>	Jeng <i>et al.</i> (2003)
pAD <i>sagA</i>	pDC125 + NZ131 <i>sagA</i> gene + the native <i>sag</i> promoter	This study
pAD <i>sagB</i>	pDCerm + NZ131 <i>sagB</i> gene behind vector promotion	This study
pAD <i>sagC</i>	pDCerm + NZ131 <i>sagC</i> gene behind vector promotion	This study
pAD <i>sagD</i>	pDCerm + NZ131 <i>sagD</i> gene behind vector promotion	This study
pAD <i>sagE</i>	pDCerm + NZ131 <i>sagE</i> gene behind vector promotion	This study
pAD <i>sagF</i>	pDCerm + NZ131 <i>sagF</i> gene behind vector promotion	This study
pAD <i>sagG</i>	pDCerm + NZ131 <i>sagG</i> gene behind vector promotion	This study
pAD <i>sagA</i> .P21A	pAD <i>sagA</i> with site-directed mutation of proline 21 to alanine	This study
pAD <i>sagA</i> .G23A	pAD <i>sagA</i> with site-directed mutation of glycine 23 to alanine	This study
pAD <i>sagA</i> .C24A	pAD <i>sagA</i> with site-directed mutation of cysteine 24 to alanine	This study
pAD <i>sagA</i> .C27A	pAD <i>sagA</i> with site-directed mutation of cysteine 27 to alanine	This study
pAD <i>sagA</i> .K53A	pAD <i>sagA</i> with site-directed mutation of lysine 53 to alanine	This study
pSagLocus	pDC123 + 9440 bp PCR amplicon of entire nine-gene <i>sag</i> operon	Nizet <i>et al.</i> (2000)
pSagLocusΔE	pSagLocus with <i>SagE</i> deleted leaving <i>SagF</i> ribosome binding site intact	This study
pDL278	<i>E. coli</i> /streptococcal shuttle vector, pVA308-1 replicon, Spc ^R	LeBlanc <i>et al.</i> (1992)
pDL278- <i>sagE</i>	pDL278 + NZ131 <i>sagE</i> gene behind vector promotion	This study

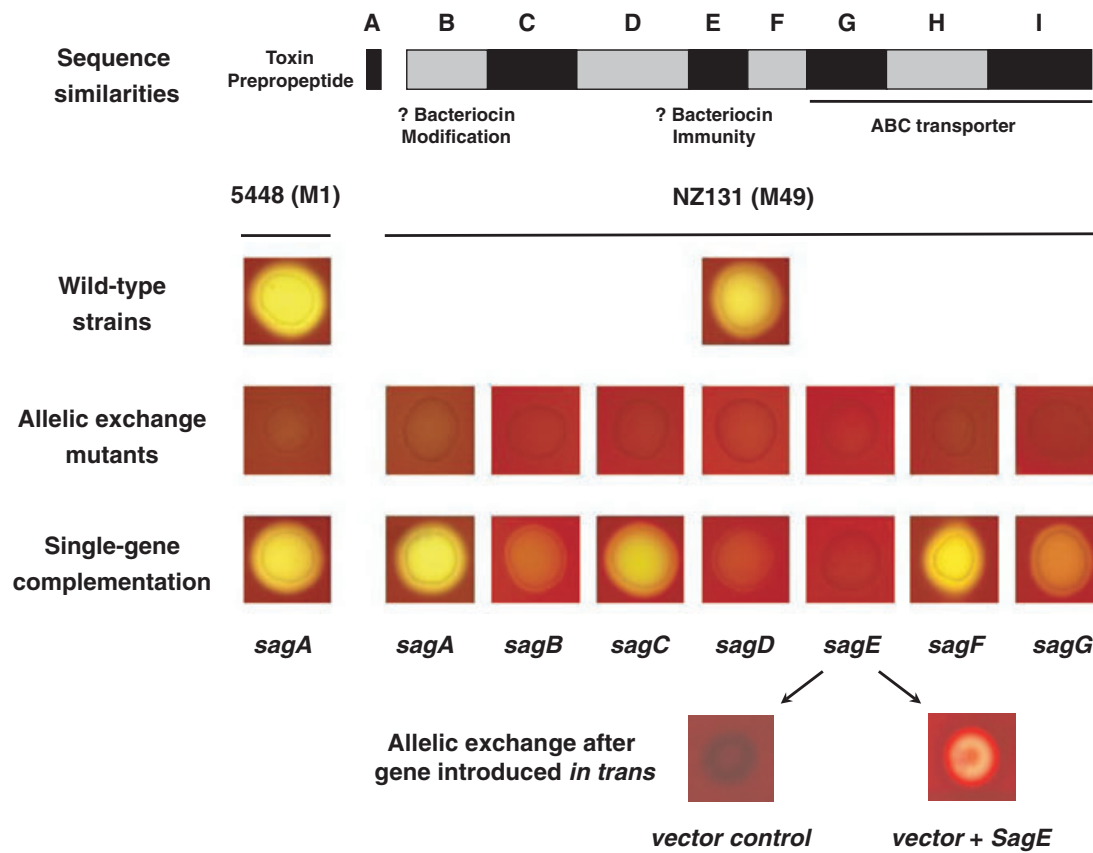


Fig. 1. Genetic analysis of the GAS *sag* operon for SLS biosynthesis. Precise in-frame allelic replacement of individual genes was achieved with a chloramphenicol acetyltransferase (*cat*) cassette. Single-gene complementation analysis *in trans* was performed under control of the native *sag* promoter for *sagA* and vector promoters for downstream genes. Allelic replacement of *sagE* in the GAS chromosome was performed alternatively before and after introduction of the complementation vector.

sagE alone *in trans* could not restore the SLS phenotype. To address this issue experimentally, we performed allelic exchange mutagenesis of *sagE* in the chromosome after pre-transforming GAS with a vector expressing *sagE*. Allelic replacement of *sagE* with *cat* yielded SLS-positive GAS when the chromosomal gene substitution was performed in bacteria harbouring a second copy of *sagE* *in trans* throughout (Fig. 1). These results strongly suggest an immunity function encoded by *sagE*. It remains unproven whether the *sagE* gene itself is also required for SLS biosynthesis. Also, it appears that *sagE*-mediated immunity is related only to the endogenous production of SLS and not exogenous exposure to SLS. Colonies of the *sagE* allelic exchange mutant could grow normally within a zone of β -haemolysis surrounding a wild-type colony, and equivalent growth of wild-type and *sagE* mutant GAS occurred in mixed liquid culture (data not shown).

Heterologous expression of individual *sag* genes

The complete *sag* operon cloned in an expression vector (pSagLocus) can confer SLS production to *Lactococcus*

lactis. When the complementation vectors produced in this study were used to transform *L. lactis*, no single gene from *sagA* to *sagG* was sufficient for SLS production (not shown). A construct harbouring a deletion of *sagE* alone from plasmid pSagLocus (pSagLocus Δ E) also failed to produce SLS when expressed in *L. lactis*, suggesting the role of SagE in immunity and/or production of SLS is also required for heterologous expression.

