A role for Trigger Factor and an Rgg-like regulator in the transcription, secretion and processing of the cysteine proteinase of *Streptococcus pyogenes*

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The ability of numerous microorganisms to cause disease relies upon the highly regulated expression of secreted proteinases. In this study, mutagenesis with a novel derivative of Tn4001 was used to identify genes required for the expression of the secreted cysteine proteinase (SCP) of the pathogenic Gram-positive bacterium *Streptococcus pyogenes*. Designated as Rop loci (regulation of proteinase), *ropB* is a *rgg*-like transcriptional activator required for transcription of the gene which encodes the proteinase. In contrast, *ropA* contributes post-transcriptionally to the secretion and processing of SCP and encodes a homologue of Trigger Factor, a peptidyl-prolyl isomerase and putative chaperone which is highly conserved in most bacterial species, but of unknown function. Analysis of additional *ropA* mutants demonstrated that RopA acts both to assist in targeting SCP to the secretory pathway and to promote the ability of the proprotein to establish an active conformation upon secretion. This latter function was dependent upon the peptidyl-prolyl isomerase domain of RopA and mutants that lacked this domain exhibited a bipartite deficiency manifested as a kinetic defect in autologous processing of the proprotein to the mature proteinase, and as a catalytic defect in the mature proteinase. These results provide insight into the function of Trigger Factor, the regulation of proteinase activity and the mechanism of secretion in Gram-positive bacteria.

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Introduction

Extracellular proteinases play key roles in many essential processes, including angiogenesis, wound healing, inflammation, development and fibrinolysis (Masure and Opdenakker, 1989; Chung *et al.*, 1996; Pepper *et al.*, 1996; Kidd, 1998; Wong, 1998). Many pathogenic microorganisms also produce extracellular proteinases which contribute to the ability of these microorganisms to cause disease (Goguen *et al.*, 1995; Lantz, 1997). A common theme in the biology of both host and pathogen extracellular proteinases is that the expression of proteolytic activity is highly regulated, both at the level of expression and secretion of the proteinase, and at the level of the pro-

cessing of an inactive secreted precursor to its active form (Wandersman, 1989; Corcoran *et al.*, 1995). These multiple layers of regulation are required to ensure that expression of extracellular proteolytic activity is tightly controlled in the appropriate temporal pattern and restricted to the appropriate spatial pattern.

Pathogen extracellular proteinases present interesting models for studying the regulation of proteinase activity, since it appears that many of these proteinases contribute to the pathogenesis of disease by acting to disrupt the normal pattern of host proteinase activation or by mimicking the effector function of an activated host proteinase in an unregulated fashion (Goguen *et al.*, 1995). An example of a microbial proteinase which has these properties is the cysteine proteinase produced by *Streptococcus pyogenes* (group A streptococcus) (Kapur *et al.*, 1993a). This pathogenic Gram-positive bacterium is the causative agent of numerous suppurative infections of the pharynx (e.g. 'strep throat') and soft tissues (impetigo, cellulitis and necrotizing fasciitis), as well as several systemic diseases which can result from toxigenic (scarlet fever and toxic shock-like syndrome) or immunopathological (rheumatic fever) processes (Bisno and Stevens, 1996). The secreted cysteine proteinase (SCP) of *S.pyogenes* is also known as the streptococcal pyrogenic exotoxin B (SpeB) (Gerlach *et al.*, 1983) and the gene that encodes the proteinase, which is referred to as *speB* to reflect the fact that its sequence was found to be identical to that previously determined for SpeB (Gerlach *et al.*, 1983), is found highly conserved in almost all clinical isolates of *S.pyogenes* (Yu and Ferretti, 1991; Talkington *et al.*, 1993). Considerable evidence has accrued which suggests that SCP makes important contributions to the pathogenesis of streptococcal infections, including the observations that insertional inactivation of *speB* can reduce the virulence of *S.pyogenes* following intraperitoneal challenge of mice (Lukomski *et al.*, 1997, 1998) and that vaccination with the proteinase can provide protection in the same model of infection (Kapur *et al.*, 1994).

