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Invasive M1T1 group A *Streptococcus* undergoes a phase-shift *in vivo* to prevent proteolytic degradation of multiple virulence factors by SpeB

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Summary

A globally disseminated strain of M1T1 group A *Streptococcus* (GAS) has been associated with severe infections in humans including necrotizing fasciitis and toxic shock syndrome. Recent clinicoepidemiologic data showed a striking inverse relationship between disease severity and the degree to which M1T1 GAS express the streptococcal cysteine protease, SpeB. Electrophoretic 2-D gel analysis of the secreted M1T1 proteome, coupled with MALDI-TOF mass spectroscopy, revealed that expression of active SpeB caused the degradation of the vast majority of secreted GAS proteins, including several known virulence factors. Injection of a SpeB$^+$/SpeA$^-$ M1T1 GAS strain into a murine subcutanous chamber model of infection selected for a stable phase-shift to a SpeB$^-$/SpeA$^+$ phenotype that expressed a full repertoire of secreted proteins and possessed enhanced lymphocyte-stimulating capacity. The proteome of the SpeB$^-$ *in vivo* phase-shift form closely matched the proteome of an isogenic *speB* gene deletion mutant of the original M1T1 isolate. The absence or the inactivation of SpeB allowed proteomic identification of proteins in this M1T1 clone that are not present in the previously sequenced M1 genome including SpeA and another bacteriophage-encoded novel streptodornase allele. Further proteomic analysis of the M1T1 SpeB$^+$ and SpeB$^-$ phase-shift forms in the presence of a cysteine protease inhibitor demonstrated differences in the expression of several proteins, including the *in vivo* upregulation of SpeA, which occurred independently of SpeB inactivation.

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Introduction

Group A Streptococci (GAS) are important human pathogens commonly associated with superficial infections such as pharyngitis and impetigo (Cunningham, 2000). However, during the last 20 years, there has been a marked resurgence of severe, invasive and potentially fatal GAS infections, including necrotizing fasciitis (NF) and streptococcal toxic shock syndrome (STSS) (Stevens, 1992; Davies *et al*., 1996; Low *et al*., 1997). Although studies have provided evidence that host factors play an important role in determining the outcome of invasive GAS infections (Norrby-Teglund *et al*., 1996, 2000; Basma *et al*., 1999; Kotb *et al*., 2002), it is unlikely that changes in host factors alone could account for this dramatic change in epidemiology. The rapid evolution of bacteria suggests a more likely scenario in which certain GAS strains have acquired a repertoire of virulence factor(s) that can, in the susceptible host, trigger severe and potentially fatal diseases.

GAS possess a large number of virulence factors. The bacteria produce several molecules that block phagocytosis, inhibit complement activation, degrade immunoglobulins and facilitate adherence to, and invasion of, human tissues (Cunningham, 2000; Norrby-Teglund and Kotb, 2000). Streptolysin O (SLO) and streptolysin S (SLS) are potent cytotoxins and lysins. Two cysteine proteases, SpeB (Streptococcal pyrogenic exotoxin B) and IdeS (Immunoglobulin G-degrading enzyme of *S. pyogenes*), are believed to play important roles in pathogenesis by degrading host proteins. SpeB has been shown to cleave many host proteins including cytokine precursors, certain cell receptors and immunoglobulins (Kapur *et al*., 1993; Musser, 1997; Ashbaugh and Wessels, 2001; Collin and Olsen, 2003; von Pawel-Rammingen and Björck, 2003). GAS also produce potent immune stimulatory molecules

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such as superantigens (SAgs), lipoteichoic acid and peptidoglycans (Cunningham, 2000; Kotb, 1995). GAS SAgs are instrumental to the pathogenesis of STSS, and include the streptococcal pyrogenic exotoxins (Spes), some of which are chromosomally encoded (e.g. SmeZ 1-24, SpeG and SpeF/MF), while others are phage-encoded (e.g. SpeA, SpeC, SpeL) (Kotb, 1995; Schlievert *et al*., 1995; Proft *et al*., 1999).

A globally disseminated clonal M1T1 GAS strain has persisted for over 20 years as the most prevalent isolate from both invasive and non-invasive GAS infections. This clone harbours the *speA*, *speB*, *speF*, *speG* and *smeZ* genes (Chatellier *et al*., 2000). In invasive M1T1 infections, a distinct inverse relationship exists between expression of the SpeB cysteine protease and disease severity (Chatellier *et al*., 2000; Kansal *et al*., 2000; Eriksson and Norgren, 2003); the majority of isolates recovered from severe cases showed little or no SpeB expression. SpeB can degrade the GAS surface M protein, increasing the susceptibility of the bacterium to phagocytosis (Raeder *et al*., 1998; Kansal *et al*. 2003). SpeB can also selectively degrade GAS SAgs, reducing the proliferative and T cell Vβ clonal responses elicited by these molecules (Kansal *et al*., 2003). It is possible that the degradation of GAS virulence components by SpeB may explain the inverse correlation between the level of SpeB expression and human disease severity.

To study the behaviour of GAS *in vivo*, we developed a mouse tissue-chamber infection model. In this model, the bacteria are confined to the tissue-sealed subcutaneous Teflon chamber, but secreted bacterial components (e.g. SAgs) can diffuse out to the systemic circulation and migrating host phagocytic cells can enter the chamber by diapedesis. An M1T1 strain introduced into this model demonstrated a steady change from a $SpeB^+/SpeA^-$ phenotype to a $SpeB^-/SpeA^+$ phenotype over time (Kazmi *et al*., 2001). Isolated colonies with the *in vivo*-derived $SpeB^-/SpeA^+$ phenotype remained stable upon repeated passage *in vitro*, suggesting that the bacteria underwent a stable ‘phenotypic phase-shift’ or a form of ‘phase-variation’. In the present study, we used proteomics and mass spectroscopy to understand the changes of GAS protein expression induced *in vivo*, and the significance of the SpeB protease downregulation on the entire M1T1 virulence repertoire. The expressed protein profile of the globally disseminated M1T1 strain was also compared with the published GAS genomes to gain insight into the spread and persistence of this disease-associated clone.