Site-directed mutagenesis of the *SagA* propeptide

Several structural features of the GAS *sag* operon suggest that SLS is related to the bacteriocin family of small peptide toxins (Nizet *et al.*, 2000; Ferretti *et al.*, 2001; Wescombe and Tagg, 2003). The structural gene *sagA* encodes a 53-amino-acid product (Fig. 2) containing a predicted glycine–glycine cleavage characteristic of non-lanthionine-containing bacteriocins of Gram-positive bacteria (Jack *et al.*, 1995) as well as certain lantibiotics such as salivaricin A (Ross *et al.*, 1993). Cleavage at this site would remove a 23-amino-acid leader peptide from a mature 30 SLS propeptide matching the predicted size

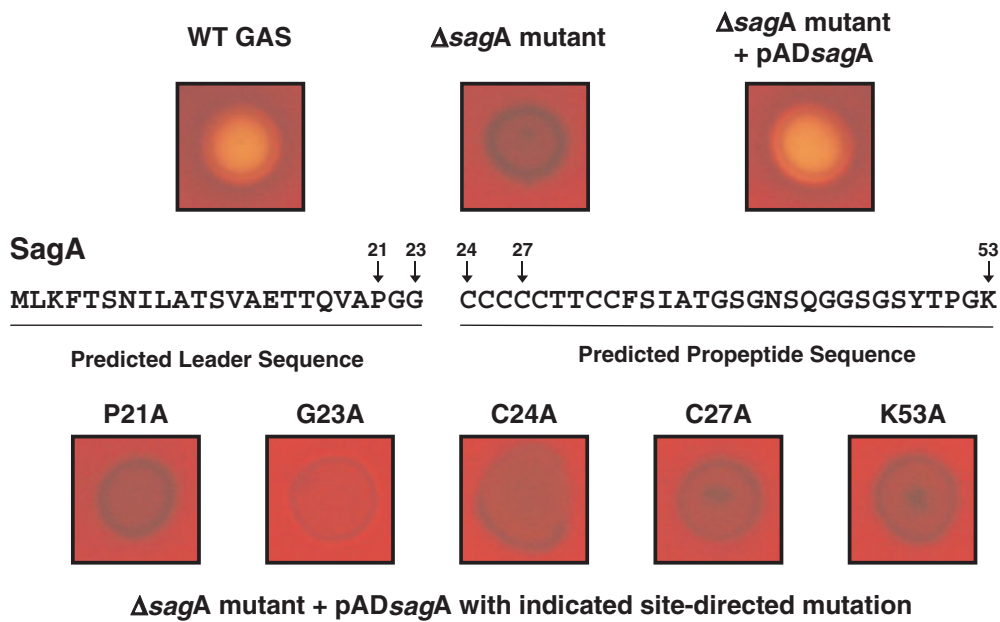


Fig. 2. Site-directed mutagenesis studies of the SagA gene product. Using the complementation vector bearing *sagA* under its native promoter, the codons for the specified amino acids were mutated into a codon for alanine. The respective plasmids were used to transform the GAS *sagA* allelic replacement mutant to screen for streptolysin S phenotype on blood agar.

of SLS (2.9 kDa) from earlier biochemical analyses (Koyama, 1963; Bernheimer, 1967). The SagA sequence also shares a proline at the -3 position from the cleavage site with the leader peptide of microcin B17 of *Escherichia coli* (Madison *et al.*, 1997). We found that mutation of this SagA proline (21) to alanine led to complete loss, and mutation of SagA glycine (23) to alanine led to a significant decrease in the haemolytic activity of GAS on SBA (Fig. 2). High cysteine content is a common feature of bacteriocin propeptides, and these residues can link to dehydrated serine and threonine residues (in lantibiotics), to dehydrated glycine residues (as in Microcin B17), or to one another (as in the cystibiotics pediocin PA-1 and leucocin A) (Jack *et al.*, 1995). We found changing either cysteine (24) or cysteine (27) to alanine led to complete abolishment of SLS activity (Fig. 2). Finally, the very C-terminal residue of several lantibiotic (e.g. nisin, subtilin) and non-lantibiotic (e.g. sakacin, lactacin) bacteriocin propeptides is lysine as in SagA. Mutation of lysine (53) to alanine eliminated SLS haemolytic activity (Fig. 2). Our limited analysis of SagA by site-directed mutagenesis of conserved residues supports its assignment as bacteriocin-like peptide toxin.

Elimination of sagA does not produce significant pleiotrophic effects

In standard Todd-Hewitt broth (THB) media, the seven individual M49 mutants in *sagA-sagG* genes grew equally well as parent strain NZ131, as did allelic exchange

mutant 5448:*sagAΔcat* compared with M1 parent strain 5488. An earlier study of a transposon mutant disrupting *sagA* associated the mutation with loss of SLS expression but also with decreased expression of the genes encoding M protein and cysteine protease SpeB (Li *et al.*, 1999). Another study using an insertion duplication mutation of *sagA* associated the mutation with loss of SLS expression and with decreased surface expression of the M protein (Biswas *et al.*, 2001). Our precise in-frame allelic exchange mutant of *sagA* in the M1 background is free of polar effects the downstream operon (as verified by single-gene complementation), and did not show differences in *emm* gene transcription nor M protein expression as determined by reverse transcription polymerase chain reaction (RT-PCR) (Table 2) and whole-cell dot blot analysis (Fig. 3A) respectively. Removal of *sagA* also did not affect transcription of the *speA* gene encoding the GAS pyrogenic exotoxin A (Table 2). The *ΔsagA* mutant had slightly increased levels (fourfold) of mRNA production for the *speB* gene (Table 2) and immunodetectable SpeB protein (Fig. 3A), but these differences did not translate into a significant increase in the amount of cysteine protease activity exhibited by the whole bacterium (Fig. 3B). Finally, procedures for genetic manipulation of GAS may inadvertently select mutants with decreased expression of the surface hyaluronic acid capsule, leading to potential misinterpretation of experimental results (Ashbaugh *et al.*, 1998). We found that *sagA* mutagenesis did not affect GAS expression of hyaluronic acid capsule (Fig. 3C). The lack of pleiotrophic effects on M protein and capsule are

Table 2. Real-time RT-PCR analysis of gene expression in wild-type and $\Delta sagA$ mutant GAS.

GAS strain	RecA		SpeA		M protein		SpeB	
	No. of molecules ($\times 10^6$)	No. of molecules ($\times 10^4$)	Normalized ratio	No. of molecules ($\times 10^6$)	Normalized ratio	No. of molecules ($\times 10^6$)	Normalized ratio	
Wild-type 5448 (M1)	11.1	5.76	0.0053	14.75	1.3	14.11	1.3	
SLS- mutant 5448:sagA Δ cat	12	6.13	0.0050	14.1	1.18	68.44	5.7	

especially important as each is known to contribute to GAS tissue necrosis and virulence in the murine model (Ashbaugh *et al.*, 1998).