A number of potential targets of SCP activity in infection have been identified. For example, SCP can cleave proteins of the extracellular matrix such as fibronectin and vitronectin (Kapur *et al.*, 1993b) and can mimic the action of host interleukin-1 β (IL-1 β)-converting enzymes by cleaving the inactive IL-1 β precursor to generate its active form (Kapur *et al.*, 1993a). The streptococcal proteinase can also mimic the action of host kallikreins and can liberate active kinins through cleavage of kininogen (Herwald *et al.*, 1996). Also, through an ability to cleave and activate matrix metalloproteinases, SCP may contribute to tissue damage by disrupting the normally tightly restricted pattern of expression of these host proteinases (Burns *et al.*, 1996). In addition to targets derived from the host, SCP may also act on the microbial surface to release biologically

active fragments of streptococcal proteins normally associated with the cell surface, including immunoglobulin Fc region-binding activities, fibrinogen-binding activities and an active fragment of a proteinase which inactivates the chemotactic C5a component of complement (Berge and Björck, 1995).

Greater insight into how these various activities of SCP may contribute to pathogenesis would be facilitated by a greater understanding of the factors involved in the regulation of the expression, secretion and processing of the proteinase precursor. The primary translation product of *speB* is a 43 kDa preproprotein, which is secreted extracellularly as a 40 kDa proprotein (Hauser and Schlievert, 1990). Under reducing conditions, the proprotein can undergo autocatalytic cleavage to the mature active 28 kDa form. Consistent with a requirement for reducing conditions, mutation of the single cysteine residue of the proprotein blocks the ability of the resulting mutant protein to undergo autocatalytic processing (Musser *et al.*, 1996). This level of processing suggests the involvement of a complex secretion mechanism. However, additional details of the export, folding and processing pathways are not well understood. In fact, the details of protein secretion in Gram-positive bacteria are generally only poorly understood in comparison with Gram-negative bacteria, but are likely to be quite different due to distinct differences in cellular architecture. For example, Gram-positive bacteria have a much thicker cell wall, lack an outer membrane and lack the accessory cellular compartments of Gram-negative bacteria, including the periplasmic space. Similarly, the control of expression of *speB* is also not well understood, although it is clear that this process is highly regulated, both temporally such that expression is maximal during stationary phase of growth and in response to nutrient and carbon source limitation (Chaussee *et al.*, 1997). Insertional inactivation of an oligopeptide transporter in *S.pyogenes* generated a mutant strain with a diminished expression of SCP (Podbielski *et al.*, 1996), further suggesting a relationship between nutrient acquisition and expression of the proteinase.

The aim of the present study was to identify additional genes which effect the expression, secretion and processing of SCP. In addition to providing insight into the role of SCP in the pathogenesis of streptococcal infections, this analysis can also provide a model for the study of secretion pathways in Gram-positive bacteria. To accomplish this, we developed a novel derivative of the transposon Tn4001 which addresses many of the problems that have previously been associated with the use of this element for insertional mutagenesis (Mahairas *et al.*, 1989; Lunsford, 1995). The resulting derivative was found to insert into the streptococcal chromosome at high frequency and with a high degree of randomness, which allowed us to identify several mutants defective in production of SCP. Detailed analyses of two of these mutants revealed that one functions as a positive regulator of the transcription of *speB*, while the second was found to be involved in the secretion and processing of the proteinase to its active form. Interestingly, this latter gene was identified as a homologue of Trigger Factor, an enigmatic protein variously reported to function as a peptidyl-prolyl isomerase, a folding catalyst and/or as a molecular chaperone in various *in vitro* assays (Lecker *et al.*, 1989; Stoller *et al.*, 1995). However,

Fig. 1. Structure of the novel transposon TnSpc. A map of the TnSpc element with relevant features is shown. Ends of the transposon are indicated by the right and left inverted repeats (IR_R, IR_L), which surround a transposase (*tnp*) gene and spectinomycin resistance marker (*aad9*). Relative orientations of the individual genes within the 2450 bp long transposon are shown. Several restriction sites internal to the transposon are indicated: C, *Cla*I; Ev, *Eco*RV; H, *Hind*III; P, *Pst*I; Sp, *Sph*I; Hn, *Hinc*II. The *Eco*RV sites inactivated by the insertion of *aad9* are enclosed by parentheses.

depletion of Trigger Factor has not previously been shown to have any effect on the production or secretion of any known polypeptide *in vivo* (Guthrie and Wickner, 1990).

Results

Identification of additional genes required for expression of SCP activity

SCP is one of the most abundant proteins secreted by *S.pyogenes* in stationary phase, and at least one study which demonstrated that a cysteine proteinase inhibitor could inhibit the growth of *S.pyogenes* both *in vitro* and *in vivo* has suggested that SCP is essential for cell viability (Björck *et al.*, 1989). However, while most isolates of *S.pyogenes* possess *speB*, not all isolates express the gene *in vitro* (Talkington *et al.*, 1993). Furthermore, several groups have reported the successful insertional inactivation of *speB* with no deleterious effect on the viability of the organism (Chaussee *et al.*, 1993; Tsai *et al.*, 1998). These latter observations suggested that a strategy of transposon mutagenesis to identify mutants defective in proteinase activity could be useful for the identification of additional genes required for the production of SCP activity.