Results

Isolation of the $SpeB^+/SpeA^-$ and the $SpeB^-/SpeA^+$ phenotypes of the M1T1 Clone

We have previously shown that when clonal $SpeB^+/SpeA^-$ M1T1 GAS isolates were introduced into the mouse tissue chamber model, the bacterial population 7 days later consisted entirely of $SpeB^-/SpeA^+$ bacteria. Like the original $SpeB^+/SpeA^-$ parent phenotype, the $SpeB^-/SpeA^+$ phase-shift phenotype remained stable for at least 21 passages *in vitro* (Kazmi *et al*., 2001). Here we performed a temporal analysis of the kinetics of the phenotypic phase-shift in SpeA and SpeB expression. Figure 1A shows that GAS recovered from the chambers on day 5 post-inoculation were a mixture of a few (original) $SpeB^+/SpeA^-$ colonies and a majority of (phase-shifted) $SpeB^-/SpeA^+$ colonies. Single GAS colonies recovered on day 5 were screened by colony blot to identify a $SpeB^+/SpeA^-$ parent phenotype and another $SpeB^-/SpeA^+$ phase-shift phenotype (Fig. 1A–B). The $SpeB^-/SpeA^+$ isolate retained its phenotype when re-inoculated into mice while the re-inoculated day 5-isolated $SpeB^+/SpeA^-$ GAS subsequently underwent phase-shift to $SpeB^-/SpeA^+$ (Fig. 1C). These observations suggest that the shift to the $SpeB^-/SpeA^+$ phenotype is favourable to the survival of the bacteria *in vivo*.

Differences in immune stimulation activity of secreted proteins from the $SpeB^+$ and $SpeB^-$ phenotypes

Lymphocytes were stimulated with optimal dilutions of overnight supernatants from cultures of the $SpeB^+$ and $SpeB^-$ bacteria. The proliferative response was significantly lower in the cultures stimulated with $SpeB^+$ supernatants compared with those stimulated with the $SpeB^-$ supernatants (Fig. 2, inset table). Growing the $SpeB^+$ bacteria in the presence of the cysteine protease inhibitor, E-64, restored the mitogenic activity to a level similar to what was seen with the $SpeB^-$ supernatants. Similarly, a marked difference in the levels of inflammatory cytokine responses was noted when we knocked out the *speB* gene by allelic exchange mutagenesis. In cultures stimulated with isogenic *speB*-deletion mutant (Δ*speB*) supernatants, the levels of IFNγ, IL2 and TNFα were higher. When active recombinant SpeB (rSpeB) was included in growth cultures of the Δ*speB* mutant, the cytokine response was reduced to a level similar to that seen in lymphocyte cultures stimulated with the parent $SpeB^+$ bacteria (Fig. 2, diagram).

Two-dimensional gel electrophoretic analysis of the secreted proteomes of the parent $SpeB^+/SpeA^-$ strain and the in vivo *$SpeB^-/SpeA^+$ phase-shift isolates*

Two-dimensional gel analysis followed by MALDI-TOF MS revealed that the major protein expressed by the parent M1T1 $SpeB^+/SpeA^-$ isolate was the enzymatically active 28 kDa form of SpeB (see below). Other than breakdown products of SpeB, and a few fragments of undefined proteins, we could not visualize other higher molecular weight

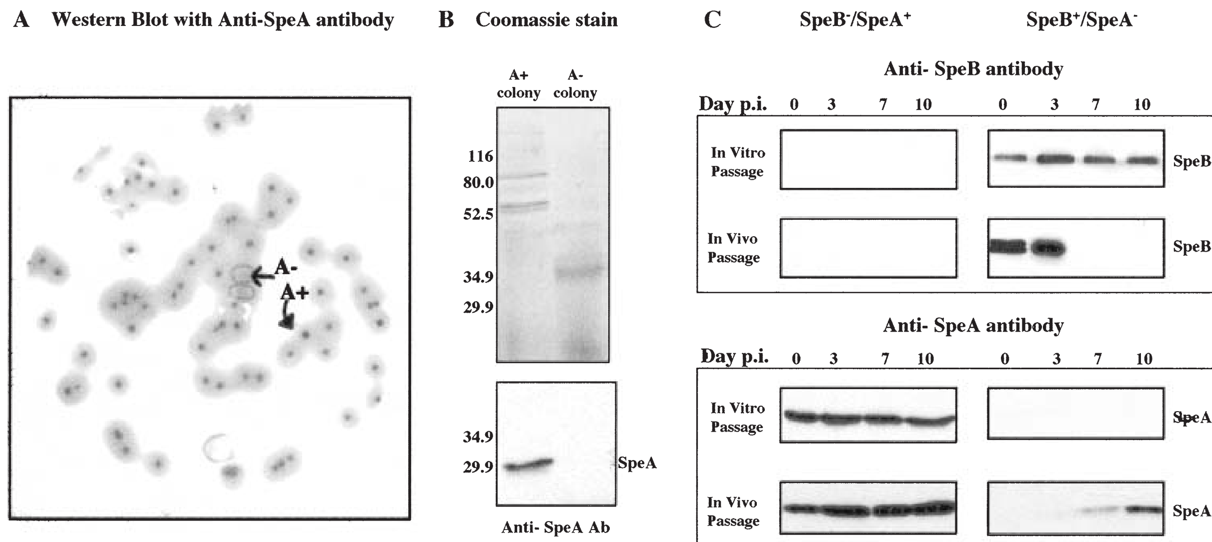

Fig. 1. Isolating isogenic variants having different SpeB/SpeA phenotypes.
A. Colony lift Western blot showing SpeA expression levels by colonies that were sampled on day 5 post-inoculation and spread on a blood agar plate. SpeA$^+$ and SpeA$^-$ colonies were isolated.
B. Their culture supernatants were analysed by SDS–PAGE and the gel was stained with Coomassie blue (upper panel) to show the differences in the proteins secreted by phenotypes. An immunoblot was performed on a duplicate gel (lower panel) confirming the variability of SpeA expression between the two isogenic colonies.
C. The stability of the parent SpeB$^+$/SpeA$^-$ and the *in vivo*-selected SpeB$^-$/SpeA$^+$ phenotypes was analysed by Western blots on different days following both *in vitro* and *in vivo* passage. Both phenotypes were stable after multiple *in vitro* passages, but *in vivo* the parent phenotype shifted to the *in vivo-stable* SpeB$^-$/SpeA$^+$ phenotype.

proteins in the supernatant of the parent SpeB$^+$/SpeA$^-$ strain (Fig. 3A). In contrast, identical analysis of the secreted proteome of the SpeB$^-$/SpeA$^+$ *in vivo* phase-shift isolate revealed many high molecular weight secreted streptococcal proteins (Fig. 3C). We hypothesized that the paucity of secreted proteins in the parent isolate was attributable to the potent proteolytic activity of the SpeB cysteine protease. To test this hypothesis, we performed similar analysis of the secreted proteomes of the parent SpeB$^+$/SpeA$^-$ strain grown in the presence of cysteine protease inhibitor E-64 (Fig. 3B) and an isogenic SpeB knockout mutant of the parent strain (Fig. 3D). In both cases, a large number of high-molecular weight secreted GAS proteins were now identified. Because the pattern of the secreted proteome was nearly indistinguishable upon chemical (Fig. 3B) or genetic (Fig. 3D) inactivation of SpeB, we conclude that SpeB is capable of degrading essentially all abundant proteins in the secreted GAS M1T1 proteome. The secreted proteome of the *in vivo*-selected SpeB$^-$/SpeA$^+$ phase-shift form (Fig. 3C) shared many spots with the SpeB-inhibited (Fig. 3B) parent strain or *speB* knockout mutant (Fig. 3D), but also showed some obvious differences. These observations suggested that (i) *in vivo* downregulation of SpeB frees many secreted proteins from proteolytic degradation, and (ii) certain secreted proteins are upregulated *in vivo* independently of SpeB suppression.