SLS is important for the pathogenesis of M1 GAS necrotizing soft tissue infection

M1 strains are the most common isolates associated with the recent resurgence of NF and other invasive GAS infections. To determine the importance of SLS for disease progression, we injected mice subcutaneously with 10^7 colony-forming units (cfu) of wild-type strain M1 GAS 5448 or its isogenic SLS-negative mutant 5448:sagA Δ cat. Six of eight mice infected with the wild-type strain developed large necrotic ulcers beginning between 24 and 48 h, while only one of eight SLS-negative mutant ulcer, itself very small (Fig. 4A). Mice were sacrificed at 96 h with collection of lesion (or inoculation site) biopsies and blood for quantitative culture. Consistent with larger lesions ($P < 0.002$), the wild-type GAS strain was present in much higher concentrations in the skin (mean cfu g $^{-1}$ = 1×10^9) than the SLS-negative mutant (mean cfu g $^{-1}$ = 4×10^5) ($P < 0.001$) (Fig. 4B). Seven of eight mice infected with the wild-type strain were bacteremic at 96 h (mean cfu ml $^{-1}$ = 2×10^4) compared with three of eight mice infected with the SLS-negative mutant

(mean cfu ml $^{-1}$ = 1×10^2) ($P < 0.001$). Histopathologic studies were performed on excised skin and subcutaneous tissue with representative light micrographs shown in Fig. 5. Extensive necrosis of the skin and subcutaneous fatty tissues was seen in the six mice infected with wild-type M1 GAS that developed ulcers and thrombosis of bridging blood vessels, with abundant bacteria and dense neutrophilic infiltrates throughout the ulcer margin (Fig. 5B and C). In two of these mice, deeper involvement with myonecrosis was seen (Fig. 5D). In contrast, mice infected with the SLS-negative mutants had minimal inflammation without necrosis and mild perivascular neutrophilic infiltrates (Fig. 5E and F), findings that resembled the histopathology of mock-infected mice injected with sterile Cytodex beads alone (Fig. 5A). The *in vivo* studies demonstrate that SLS is an important virulence factor in M1 GAS skin and soft tissue infection, contributing to tissue injury and promoting systemic spread of the bacterium.

SLS does not contribute to epithelial cell invasion or cathelicidin resistance

It has been proposed that the increased virulence of M1 GAS strains is associated with a capacity for high frequency intracellular invasion of human epithelial cells (LaPenta *et al.*, 1994; Cleary *et al.*, 1998). We found that

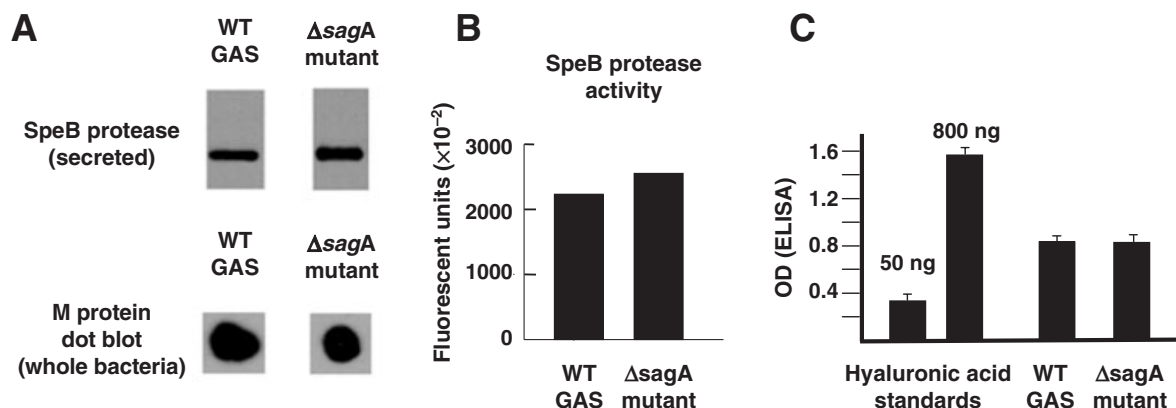


Fig. 3. Analysis of other virulence phenotypes in wild-type and *sagA* mutant GAS. A. Immunoblot analyses for cysteine proteinase SpeB and M protein. B. Assay for cysteine protease enzymatic activity. C. ELISA for hyaluronic acid capsule production.

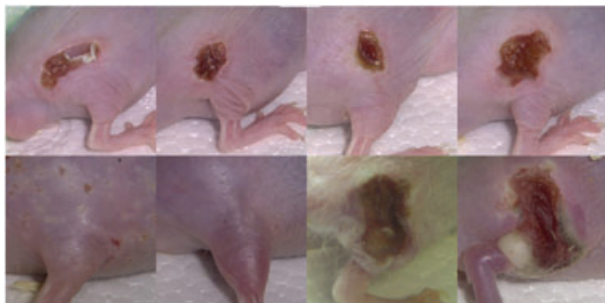
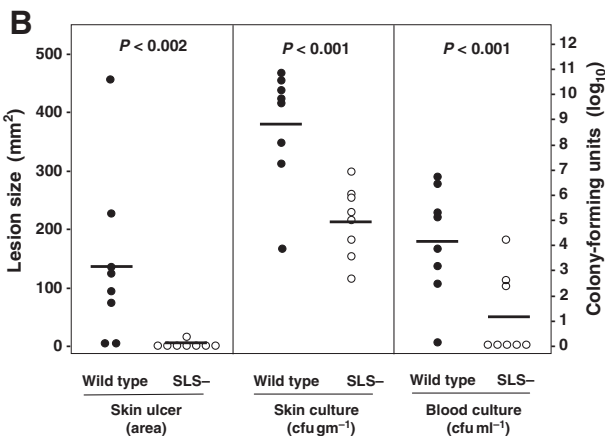
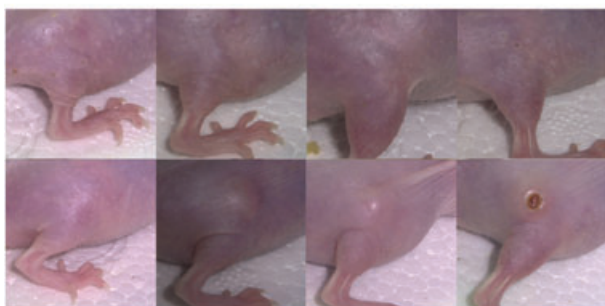
A Wild-type GAS M1 strain 5448**SLS-mutant 5448:sagAΔcat**

Fig. 4. Contribution of streptolysin S to M1T1 GAS necrotizing soft tissue infection. Four-week-old hairless *cr1:SKH1(hrhr)Br* mice were challenged subcutaneously with 10^7 colony-forming units of wild-type or *sagA* mutant GAS.