The transposon mutagenesis strategy we conducted utilized Tn4001, a class I composite-type transposon originally isolated from *Staphylococcus aureus* which contains two identical copies of the insertion sequence IS256 that flank a central aminoglycoside resistance determinant (Byrne *et al.*, 1989). This element has previously been used for the generation of mutations in a number of mycoplasma and Gram-positive bacterial species where it appears to insert with a high degree of randomness (Cao *et al.*, 1994; Lunsford and Roble, 1997). However, its usefulness has been limited by the fact that multiple events frequently accompany insertion of the element into a chromosome, including: (i) independent transposition of IS256 and (ii) integration of the entire plasmid delivery vector into the host chromosome (Lunsford, 1995). To address these limitations, we constructed a novel derivative of Tn4001 which essentially consists of a single copy of IS256 into which a spectinomycin resistance gene has been inserted adjacent to one of the inverted repeats that defines the ends of the insertion sequence (Figure 1). The resulting element, Tn4001.spc (which for simplicity will be subsequently referred to as TnSpc), is placed on a plasmid that can replicate in *Escherichia coli*, but is

Table I. Bacterial strains used in this work

Strains	Relevant genotype	Characteristics	Reference
<i>E.coli</i>			
DH5 α	<i>recA1 endA1 hsdR17</i>		BRL ^a
HB101	<i>recA13 proA2</i>		Boyer and Roulland-Dussoix (1969)
<i>S.pyogenes</i>			
HSC5	wild-type		Hanski <i>et al.</i> (1992)
HSC16 ^b	HSC5 Ω pMGC50	<i>prtF::lacZ</i> promoter fusion	C.M.Gibson and M.G.Caparon, unpublished work
HSC100	HSC16 <i>ropA1::TnSpc</i>	<i>ropA</i> inactivated	this work
HSC101	HSC16 <i>ropB1::TnSpc</i>	<i>ropB</i> inactivated	this work
HSC102	HSC16 <i>ropC1::TnSpc</i>	<i>ropC</i> inactivated	this work
HSC105 ^b	HSC5 <i>ropB</i> Ω pBL42	<i>ropB</i> inactivated	this work
HSC110	HSC5 <i>ropA::TnSpc</i> Δ <i>tnp</i>	<i>ropA</i> inactivated	this work
HSC120 ^b	HSC5 <i>ropA</i> Ω pBL22	polar insertion of pBL22	this work
HSC130	HSC5 <i>ropA</i> Δ ₈₂₋₂₉₇	in-frame deletion of <i>ropA</i>	this work

^aGibco-Bethesda Research Laboratories Inc., Gaithersburg, MD.

^bStrain derived through transformation of designated strain with the integrational plasmid shown.

incapable of replication in Gram-positive hosts. Thus, when the chimeric plasmid (pMGC57-*spc*) is introduced into *S.pyogenes*, spectinomycin-resistant transformants arise as a result of transposition of Tn*Spc* into the streptococcal chromosome. Transformants were obtained at frequencies between 10³ and 10⁵/μg of pMGC57-*spc* DNA depending on the streptococcal host strain employed and the resulting libraries contained only insertions of the modified transposon, since the construction of Tn*Spc* prevents independent transposition of IS256. Furthermore, integration of the entire pMGC57-*spc* delivery vehicle was not detected at a frequency >0.1%.

A total of 7000 spectinomycin-resistant colonies from three independent transformations of the proteinase-producing strain HSC16 were examined for proteinase production on skimmed milk agar plates. A screen for a complete defect in proteinase production, as defined by the total loss of the clear zone which surrounds proteinase-proficient colonies caused by the proteolytic cleavage of casein, resulted in the identification of five mutants. A Southern blot analysis revealed that two of these mutants had at least two insertions of Tn*Spc* and that the remaining three mutants (designated HSC100, HSC101 and HSC102) (Table I) possessed a single insertion of the transposon (data not shown). None of these five original mutants possessed an insertion in *speB* itself, as was shown through the use of a *speB*-specific probe (data not shown).