MALDI-TOF mass spectrometric analysis of differences in the SpeB$^+$/SpeA$^-$ parent and the SpeB$^-$/SpeA$^+$ in vivo *phase-shift strains*

Differences in the secreted proteomes of the SpeB$^+$/SpeA$^-$ parent and the SpeB$^-$/SpeA$^+$ phase-shifted form proteomes were analysed by MALDI-TOF MS (*Supplementary material*, Table S1 and Fig. 3A–D). Protein spots of interest were excised from the 2D gels and digested with trypsin for analysis. The masses of the experimental digests were compared with the theoretical tryptic digests of proteins found in databases using PeptIdent (SWISSPROT) and MSFIT (UCSF). In this fashion, we determined the identity of many of the visible protein spots on the 2D gels.

Of the more than 150 spots analysed, we could readily determine the identity of 28 proteins whose expression was consistent in three separate experiments, where each sample was run on triplicate gels. The majority of these proteins were targets of SpeB degradation based on the following criteria: (i) absent in the proteome of the parent SpeB$^+$ isolate, (ii) present in the SpeB$^-$ *in vivo* phase-shift form, (iii) present in the proteome of parent isolate grown in the presence of E-64, and (iv) present in the proteome of the SpeB knockout mutant of the parent isolate. These proteins included 15 known GAS virulence factors implicated in bacterial

Supernatant	SpeB$^+$/SpeA$^-$	SpeB$^-$/SpeA$^+$	SpeB$^+$/SpeA$^-$ +E-64
Dilution	**PBMCs proliferation (mean±S.E.) x10^{-3} cpm**		
1:1000	26.6 ± 0.4[a]	49.7 ± 0.2[a]	42.9 ± 3.5
1:2000	15.9 ± 2.5[b]	40.3 ± 2.1[b]	36.9 ± 1.5
1:4000	12.8 ± 1.4	33.2 ± 2.8	31.1 ± 2.5

[a]p value= 8.35×10^{-6} (n=3, student t test). [b]p value = 0.001 (n=3, student t test).

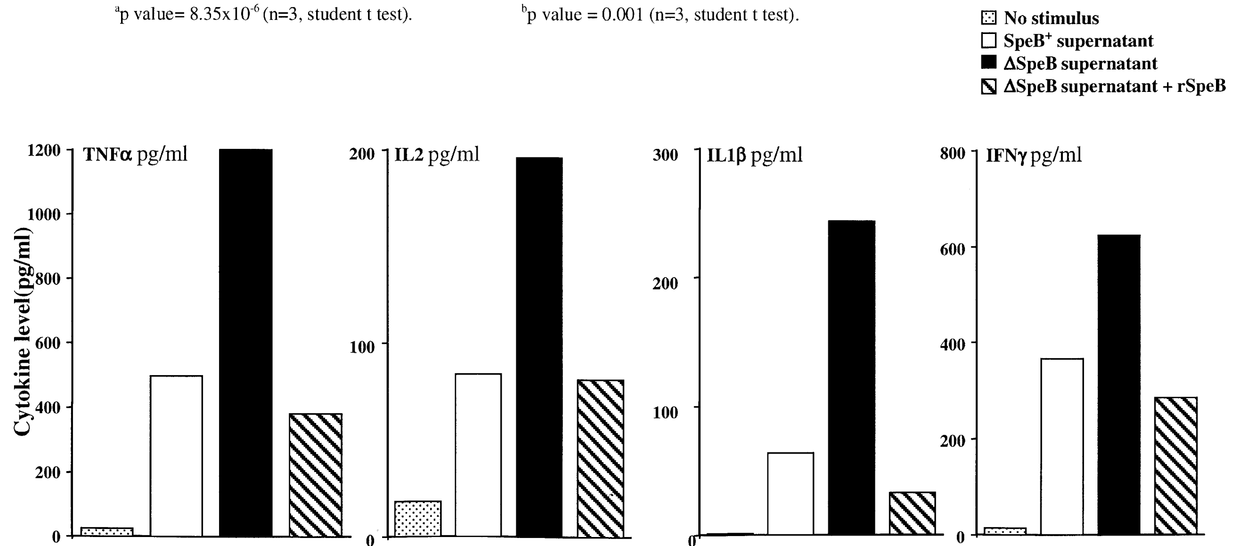

Fig. 2. Immune stimulation activity of secreted proteins from the SpeB$^+$, SpeB$^-$ and ΔspeB bacteria. The table provides data on the lymphocyte proliferative activity of SpeB$^+$ and SpeB$^-$ supernatants. The proliferative response of human peripheral blood mononuclear cells (PBMCs) (2×10^6 cells ml^{-1}) stimulated with the indicated dilutions of partially purified overnight culture supernatants from the SpeB$^+$ or the SpeB$^-$ bacteria for 72 h, was measured as described in *Experimental procedures*. The third column shows the effect of adding the cysteine protease inhibitor E-64 to the SpeB$^+$ cultures. The diagram illustrates the cytokine production by human PBMCs (2×10^6 cells ml^{-1}) stimulated for 72 h with either THY medium (dotted columns) or with 1:500 dilution of the overnight culture supernatants from the SpeB$^+$ (open columns) or the ΔspeB bacteria (solid columns). The addition of recombinant SpeB protein (rSpeB) at 200 μg ml^{-1} to the ΔspeB supernatants (hatched columns) reduced cytokine production to a level that was similar to that seen with the SpeB$^+$ supernatants.

invasion or avoidance of immune clearance, as well as several toxins and superantigens (Table 1). Prominent among these were the extensively studied and proven virulence factors M1 protein, streptolysin O and streptokinase. In addition, we were able to identify seven glycolytic enzymes, two of which are themselves involved in bacterial virulence. Three cytosolic 'housekeeping' proteins and four proteins sharing significant homology to known enzymes found in other organisms were also identified. In this study, endoglycosidase (EndoS) and streptodornase D-like protein (SdaD) were identified for the first time in the M1T1 GAS proteome (Tables 1 and S1). Only one protein (Spy0136) with no known function or structural homology was identified. Other protein spots could not be identified because the recovered material was below the sensitivity limit of detection by MALDI-TOF MS. These results indicate that SpeB can degrade several GAS virulence factors, and that expression of these virulence proteins may become more abundant after *in vivo* selection for the M1T1 phase-shift to a SpeB$^-$ phenotype.