A. Gross appearance of mice at 96 h post inoculation.

B. Measured size of necrotic skin lesions and GAS colony-forming units recovered from skin and blood at 96 h post inoculation.

the *sagA* allelic exchange mutant was not significantly different from the wild-type parent strain in its ability to invade or adhere to monolayers of A549 human lung epithelial cells. Calculated values were $12 \pm 0.5\%$ (wild type) versus $12 \pm 0.4\%$ (Δ *sagA*) of the input inoculum for A549 cell adherence, $3 \pm 0.2\%$ versus $3 \pm 0.3\%$ of the input inoculum for A549 cell invasion. An important component of innate host defence against invasive GAS skin infection is keratinocyte and neutrophil production of

the cathelicidin class of cationic anti-microbial peptides (Dorschner *et al.*, 2001; Nizet *et al.*, 2001). We found no difference in the susceptibility of the M1 wild-type strain and its isogenic Δ *sagA* mutant to the murine cathelicidin mCRAMP [minimum inhibitory concentration (MIC) = $16 \mu\text{M}$ for both]. Likewise, no difference in cathelicidin susceptibility was observed when comparing M49 parent strain NZ131 to its isogenic Δ *sagA*, Δ *sagB*, Δ *sagC*, Δ *sagD*, Δ *sagE*, Δ *sagF* and Δ *sagG* mutants (not shown).

SLS promotes resistance to phagocytic killing

One mechanism by which SLS expression could contribute to bacterial proliferation *in vivo* is by impairing host phagocytic clearance mechanisms. Killing assays were performed on M1 and M49 wild-type and *sagA* allelic exchange mutant GAS using fresh human blood from several donors. While the wild-type strains proliferated in human blood over the 2 h assay period, the SLS-negative mutants were effectively cleared (Fig. 6A). Additional studies were performed with purified human neutrophils using M1 GAS opsonized in 10% autologous serum. Again, the wild-type GAS strain proliferated to a much greater extent than the SLS-negative mutant strain in the neutrophil bactericidal assay (Fig. 6B). These results indicate that SLS is an important component of GAS resistance to phagocytic clearance.

SLS contributes to direct host cell injury

Partially purified preparations of SLS have been reported to show potent cytolytic activity against a broad range of eukaryotic cell membranes. To determine the contribution of SLS to cytotoxicity in the context of GAS-epithelial cell interaction, we exposed the human keratinocyte cell line HaCat to wild-type and SLS mutant GAS and assessed cell death by trypan blue nuclear staining. Figure 7A shows that the SLS- mutant was associated with drastically reduced epithelial cell killing compared with wild-type (6% versus 72%), while restoration of SLS activity to the mutant with a *sagA* complementation vector restored the wild-type cytolytic phenotype. Representative Trypan blue-stained epithelial monolayers from these experiments are seen in Fig. 7B. We hypothesized that the M protein of GAS may facilitate close bacterial-epithelial cell contact that would enhance SLS-mediated cytotoxicity. Figure 7C shows experiments performed with A549 human lung epithelial monolayers exposed to wild-type GAS and isogenic single and double mutants in SLS and M protein in which cell injury was measured by lactate dehydrogenase (LDH) release. We found that both SLS and M protein contributed significantly to GAS cytotoxicity (~50% reduction in LDH release in single mutants), and that a further reduction (~70% reduction from wild type)

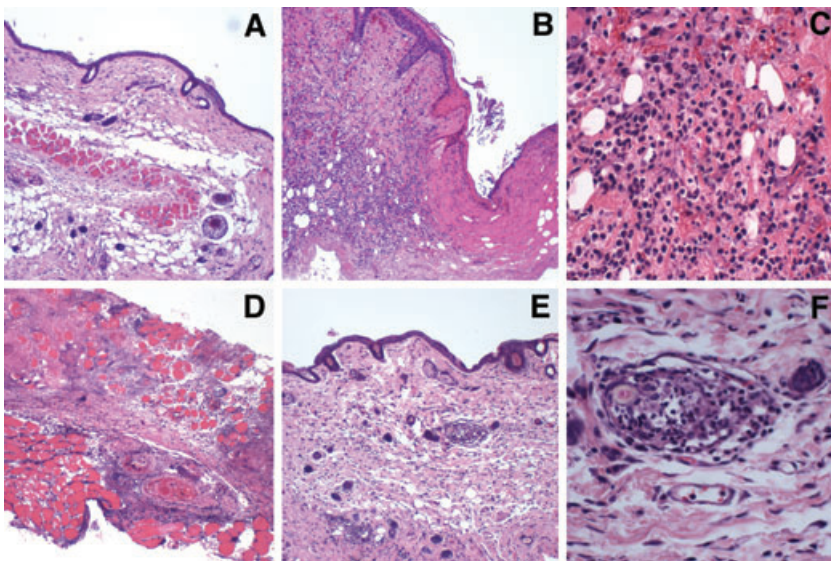


Fig. 5. Histopathology of skin and subcutaneous tissues at 96 h post challenge with M1T1 GAS.

A. Normal histopathology seen in a mock-infected animal.
 B and C. In animals challenged with wild-type GAS there is evidence of necrotic ulcer formation with thrombosis (B) and under higher power neutrophilic inflammatory infiltrates (C).
 D. Myonecrosis was seen in two animals.
 E, F. Mice challenged with the *saga* mutant did not develop skin necrosis and had only mild perivascular inflammatory cell infiltrates.

was seen in a mutant lacking both virulence determinants. In a parallel experiment using isogenic single and double mutants of SLS and the GAS pore-forming cytolysin SLO, we found that SLS was a more important contributor to GAS cytolytic action, and that a mutant lacking both toxins had a further reduction of cytolysis to very low levels (Fig. 7C). Note that these experiments were performed at atmospheric oxygen concentrations with 5% CO₂, and SLO is known to be oxygen labile.

Discussion

Although β -haemolysis is often applied synonymously to the GAS bacterium, the SLS toxin responsible for this signature phenotype remains enigmatic and incompletely understood. Earlier work has shown that the nine-gene GAS operon *sagA*–*sagI* is necessary and sufficient for SLS biosynthesis (Betschel *et al.*, 1998; Nizet *et al.*, 2000), but the use of transposon or plasmid integrational mutagenesis techniques with potential for polar mutations in these studies failed to provide definitive proof of the role of individual *sag* genes in toxin production. Here we use

precise, in-frame allelic replacement and corresponding single-gene complementation to demonstrate that the *sagA*, *sagB*, *sagC*, *sagD*, *sagF* and *sagG* genes are specifically required for SLS production, and to provide strong suggestive evidence that the *sagE* gene encodes an immunity function against SLS autotoxicity.

The *sag* operon has many features reminiscent of a biosynthetic apparatus for a bacteriocin-like small peptide toxin. By sequence homology, these features include a probable prepeptide structural gene (*sagA*) and ATP-binding cassette-type export apparatus (*sagG*–*I*), and with considerably less certainty, genes encoding a candidate bacteriocin modifying enzyme (*sagB*) and bacteriocin immunity protein (*sagE*) (Nizet *et al.*, 2000). Without significant GenBank homologies to genes of known function, *sagC* and *sagF* are predicted to be membrane proteins, and *sagD* a cytoplasmic protein, by the PSORT localization algorithm (Nakai and Horton, 1999). Our allelic replacement and single-gene complementation of *sagC*, *sagD* and *sagF* confirm the requirement of these individual genes for SLS production, but their precise functions in the toxin biosynthetic or export process

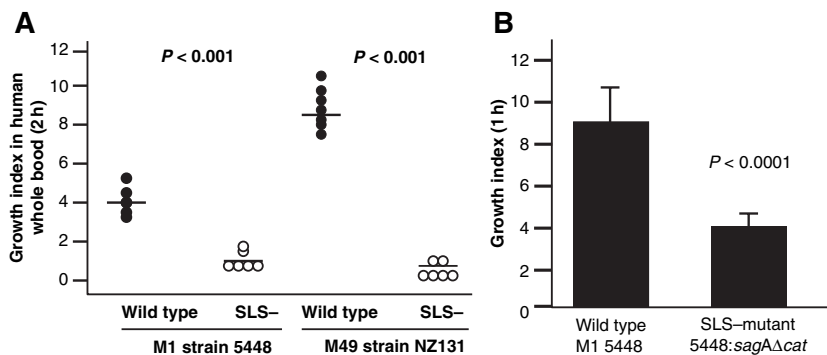


Fig. 6. Anti-phagocytic properties of streptolysin S.