Differentially expressed proteins by the SpeB$^+$ and the SpeB$^-$ phenotypic forms

We hypothesized that the expression of certain GAS proteins would be upregulated *in vivo* independent of SpeB degradation. To test this hypothesis, we analysed differences in the secreted proteome of the SpeB$^+$ parent strain and the SpeB$^-$ *in vivo*-selected phase-shift form, both grown in the presence of the cysteine protease inhibitor E-64. The majority of the proteins firmly identified by 2D GE/MS analysis were present in both the SpeB$^+$ and the SpeB$^-$ secreted proteomes in comparable amounts with the exception of several proteins listed in Table 2, whose expression levels were consistently different between the two phenotypic forms in at least three replicate experiments.

Most strikingly, the active and zymogen forms of SpeB

Table 1. Functional classification of the identified proteins.[a]

Category	Proteins
Known virulence proteins	
Toxins	Streptolysin O (SLO), CAMP factor, NAD-Glycohydrolase (NADGH)
Pyrogenic exotoxins	SpeA, SpeB (cysteine protease), SpeF (MF or Spd)
Other invasive proteins	Streptokinase (Ska), Streptodornase D-like protein (Sda)
Immune evasion proteins	M1 Protein, EndoS (secreted endoglycosidase), SIB (secreted Ig-binding protein), SIC (secreted inhibitor of complement), ISP2 (Immunogenic-secreted protein ISP homologue)
Plasmin(ogen)-binding glycoytic enzymes	Enolase [D1], GAPDH
Other glycolytic enzymes	Fructose-bisphosphate aldolase [A4], Triosephosphate isomerase (TIM) [A5], Phosphoglycerate mutase [A70], Phosphoglycerate kinase [D5], Pyruvate kinase [D6]
Other putative enzymes	Peptidoglycan hydrolase (Lysozyme) [A6], Cyclomaltodextrin glucanotransferase (amylase) [Amy], Putative DNase (MF3) [A3], Putative manganese-dependent inorganic pyrophosphatase [A33]
Housekeeping proteins	Elongation factor EF-tu [D2], Elongation factor EF-G [D4], dnaK (HSP 70) [D3]

a. Spot numbers are shown between square brackets when different from standard protein abbreviations.

were absent or greatly reduced, whereas the expression of SpeA was considerably higher in the *in vivo*-selected phase-shift form (Fig. 3B and C). The downregulation of SpeB and the upregulation of SpeA were confirmed to occur at the transcriptional level using real-time RT-PCR (Fig. 4). Analysis of the secreted proteome of the Δ*speB* isogenic mutant before and 10 days after *in vivo* inoculation in the mouse tissue chamber showed similar increases in SpeA expression (data not shown), further indicating that upregulation of SpeA *in vivo* is independent of SpeB expression. These data were also confirmed by Western blot analyses of 2D-GE of the secreted proteomes of the E-64-treated SpeB$^+$ parent strain and the SpeB$^-$ *in vivo* phase-shift form (Fig. 5). EndoS, a well-characterized protein known to degrade IgG (Collin and Olsen, 2001), was also slightly increased in the supernatant of the *in vivo*-selected SpeB$^-$ form (Fig. 3C). Another protein, SIC, showed considerable intra-experimental variation consistent with its known high mutagenic rate (Matsumoto *et al.*, 2003). However, the isoelectric point (pI) of SIC was consistently higher in the SpeB$^-$ *in vivo*-selected phase-shift form.

Table 2. Proteins that are consistently differentially expressed by the original and *in vivo* phase-shift forms of M1T1 GAS.[a,b]

Proteins relatively up-regulated in the SpeB$^+$/SpeA$^-$ form (or the DspeB/SpeA$^-$)	Proteins relatively upregulated in the SpeB$^-$/SpeA$^+$ form
SpeB[c]	SpeA[c]
GAPDH	Spy0136 (C25)[c]
CAMP factor	EndoS
Cyclomaltodextrin glucanotransferase (Amylase)	SLO
	NADGH

a. Proteins listed are those whose expression levels were consistently different between the two phenotypic forms in at least three replicate experiments.
b. Protein accession numbers are provided in Table S1 (see *Supplementary material*).
c. Differential expression was confirmed by real-time RT-PCR to occur at the transcriptional level.

Finally, a protein spot with a molecular weight of ~25 kDa and pI of ~7.3, which we have designated C25, was significantly and reproducibly upregulated in the *in vivo*-selected SpeB$^-$ form (Fig. 6); changes that were confirmed to occur at the mRNA level by real-time RT-PCR (Fig. 4). This protein matched the Spy0136 open reading frame (ORF) coding for a hypothetical protein reported in the genomic databases of M1GAS SF370 with 12 tryptic peptide identity. BLAST homology searches showed no shared sequences with any other known or unannotated protein in all public databases. Expression and characterization of this protein is currently ongoing in our laboratory.

Discussion

An M1T1 strain of GAS has disseminated globally and is recognized as the most frequent cause of both invasive and non-invasive human infections. In earlier studies, we have proven the clonality of this strain by showing identical pulsed-field gel electrophoretic (PFGE) patterns and identical sequence of several virulence genes (Chatellier *et al.*, 2000). Our epidemiological studies showed that the SpeB$^-$ phenotype of the M1T1 clonal strain was more frequently isolated from the more severe invasive GAS infection cases. Also, using our mouse chamber model of infection, we showed that the SpeB$^+$ bacteria shift to the SpeB$^-$ phenotype *in vivo* (Kazmi *et al.*, 2001). Our working hypothesis has been that the absence of active SpeB preserves the integrity of important GAS virulence proteins required for bacterial survival *in vivo*. Rasmussen and Björck (2002) suggested that GAS might regulate the production and proteolytic activity of this protein depending on the site and stage of infection. Our studies support the notion that it may be advantageous to the bacteria to downregulate its major secreted protease in order to preserve the integrity of several other proteinaceous virulence components. We and others have shown that SpeB degrades the M protein that confers resistance to phago-

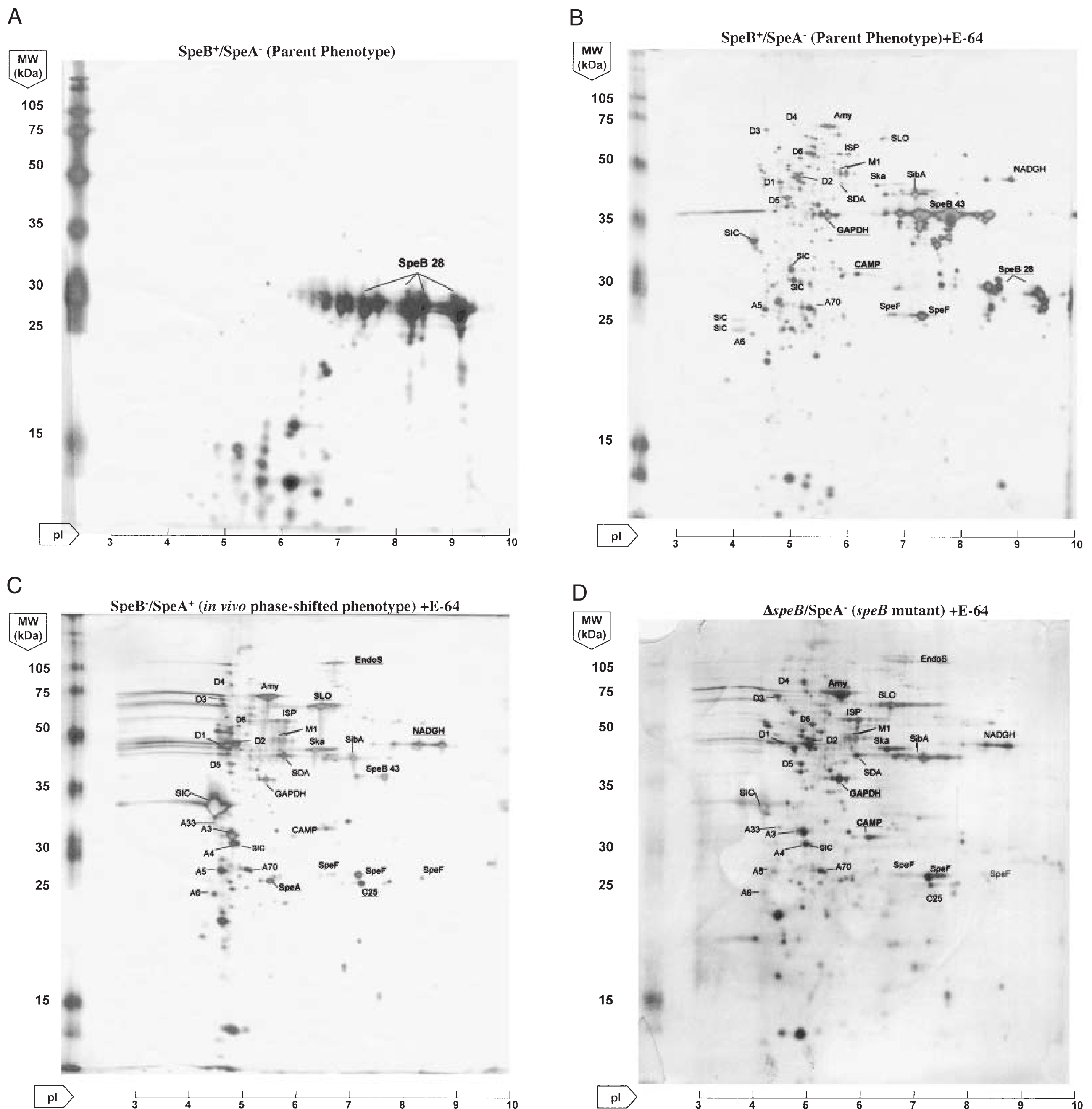

Fig. 3. Proteomic analysis of M1T1 GAS culture supernatant. The supernatants of GAS M1T1 isogenic bacteria were analysed by 2D-gel electrophoresis followed by MALDI-TOF MS to identify spots of interest. (A–D) show silver-stained 2D-gels from different bacteria, grown in presence (B–D) or absence (A) of the cysteine protease inhibitor E-64. The proteins that were identified are marked either by abbreviations or by spot numbers (see Tables 1 and S1). Molecular weights are shown in kDa on the vertical axis and approximate isoelectric point (pI) values are shown on the horizontal axis of each gel. Gels displayed are representative from duplicate gels of at least three different experiments. Differentially expressed proteins are underlined.

cytosis (Raeder *et al.*, 1998; Kansal *et al.*, 2003). This protease also selectively degrades Strep SAgs, which elicit deleterious immune responses in the host and are primarily responsible for the hypotension and multiple organ failure associated with STSS (Kotb, 1998; Kansal *et al.*, 2003). In this study, we showed whole-scale degradation of secreted proteins in the SpeB$^+$ parent form, and this degradation was associated with a significant decrease in the lymphocyte stimulating capacity of the bacterial supernatant. Recent studies by Kansal *et al.* show that SpeB expression also reduces systemic inflammatory responses *in vivo* (pers. comm.).

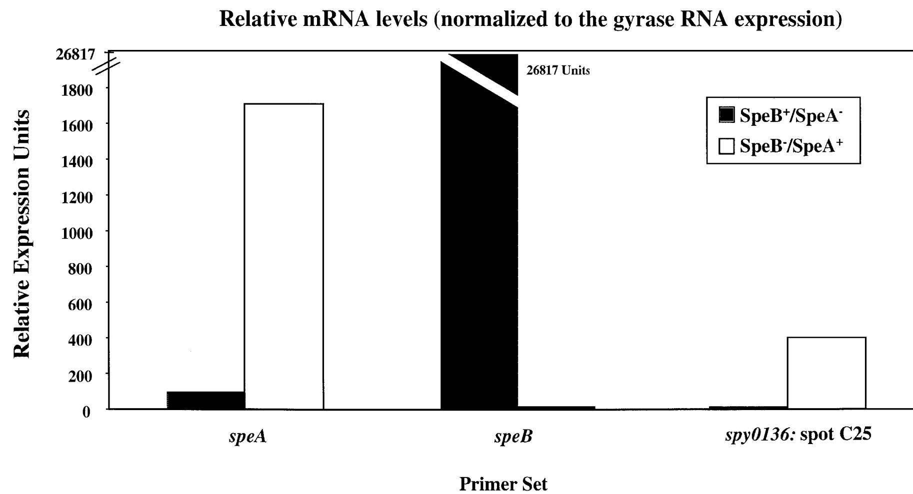

Fig. 4. Relative expression levels of SpeA, SpeB and Spy0136 mRNA obtained from both phenotypic forms. RNA was extracted from the two phenotypic forms of GAS M1T1 strain. RNA was quantified using real-time fluorogenic RT-PCR. RNA amounts were first normalized to gyrase (to correct for variability in total RNA) then relative amounts were determined for expression levels of *speA, speB and spy0136* genes. SpeA and Spy0136 are upregulated in $SpeB^-/SpeA^+$ form, while SpeB is downregulated. mRNA levels correspond to the proteomic analysis results (see Figs 5,6).