A. Relative survival in human whole blood of wild-type and *sagA* mutant GAS, using eight different donors. Growth index = surviving cfu divided by cfu in the input inoculum.
 B. Similar comparison in an assay using purified human neutrophils and autologous serum.

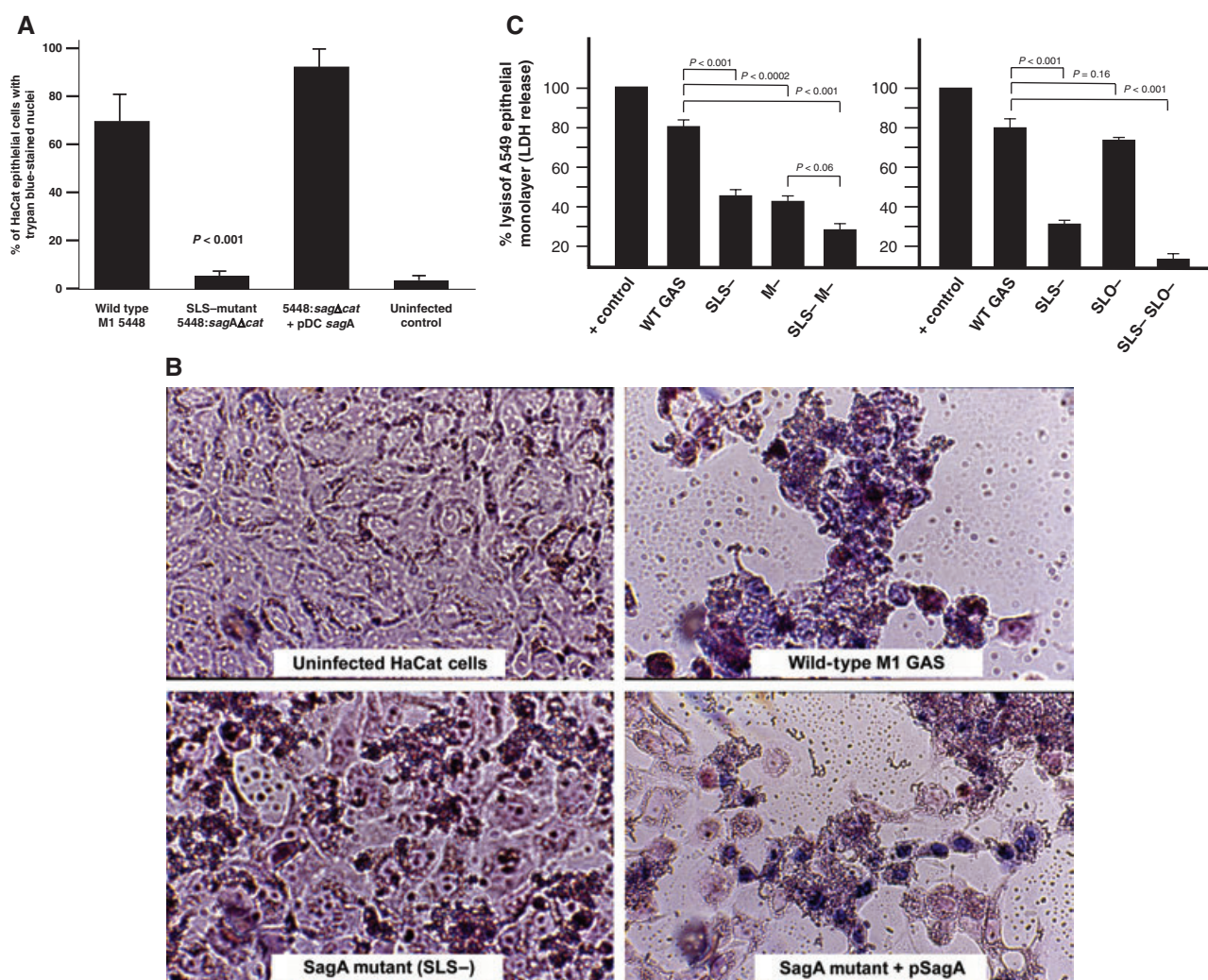


Fig. 7. Cytolytic activity of streptolysin S (SLS) against human cells. A and B. SLS-mediated injury to HaCat human keratinocytes measured by trypan blue nuclear staining. C. Contribution of SLS, M protein and streptolysin O to injury of A549 human lung epithelial cells quantified by lactate dehydrogenase release.

remain undefined. Preparations of stabilized SLS are lytic to bacterial protoplasts and spheroplasts (Bernheimer, 1966), but to date no definitive bacteriocidal activities against cell-wall competent bacteria have been defined in co-culture experiments comparing wild type and SLS-negative mutant GAS (Nizet *et al.*, 2000).

The identity of SagA as the prepeptide precursor of SLS was strongly suggested by the predicted small size of mature SLS (Koyama, 1963; Bernheimer, 1967; Alouf and Loridan, 1988) and confirmed through neutralization of SLS activity with antibodies directed against synthetic peptides representing SagA C-terminal sequences (Carr *et al.*, 2001; Dale *et al.*, 2002). Our allelic replacement and single-gene complementation of *sagA* corroborated its absolute necessity for SLS production, and provided a platform for testing the requirement of several amino acid residues characteristic of the bacteriocin toxin family by

plasmid-based site-directed mutagenesis. The predicted processing site of the SLS propeptide is preceded by the sequence Pro-Gly-Gly. Pro(21) and the Gly(23) are conserved with residues in the leader peptide required for the post-translational processing of the McbA prepeptide precursor of Microcin B17 (Madison *et al.*, 1997). We found that mutation of SagA Pro(21) to Ala eliminated SLS activity while mutation of Gly(23) to Ala led to significantly diminished, but not absent, β -haemolysis on blood agar. We hypothesize that the Gly to Ala substitution is sufficiently conservative to allow partial recognition by the SLS leader peptidase, and indeed Gly-Ala processing sequences are found in a small proportion of bacteriocin precursors including the lantibiotic SA-FF22 expressed by certain GAS strains (Jack *et al.*, 1994).

The unusual initial string of five Cys residues at the start of the predicted propeptide sequence (six in the *S. iniae*

SLS homologue) was targeted by replacing Cys(24) or Cys(27) with alanine, each time eliminating SLS activity. This result suggests that thioether bond formation characteristic of the post-translational modification of other bacteriocins may be an important step in the generation of mature SLS. Future biochemical analyses beyond the expertise of our laboratory will identify whether the target residues for cyclization are the abundant Thr (29, 30, 36, 50) or Ser (34, 39, 42, 46, 48) residues that could undergo dehydration to form thioether bonds seen in lantibiotics (Sahl and Bierbaum, 1998), or the many glycine residues (38, 40, 44, 45, 47, 52) that could potentially participate in formation of thiazole or oxazole rings reminiscent of Microcin B17 (Madison *et al.*, 1997). Our site-directed mutagenesis also indicates the Lys(53) residue is essential for SLS toxin processing or activity. An immediate C-terminal Lys is also present in the lantibiotic bacteriocin nisin, and chemical blockage of that Lys results in marked reduction of the toxin's pore-forming potential (van Kraaij *et al.*, 1998).

Allelic replacement and single-gene complementation prove the requirement of two additional genes for SLS production: the *sagB* gene encoding a candidate bacteriocin modifying enzyme and the *sagG* gene encoding a protein with the signature ATP binding pocket motifs of an ABC-type transporter. These results are consistent with the processing and export pathway of a bacteriocin-type toxin. The predicted protein product of the *sagE* gene shares weak homology with a candidate immunity protein PlnP of *Lactobacillus plantarum* (Diep *et al.*, 1996). Our discovery that allelic replacement of *sagE* and single-gene complementation to restore SLS activity occurs only if a copy of *sagE* is introduced *in trans* before completing the chromosomal deletion is consistent with its gene product serving an immunity function. Little is known about the precise mechanisms of bacteriocin immunity; however, a concept based on target shielding has been recently proposed for the bacteriocin immunity protein Pepl of *Staphylococcus epidermidis* in which a hydrophobic N-terminal domain (24 of 26 non-polar residues) and a hydrophilic and net-positively charged C-terminus (NSNKKDKL) are the essential components (Hoffmann *et al.*, 2004). Curiously, the N-terminus of GAS SagE has no charged amino acids among its first 28 residues, and its C-terminus also ends in a hydrophilic and net-positively charged sequence (TKKKKEVT).

In our experiments with the M1T1 GAS parent strain, we did not observe strong pleiotropic effects of *sagA* deletion on expression of M protein, cysteine protease SpeB, pyrogenic exotoxin A or hyaluronic acid capsule. These findings are consistent with earlier analysis of transposon insertion mutants in the *sag* promoter region of M1 and M18 strains (Betschel *et al.*, 1998) and a non-polar deletion mutant of *sagB* in an M5 GAS strain (Fon-

taine *et al.*, 2003). Separate investigations have attributed global regulatory functions to the *sagA* gene itself, applying the designation *pel*, for 'pleiotropic effects locus', to the open reading frame (ORF). A M49 serotype Tn917 '*pel*' mutant with an insertion in the region of the *sag* promoter was non-haemolytic and showed decreased transcription of the genes for M-protein, SpeB and streptokinase (Li *et al.*, 1999). Passage of this mutant in mice selected for restoration of *sagA* transcription and β -haemolysis but did not reverse all of the pleiotropic effects (Eberhard *et al.*, 2001). In contrast, an insertion duplication mutation of the *sagA* gene in an M6 GAS strain was shown to have normal *emm* transcription but to express a truncated version of the M-protein that would not anchor to the cell surface (Biswas *et al.*, 2001). A recent well-designed study provides compelling evidence that the untranslated RNA sequence comprising *sagA* can act as a growth phase-dependent positive regulator of the *emm* and *sic* genes at the transcriptional level and of SpeB at the post-translational level (Mangold *et al.*, 2004). Further complexity is presented by the fact that the expression level of the *sag* locus is under transcriptional control of the GAS global regulators *covR/covS* (also known as *csrR/csrS*), *rofA*, *mga* and *fas*. (Kihlberg *et al.*, 1995; Federle *et al.*, 1999; Heath *et al.*, 1999) In the present post-genomic era of GAS research, new studies to analyse the complete transcriptome of wild type and Δ *sagA* mutants under a variety of *in vitro* and *in vivo* conditions will be helpful in elucidating the intriguing regulatory roles of the locus.

We found SLS expression to be critical for the pathogenesis of M1T1 GAS infection in a murine model of necrotizing soft tissue infection, corroborating previous studies indicating a virulence role of SLS in lesion size or mortality in GAS, GGS and *S. iniae* skin infection (Betschel *et al.*, 1998; Fuller *et al.*, 2002; Humar *et al.*, 2002; Fontaine *et al.*, 2003; Engleberg *et al.*, 2004). We further found that SLS expression was strongly correlated to viable bacterial counts in the infected skin tissue and to the ability of the organism to rapidly disseminate and produce high-grade bacteraemia. Histopathologic examination at day 4 showed severe necrosis, widespread bacterial infiltration and abundant neutrophil infiltration in the ulcer tissue of most animals infected with wild-type M1 GAS. In contrast, minimal evidence of necrosis and tissue injury was seen in mice challenged with the SLS mutant. The necrotic tissue damage is likely to reflect a potent direct cytolytic effect of SLS on host cell types resident in the skin and subcutaneous tissues. This finding was confirmed in our *in vitro* assays in which SLS was seen to produce direct cytolytic injury to human keratinocytes. We found that SLS may act synergistically with the oxygen-labile GAS exotoxin SLO to effect host cell injury, and that SLS cytotoxicity was also enhanced by the presence of M

protein, whose adhesin properties could facilitate the close interaction between the bacteria and target cell membrane required for optimal SLS delivery (Ofek *et al.*, 1990; Ginsburg, 1999).

Compared with the SLS mutant, several log-fold greater quantities of viable wild-type GAS were present in the infected tissue biopsies despite the presence of markedly increased numbers of neutrophils recruited to the site of infection. This potential paradox suggested that SLS must contribute in some fashion to resistance against phagocytic clearance mechanisms. Indeed our studies confirmed this to be the case as the $\Delta sagA$ mutant did not survive as well as wild-type GAS in killing assays with human whole blood and purified human neutrophils. These results contrast with recent observations for whole blood survival of an SLS-deficient *sagB* mutant of M5 GAS (Fontaine *et al.*, 2003) or neutrophil resistance of an SLS-deficient M3 strain of GAS (Sierig *et al.*, 2003) in which negligible differences were seen in comparison to the parent strains. Neutralizing antibodies against the SagA propeptide sequence were found to enhance neutrophil opsonophagocytosis of an M24 strain of GAS (Dale *et al.*, 2002). Thus it is apparent that strain differences may exist in the relative contribution of SLS to phagocyte resistance, and that other well-studied phenotypic characteristics such as the M protein or hyaluronic acid capsule may play a more prominent role in certain backgrounds. In our animal studies, a role of SLS in phagocytic resistance may facilitate bacterial proliferation in the face of a brisk neutrophilic inflammatory response elicited in response to the toxin-mediated tissue injury. Consequently, both SLS cytotoxicity- and neutrophil-mediated inflammatory damage may contribute to the observed necrotic ulcer formation.