The fact that SpeB has a strong proteolytic activity deterred many researchers from comparing the proteomes of SpeB-producing strains. Thongboonkerd *et al.* (2002) were studying the effects of fluoride on the expression of GAS proteins and compared GAS supernatants in presence or absence of NaF. They attributed the absence of certain proteins in NaF-treated bacterial supernatants to the effect of this agent on inducing the genes that encode these proteins. However, a careful inspection of these data suggests that the difference could be related

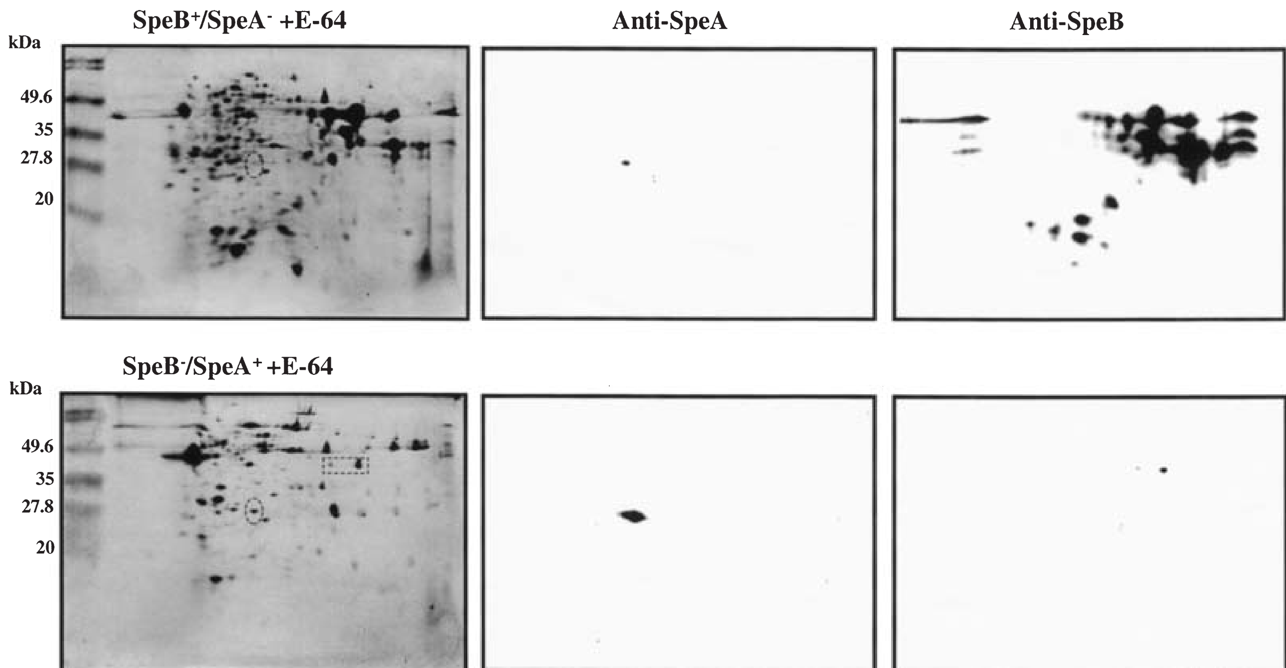

Fig. 5. Two-dimensional Western blots probed with anti-SpeA and anti-SpeB antibodies. Differential expression of SpeA and SpeB between the two phenotypic forms of the GAS M1T1 strain was confirmed by electro-transferring 2D gels to nitrocellulose membranes and probing these membranes using monoclonal anti-SpeB and polyclonal anti-SpeA antibodies. SpeB was expressed abundantly by the $SpeB^+/SpeA^-$ bacteria in different forms (28–43 kDa as well as smaller degradation products). On the other hand, the *in vivo*-selected bacteria ($SpeB^-/SpeA^+$) show significantly higher amounts of SpeA but only trace amounts of SpeB.

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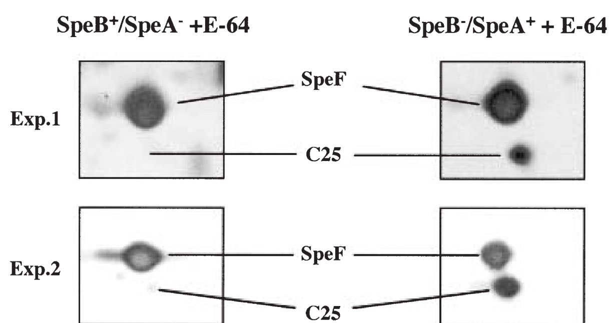

Fig. 6. Spot C25 (Protein Spy0136) is differentially expressed. Two areas of two different sets of gels showing the different levels of expression of spot C25 (see Fig. 3) between the two phenotypic forms of the GAS M1T1 strain.

to differences in the amount of SpeB expressed in NaF-treated and untreated cultures. Lei *et al.* (2000) have attempted to address the problem associated with SpeB-mediated degradation of GAS proteins by analysing the proteomes of Δ*speB* mutants in the M1 and M3 background and were able to visualize other secreted proteins. We performed paired proteomic analysis using a Δ*speB* deletion mutant as well as the parent SpeB$^+$ strain in the presence of cysteine protease inhibitor E-64. The use of E-64 efficiently restored the protein-expression profile to resemble very closely the profile of the Δ*speB* secreted proteome. By comparing the original SpeB$^+$ proteome in the presence of E-64 to the proteome of the SpeB$^–$ *in vivo*-derived phenotype we could see that SpeB was not the only protein whose expression was altered *in vivo*. SpeA and the 25 kDa Spy0136 were upregulated independently of the release from SpeB degradation. This point is important inasmuch as the upregulation of SpeA *in vivo* represents the first example of regulation of a phage-encoded virulence protein (Banks *et al.*, 2002; Canchaya *et al.*, 2003).

Several additional proteins were differentially expressed by both phenotypes. Among those, CAMP factor, cyclomaltodextrin glucanotransferase and GAPDH were relatively increased in the original phenotypic form, whereas EndoS, SLO and NADGH were considerably increased in the phase-shifted form (Table 3). It is noteworthy that a number of lower abundance proteins appeared to be upregulated or downregulated *in vivo*, but could not be definitively identified due to expression levels below the threshold of MALDI-TOF MS detection. Further quantitative comparative analyses of the Δ*speB* knockout strain proteome before and after *in vivo* passage are ongoing to produce a more comprehensive and definitive list of differentially expressed proteins *in vitro* and *in vivo* in the absence of SpeB activity.

Several pieces of evidence suggest that the M1T1 clone and the sequenced SF370M1 strain harbour different bacteriophages. The M1T1 clone studied here possesses the *speA* gene and lacks the *speC* gene found in strain SF370 (Chatellier *et al.*, 2000). In addition, we found a protein secreted by the M1T1 clone that bears considerable homology to Sda from M49 and M18, and Sdn from M3. This protein, which is not found in the SF370 strain, may represent an allelic variant of streptodornase D characterized by Podbielski *et al.* (1996). Using reverse genomics we were able to confirm the presence of Sda in the M1T1 clone (data not shown). In sequenced GAS strains, Sda and SpeA are carried on two different prophages (Ferretti *et al.*, 2001; Beres *et al.*, 2002). Future studies will examine whether these differences in phage content have contributed to the persistence and apparent increased virulence of the M1T1 clone.

Proteomic analysis has become a major tool in the field of bacterial pathogenesis. It offers new scope in the analysis of bacterial virulence proteins and their differential expression under different conditions. Here we have shown that the cysteine protease SpeB is capable of degrading most of the M1T1 GAS secreted proteome, including virulence factors such as M protein, streptokinase, streptolysin O, SIC and others. When a SpeB$^+$/SpeA$^–$ M1T1 GAS strain is passed *in vivo*, selection is exerted for a stable phase-shift to a SpeB$^–$/SpeA$^+$ strain expressing the full repertoire of secreted virulence factors (Fig. 1). This experimental finding appears to parallel the observed inverse correlation between SpeB expression and disease severity in patients with invasive M1T1 GAS disease. Future proteomic and differential microarray studies will help us to find the molecular basis of the observed phenotypic phase-shift and may identify regulatory networks controlling expression of GAS virulence factors *in vivo* and *in vitro*.

Experimental procedures

Bacterial strains and culture conditions

Two representative M1T1 isolates were obtained from patients with invasive GAS infections and determined to belong to the same M1T1 clone as detailed elsewhere (Chatellier *et al.*, 2000). Bacteria were grown routinely in Todd–Hewitt broth (Difco Laboratories) supplemented with 1.5% yeast extract (THY). Sheep blood agar (Becton Dickinson) was used as a solid medium. The creation of the *speB*-in frame allelic exchange deletion mutant was shown previously (Nizet *et al.*, 2000; Kansal *et al.*, 2003). For *speB*-mutant selection, BHI agar supplemented with chloramphenicol (2 μg ml^{-1}) was used. All bacteria were grown at 37°C in a 5% CO_2-20% O_2 atmosphere.

Murine tissue chamber infection model

The mouse model has been described previously (Kazmi *et al.*, 2001). Briefly, female 6- to 8- week-old BALB/c mice weighing 22–25 g were obtained from the Jackson Labora-

tories. Teflon-FEP (Fisher) tissue chambers, measuring 20×10 mm and perforated by 110 equally spaced 1-mm-diameter holes were manufactured by our Biomedical Instrumentation Department (UTHSC, Memphis). Two larger holes were engineered at each end to allow penetration of a 27-gauge needle. The tissue chambers were sterilized by autoclaving and implanted in the subcutaneous connective tissue on the back of the mouse (Kazmi *et al.*, 2001). After 3 weeks, the chamber pores became sealed with vascularized connective tissue and were filled with a tissue chamber fluid (TCF). Three weeks after implantation, 100 μl aliquots of TCF were cultured on sheep blood agar plates (Difco) for 24 h at 37°C to verify sterility. Sterile chambers were inoculated with 100 μl GAS suspension as described below. All protocols involving animals were approved by the Institutional Animal Care and Use Committee of The University of Tennessee, Health Sciences Center at Memphis (UTHSC).

Preparation of bacterial inoculum for infection

To prepare the bacteria for *in vivo* inoculation into mice chambers, pure colonies were isolated and cultured overnight at 37°C in standard media under static conditions. The number of colony-forming units (cfu) per millilitre was determined, and 10^5 cfu were inoculated into each tissue chamber. Bacterial culture supernatants prepared immediately prior to infection were designated day 0 supernatants.

Preparation of bacterial culture supernatant proteins for functional and proteomic studies

Bacteria were grown to stationary phase in THY medium, which had been filtered to remove proteins with MW ≥10 000. Supernatants containing GAS-secreted proteins were partially purified by overnight 100% ethanol precipitation at −20°C and by filtration through Amicon centricon (10 kDa cut off) concentrators then dialysed using Slide-A-Lyzer dialysis cassettes with 10 kDa cut off (Pierce). Preparation of supernatants for lymphocyte stimulation assays were performed as before (Kansal *et al.*, 2003). For proteomic studies, aliquots of the protein preparations were lyophilized to dryness and used for 2D-gel analyses. Several studies were performed with inactivation of SpeB by growing bacteria in the presence of 28 μM of the cysteine protease inhibitor E-64 {*N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine, from Roche}.

Immune stimulating activity of SpeB$^+$ and SpeB$^-$ culture supernatants

Peripheral blood mononuclear cells (PBMCs) were obtained from the blood of healthy individuals by ficoll-hypaque gradient centrifugation. PBMCs (2×10^6 cells ml^{-1}) were stimulated with the different bacterial culture supernatants (1:500 to 1:4000 dilutions). Cells were pulsed after 72 h in culture with 1 μCi of [^{3}H]-thymidine for 6 h, then harvested onto glass-fibre filters and counted in a β-scintillation counter (Packard). THY medium was used as negative control. PBMC culture media were used to determine the cytokines concentrations by the IMMULITE system (Diagnostic products corporation-DPC) and according to manufacturer's instructions. All supernatants used in the cytokine experiments were pre-treated with Polymyxin-B to remove residual LPS contamination that might have been introduced during the preparation.

In some experiments, recombinant SpeB (rSpeB) was added to cultures of the SpeB$^-$ bacteria to simulate the effects of native SpeB produced by the SpeB$^+$ bacteria. The generation and activity of rSpeB have been previously described (Kansal *et al.*, 2003).