In sum, we have used a molecular genetic approach to study the unique requirement of individual *sag* locus genes to SLS production and the role it plays in the pathogenesis of GAS M1T1 necrotizing skin and soft tissue infection. We hope the insights and reagents we have generated can prove useful for future studies of the biology and biochemistry of this unusual bacteriocin-like toxin.

Experimental procedures

Bacterial culture and transformation conditions

GAS were grown in THB, on Todd–Hewitt agar plates (THA) or on plates of trypticase soy agar + 5% sheep red blood cells (SBA). For antibiotic selection, 2 $\mu\text{g ml}^{-1}$ erythromycin (Em), 1 $\mu\text{g ml}^{-1}$ chloramphenicol (Cm) or 500 $\mu\text{g ml}^{-1}$ spectinomycin (Spc) was added to the media. To prepare log-phase GAS for use in tissue culture assays or animal infections, bacteria were grown to $\text{OD}_{600} = 0.4 = 10^8 \text{ cfu ml}^{-1}$, pelleted, washed, resuspended and diluted in PBS or tissue culture media to the desired concentration. *E. coli* strains were grown in Luria–

Bertani broth or on Luria–Bertani agar plates; antibiotic selection utilized 100 μg of ampicillin (Amp) per ml, 500 μg of Em per ml, or 5 μg of Cm per ml. *L. lactis* was grown in M17 broth (Difco) supplemented with 1% glucose (GM17) or on GM17 agar plates with selection of Cm or Em at 5 $\mu\text{g ml}^{-1}$. GAS were rendered transformable by electroporation through growth in THB + 0.3% glycine (M49) or THB + 1.25% glycine + 0.5 M glucose + 5% sucrose (M1), then prepared as described for *Streptococcus agalactiae* (Framson *et al.*, 1997). *L. lactis* was made transformable by growth in GM17 plus 2.5% glycine (Holo and Nes, 1989). After electroporation (Eppendorf 2510, 1.5 kilovolts), cells were incubated in THB + 0.25 M sucrose (GAS) or GM17 media + 20 mM MgCl_2 + 2 mM CaCl_2 (*L. lactis*) for 1–2 h before antibiotic selection on agar media.

Precise in-frame allelic exchange mutagenesis

PCR was used to amplify NZ131 chromosomal DNA fragments containing *sagA*, *sagB*, *sagC*, *sagD*, *sagE*, *sagF* or *sagG* with several hundred base pairs upstream and downstream sequence in each case. The primer sequences employed have been published (Nizet *et al.*, 2000) and were paired as follows: *sagUpFwd* + *sagBRev* to amplify the *sagA* gene within a 1648 bp amplicon, *sagAFwd* + *sagCRev* to amplify the *sagB* gene within a 2250 bp amplicon, *sagBFwd* + *sagDRev* to amplify the *sagC* gene within a 2556 bp amplicon, *sagCFwd* + *sagERev* to amplify the *sagD* gene within a 2146 bp amplicon, *sagDFwd* + *sagFRev* to amplify the *sagE* gene within a 2068 bp amplicon, *sagEFwd* + *sagGRev* to amplify the *sagF* gene within a 1641 bp amplicon, and *sagFFwd* + *sagHRev* to amplify the *sagG* gene within a 1925 bp amplicon. Each PCR product was T-A cloned in pCR2.1 (Invitrogen). These vectors (pSagA-TV, pSagB-TV, etc.) served as templates for inverse PCR reactions using (i) a reverse primer immediately upstream of the start codon and (ii) a forward primer immediately after the stop codon of the cloned ORF(s); these primers were designed with 25 bp 5'-extensions corresponding to the start and end of the chloramphenicol acetyltransferase gene (*cat*) respectively. The resultant linearized PCR products, containing an in-frame deletion of the individual *sag* gene, were used to transform *E. coli* Top10 together with an ~650 bp PCR amplicon of the complete *cat* gene from pACYC184. *In vivo* recombination events were identified by screening for Top10 exhibiting Amp^R + Cm^R , and verified by PCR and restriction analysis to contain an in-frame substitution of the target gene(s) with *cat*. The mutated *sagA* Δ *cat*, *sagB* Δ *cat*, etc. and flanking DNA were subcloned as *Bam*HI/*Xba*I fragments to the temperature-sensitive Em^R vector pHY304 to produce knockout vectors pSagA Δ *cat*-KO, pSagB Δ *cat*-KO, etc. M49 GAS NZ131 was transformed with each of the seven knockout plasmids, and M1 GAS 5448 was transformed with pSagA Δ *cat*-KO. Single-recombination events were identified at 37°C under Em selection. Selection was relaxed by serial passage at 30°C without antibiotics, and double cross-over events were identified as NZ131 mutants exhibiting Cm^R but Em^S . Precise, in-frame allelic exchanges of *sagA*, *sagB*, *sagC*, *sagD*, *sagE*, *sagF* or *sagG* in the M49 chromosome and *sagA* in the M1 chromosome were confirmed by (i) PCR using *cat* primers with upstream and downstream

primers and (ii) absence of amplification of the wild-type gene.

Single-gene complementation analysis

To reintroduce *sagA* and its native promoter *in trans* to the M1 and M49 GAS allelic exchange mutants 5448:*sagA* Δ *cat* and NZ131:*sagA* Δ *cat*, the PCR amplicon from pSagA-TV was subcloned as a *Bam*H1 + *Xba*1 fragment into pDC125 (Chaffin *et al.*, 1998), a streptococcal/*E. coli* shuttle vector designed with transcriptional terminators to prevent promotion from plasmid backbone. The resultant complementation vector pAD*sagA* was introduced into 5448:*sagA* Δ *cat* and NZ131:*sagA* Δ *cat* by electroporation, transformants identified by growth on THA + Cm + Em, and plasmid integrity confirmed by restriction and PCR analysis. To reintroduce individual downstream genes of the *sag* operon *in trans* to their corresponding *cat* allelic exchange mutants, we used plasmid pDCerm (Jeng *et al.*, 2003), an Em^R streptococcal/*E. coli* shuttle vector that we derived from pDC123 (Chaffin and Rubens, 1998). This vector contains constitutive promoters on the plasmid backbone to allow expression of the cloned gene. The PCR amplicons of each downstream *sag* gene were recovered from vectors pSagB-TV, pSagC-TV, etc. by digestion with *Bam*H1 + *Xba*1 and subcloned to pDCerm prepared with the compatible restriction enzymes. The single-gene complementation vectors pAD*sagB*, pAD*sagC*, etc. were introduced to the corresponding GAS M49 allelic exchange mutants by electroporation, transformants identified by growth on THA + Cm + Em, and plasmid integrity confirmed by restriction and PCR analysis. Complemented mutants were screened for SLS activity by growth on SBA with appropriate antibiotic selection.

Allelic exchange mutagenesis of *sagE*

The *sagE* gene and flanking DNA from pSagE-TV was cloned into expression vector pDL278 (LeBlanc *et al.*, 1992) to yield pDL278-*sagE*. GAS NZ131 was transformed with pSagE Δ -*cat*-KO as above, temperature shift performed, and a non-haemolytic, downstream single-crossover integration within the *sagF* ORF confirmed by PCR and Cm^R + Em^R. This single cross-over mutant was rendered electrocompetent and transformed with either pDL278 or pDL278-*sagE* with selection of Spc^R colonies. While maintaining Spc selection, the temperature was relaxed to 30°C for several passages, then plated at 37°C for identification Cm^R but Em^S colonies. Precise allelic replacement of *SagE* with *cat* in the chromosome was confirmed by PCR.

Site-directed mutagenesis of *SagA*

PCR-based site-directed mutagenesis (ExSiteTM, Stratagene) was performed on selected amino acids within the *SagA*-encoding sequence within complementation vector pAD-*sagA*. Primers were designed to replace the targeted codon with a codon for alanine. *Dpn*I was used to remove template DNA before transformation and recovery of mutated plasmids in *E. coli* MC1061. The fidelity of the site-directed mutation was confirmed by direct sequence analysis, and the modified

plasmids were used to transform NZ131:*sagA* Δ *cat* for assessment of SLS phenotype.

Real-time RT-PCR analysis

RNA was extracted using the RNeasy kit (Qiagen) from mid-log-phase GAS lysed mechanically with a FastPrep instrument (Bio 101). The samples were treated with DNase and the quality of RNA was verified spectrophotometrically. The total RNA isolated from the bacterial isolates as well as an RNA standard for each gene of interest (*emm1*, *speA*, *speB*) were converted to cDNA using random hexamers. To confirm the absence of DNA template, conventional PCR was used with or without reverse transcriptase. Gene expression was quantified using an ABI PRISM 7900HT@ sequence detection system using gene-specific primers in PCR Master Mix (Applied Biosystems, Foster City, CA) containing SYBR Green. RNA level of the housekeeping gene *recA* was also quantified. The data from the real-time PCR reaction was generated at CT values (threshold cycle at which there was a significant increase in signal generated by any given set of PCR conditions), which were then converted to number of RNA molecules using the gene-specific standard curves. These values were divided by the calculated number of housekeeping gene RNA molecules to allow for normalization of intra- and interexperimental data.

Phenotypic analysis of *GAS* virulence factors

The amount of mature SpeB secreted by each isolate and migrating at 28 kDa was determined by Western immunoblots developed with anti-SpeB antibodies as described (Kansal *et al.*, 2000). The proteolytic activity of SpeB was detected using the EnzCheck protease assay kit (Molecular Probes) as described in detail in Kansal *et al.* (2000). M protein dot blot analysis on whole GAS cells was performed with antibodies to the N-terminal domain of M protein as described (Kansal *et al.*, 2000). ELISA for hyaluronic acid capsule was performed using the HA test kit (Corigenix) per manufacturer's instructions. Minimum inhibitory concentrations for murine cathelicidin mCRAMP against GAS strains were determined as described previously (Nizet *et al.*, 2001).

Murine model of *GAS* necrotizing skin infection

Experiments were performed using a well-established model (Betschel *et al.*, 1998; Nizet *et al.*, 2001; Humar *et al.*, 2002). Briefly, GAS were grown to log phase, pelleted, washed, resuspended in PBS, and mixed 1:1 with Cytodex beads (50 mg per 100 ml, Sigma). An inoculum of 10⁷ cfu GAS in 0.2 ml was then injected into the right flank of 4-week-old male hairless crl:SKH1(hrhr)Br mice (*n* = 8 per group). Animals were monitored daily for development of necrotic ulcers and weight loss. At 96 h, all animals were sacrificed, biopsies performed for histopathologic assessment (HandE staining), and skin, blood and spleen collected for quantitative culture.

Whole blood and neutrophil killing assays

For blood killing, the method of de Malmanche and Martin (1994) was employed. Briefly, GAS were grown to log phase and diluted in PBS to 10^2 cfu in 100 ml, then mixed with 300 ml of fresh heparinized (10 U ml^{-1}) human whole blood in siliconized tubes. The mixture was incubated at 37° for 2 h with orbital shaking, then plated on agar media for enumeration of surviving cfu. Growth index is defined as the number of surviving cfu divided by the input inoculum. Experiments were repeated a total of 10 times using six different donors. Human neutrophils were purified using the PolymorphPrep™ (Grenier Bio-One) per manufacturer's instructions, then used in a GAS bactericidal assay precisely as described by Kobayashi *et al.* (2003).

Mutagenesis of M protein and SLO genes

PCR was used to amplify internal fragments of the *emm49* gene (900 bp from codon 17 to 258) and *slo* gene (1151 bp from codon 78 to 468) from NZ131. These fragments were cloned into temperature-sensitive suicide vector pVE6007 Δ to yield targeting vectors pVE*emm49*-KO and pVE*slo*-KO respectively. These vectors were used to transform wild-type NZ131 and the *sagA* allelic exchange mutant NZ131.*sagA*::Erm (Nizet *et al.*, 2000) at the permissive temperature (30°C); Campbell-type plasmid integrational mutagenesis of *emm49* or *slo* was then achieved by shift to the non-permissive temperature (37°C) while maintaining Cm selection. PCR was used to confirm the targeted disruption of the genes yielding the single mutants NZ131:M-Pr.KO and NZ131:SLO.KO and the double mutants NZ131.*sagA*:M-Pr.KO (SLS + M protein) and NZ131.*sagA*:SLO.KO (SLS + SLO).

Cytotoxicity and cellular invasion assays

The human keratinocyte cell line HaCat (Boukamp *et al.*, 1988) was propagated in RPMI media (Gibco-BRL) supplemented with 10% fetal bovine serum and seeded in four-well chamber slides (Microtek). Newly confluent monolayers were exposed to log-phase GAS strains at multiplicity of infection (moi) of 1000:1 and incubated for 2 h at 37°C in 5% CO_2 . Excess bacteria were removed by gentle washing in PBS, monolayers stained with 0.04% trypan blue, then fixed with glutaraldehyde and counterstained with eosin for quantification of stained nuclei under light microscopy as described (Gibson *et al.*, 1999). The human lung epithelial cell line A549 (ATCC #CCL-185) was propagated in RPMI media supplemented with 10% FCS and seeded in 24-well tissue culture plates (Corning). For cytotoxicity assays, log-phase GAS were added at 10^7 cfu per well (moi = 100:1), spun at 200 g to place bacteria on the monolayer surface, and incubated for 2 h at 37°C in 5% CO_2 . LDH release was determined using a miniaturized version of the Sigma colorimetric assay (catalogue #500-C) as described (Nizet *et al.*, 1996). LDH assays were performed in quadruplicate and repeated three times. Assays for GAS intracellular invasion of A549 cells were performed as previously described for *S. agalactiae* (Doran *et al.*, 2002) using moi = 1:1 for 2 h.

Statistical analyses

Data sets were compared by Student's *t*-test using the Microsoft Excel statistical package; a *P*-value <0.05 was considered significant.

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