Two-dimensional gel electrophoresis

In the first-dimension, proteins were separated by isoelectric focusing using the PROTEAN IEF system and ReadyStrip IPG strips (7 and 17 cm) with a linear immobilized pH gradient of 3–10 (Bio-Rad Laboratories Inc.). The sample was prepared by reconstituting the lyophilized protein preparation in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM dithiothreitol and 0.16% of 40% Bio-Rad ampholytes). The sample (125 μl when using the 7 cm strip, and 300 μl when using the 17 cm strips), gel strip and mineral oil were sequentially added to a plastic tray in which the strips were allowed to rehydrate for ≥12 h at room temperature. Heating was avoided to prevent carbamoylation of proteins, which alters their isoelectric points. The proteins were focused at a constant temperature (18–20°C) in three steps starting with a conditioning stage (250 V for 15 min) followed by a linear voltage ramping stage, and ending with a final focusing stage (4000 V for the 7 cm strips and 10 000 V for the 17 cm strips). The apparatus was set at a maximum current of 50 μA per strip and a maximum run time to achieve 20 000 and 60 000 Vh for the 7 cm and 17 cm strips respectively. Proteins were then separated in the second dimension (SDS–PAGE) using the DODECA system (Bio-Rad). After isoelectric focusing, the gel strips were rocked gently in the SDS equilibration buffer (0.375 Tris-Cl pH 8.8, 6 M urea, 20% glycerol and 2% SDS) for 10 min in the presence of 130 mM dithiothreitol then for 10 min in the same buffer with to which iodoacetamide was added to a final concentration of 25 mg ml^{-1}. The equilibrated strips were gently layered onto the SDS-polyacrylamide gel, and electrophoresis was conducted at 200 V for 7 h in case of large gels, or 1 h in case of mini-gels. The gels were stained with silver or Gel Code Blue (Pierce) stains to visualize the separated protein spots. Image analysis and gel comparisons were performed using the PDQUEST software (Bio-Rad) versions 6 and 7. In some experiments, the proteins were transferred to a nitrocellulose membrane for Western blotting as detailed below.

Protein identification using matrix-assisted laser-desorption time-of-flight mass spectroscopy (MALDI-TOF MS) and bioinformatics

Individual protein spots excised from the 2-D gels were placed in siliconized centrifuge tubes, de-stained, washed twice with distilled water and then dried under vacuum for 30 min. Dried gel pieces were incubated with a solution of ~10 units of sequencing-grade trypsin (Promega) in 50 mM ammonium bicarbonate buffer at 37°C for 4 h or longer. Tryp-

tic peptides were extracted by treating the gel pieces with an aqueous solution of 60% acetonitrile and 5% trifluoroacetic acid (TFA) for 20 min twice in an ultrasonic water bath. The peptide extract was dried under vacuum, resuspended in 0.1% TFA, and purified using C18 Zip tips (Millipore). The bound peptides were then eluted in 12.5 mg ml^{-1} matrix (α-cyano-4-hydroxycinnamic acid) dissolved in 50% acetonitrile in 0.1% TFA acid, then applied to a MALDI sample plate.

We performed MALDI-TOF MS analyses using a Voyager DE RP MALDI-TOF mass spectrometer (ABI) in the Stout Neuroscience Laboratory (UTHSC proteomic facility, Memphis, TN). Peptide masses were matched using PeptIdent (URL http://www.expasy.ch/tools/peptident.html) and MSFIT (URL http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm).

Immunoblotting and 2-D Westerns

Proteins separated by 1D or 2D gel electrophoresis were transferred to nitrocellulose membranes (Amersham-Pharmacia) using a Mini Trans-Blot cell (Bio-Rad Laboratories) at 100 V for 60 min. The membranes were blocked with 6% non-fat milk, probed with primary and secondary antibodies as previously detailed (Chatellier *et al*. 2000). To detect the SpeA protein, we used rabbit polyclonal antibodies raised against recombinant SpeA (rSpeA) generated as previously reported (Norrby-Teglund *et al*., 1997). SpeB was detected by mouse monoclonal anti-SpeB antiserum that was a gift from James Musser (National Institute of Allergy and Infectious Diseases, Hamilton, Mont.). For signal detection, we used luminol-chemiluminescence detection reagents (ECL; Amersham). The processed blots were exposed to X-ray films, and the autoradiograms were analysed.

Quantitative transcript analysis using real-time fluorogenic RT-PCR

RNA was extracted from stationary-phase bacterial cell lysates using the Qiagen RNeasy kit (Qiagen). Bacterial pellets were first sheared in the BIO101 FastPrep FP120 instrument (Qbiogene) using FastPrep lysing matrix B (Qbiogene). The extracted RNA was treated twice with DNase I (Qiagen) to remove contaminating genomic DNA and further purified using the Qiagen RNeasy kit. Removal of contaminating DNA was verified by regular PCR amplification of the *speB* and/or the *speA* genes in the absence of reverse transcriptase. The DNA-free RNA was converted to cDNA prepared using AMV reverse-transcriptase and random hexamers (Promega).

Quantitative Real-time PCR was performed using the fluorogenic SYBR Green RT-PCR system and the ABI prism 7900 Sequence BioDetector (PE Biosystems). The primers used for amplification of *speA, speB, spy0136* and gyrase were, respectively: 5′speA(real) ttttttgttttagtgacatttcttgga, 3′speA(real) ctagatctgtgaagttggcttgga, 5′speB(real) cgcac taaacccttcagctctt, 3′speB(real) acagcactttggtaaccgttg, Spy0136+314 gggacggtacagttcgttctga, spy0136+375 gcttttc caatcataatgccatt, 5′gyr cgacttgtctgaacgccaaa, 3′gyr ttatcacg ttccaaaccagtcaa. The PCR program was set for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The reaction mixture contained 25 μl SYBR Green PCR Master mix (Applied Biosystems), 1.5–12 pmol of each primer and 2 μl of the template cDNA in a final volume of 50 μl. We optimized the primer concentrations for each gene being amplified, and we also carried out dissociation curve analyses for all the amplifications to confirm the presence of a single PCR product per reaction.

The relative amounts of target RNAs were determined using the ΔΔCt method. Briefly, the differences in the threshold cycle numbers or Cts (i.e. the cycle number at which the fluorescence reaches an arbitrary chosen value) were calculated after being normalized to the Ct values of the housekeeping gyrase gene. The amount of mRNA in one sample was arbitrarily set at one and all other transcript levels were compared to it.

Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi3797/mmi3797sm.htm
Table S1. Protein spots identified by MALDI-TOF MS and bioinformatics.

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