Characterization of the insertions in HSC100 and HSC101

Further analysis concentrated on two of the mutants which contained a single insertion of Tn*Spc* (HSC100 and HSC101). A combination of direct cloning of chromosomal DNA with selection for the Tn*Spc* spectinomycin-resistance determinant in *E.coli* and an inverse PCR technique was used to obtain clones of the DNA regions adjacent to the transposon insertions in each of these strains. The DNA sequence information obtained from these clones was then compared with the sequence information available from the University of Oklahoma streptococcal genome sequencing project (<http://www.genome.ou.edu/strep.html>). This analysis revealed that the insertions had each disrupted a large open reading frame (ORF), which were designated *ropA* and *ropB* (regulator of proteinase) for HSC100 and HSC101, respectively.

Characterization of *ropB*

Comparison of the predicted 280 amino acid translation product of *ropB* versus the DDBJ/EMBL/GenBank database indicated a high degree of homology (22% identical, 44% similar) between RopB and Rgg, a transcriptional activator of *gtfG* which encodes the extracellular glucosyl-transferase of *Streptococcus gordonii* (Vickerman *et al.*, 1997). In *S.gordonii*, *rgg* is located immediately adjacent to *gtfG* (Sulavik *et al.*, 1992). An examination of the sequences adjacent to *ropB* in *S.pyogenes* revealed that like *rgg*, *ropB* is also located immediately adjacent to its regulatory target (*speB*). However, in contrast to *rgg*, *ropB* is transcribed in a divergent manner from its regulatory target (Figure 2). A prominent feature of the intergenic region between *rgg* and *gtfG* is a large inverted repeat which overlaps the promoter for *gtfG*, which has been postulated to be a site for interaction with Rgg (Sulavik *et al.*, 1992; Figure 2). Whilst not demonstrating any obvious homology with the *gtfG* inverted repeat or each other, two distinct sets of inverted repeats are present in the *ropB*-*speB* intergenic region. Similar to *gtfG*, the -35 region of the putative promoter for *speB* is included in an inverted repeat. The importance of *ropB* for expression of SCP was confirmed by additional mutagenesis using an integrational plasmid strategy to insertionally inactivate *ropB* through homologous recombination (see Materials and methods). For 20 out of 20 isolates examined, insertional inactivation of *ropB* resulted in a complete loss of proteolytic activity (data not shown).

Additional characterization of *ropA*

Comparisons of *ropA* to sequences in DDBJ/EMBL/GenBank indicated that RopA was highly homologous to Trigger Factor from *Bacillus subtilis* (TigBS; Figure 3). TigBS is a 42 kDa molecular chaperone which is also a member of the FK506-binding family of peptidyl-prolyl *cis-trans* isomerases (Göthel *et al.*, 1997). The predicted polypeptide of RopA is nearly identical in size to TigBS and the signature residues for FK506 binding in TigBS are highly conserved in RopA (Figure 3). However, depletion of Trigger Factor has not previously been associated with a specific defect for secretion of any known protein *in vivo* (Guthrie and Wickner, 1990). Thus, it was important to confirm that disruption of *ropA* was responsible for the proteinase-deficient phenotype of

*Streptococcus gordonii**Streptococcus pyogenes*

Fig. 2. Comparison of the chromosomal regions encompassing *rgg* and *ropB*. The chromosomal structure of *rgg* (DDBJ/EMBL/GenBank accession No. M89776) and *ropB* (DDBJ/EMBL/GenBank accession No. AF073923) along with their target genes, *gtfG* and *speB*, respectively, and host organisms are shown. In contrast to *rgg*, *ropB* is transcribed in a manner divergent from its regulatory target. The -10 and -35 regions and the transcriptional start site (indicated by a bent arrow) for *gtfG* and the predicted promoter for *speB* (Hauser and Schlievert, 1990) are shown in the expanded views below their respective chromosomes. Inverted repeats overlapping the promoter region of *gtfG* (DDBJ/EMBL/GenBank accession No. M89776) [IR_L(bp 1063–1091) and IR_R(bp 1131–1103)] and the putative promoter regions of *speB* (DDBJ/EMBL/GenBank accession No. U63134) [IR_{1L}(bp 1034–1047), IR_{1R}(bp 1074–1061), IR_{2L}(bp 1490–1506) and IR_{2R}(bp 1540–1524)] are indicated by the arrows.

TnSpc	
▽	
RopA:	1 MSTSFENKATNRGVITFTISQDKIKPALDKAFNKIKKDLNAPGFRKGHMPRPVFNQKFGE 60
TigBS:	1 MS +E + N GV+T + + K ALD AF K+ K ++ PGFRKG +PR +F Q+FG 60
RopA:	61 EVLYEDALNIVLPEAYBAAVTELGLDVVAQPKIDVVSMEKGKEWTLSEAEEVTKPEVKLGD 120
TigBS:	61 E LY+DAL+I+LP Y AV E G++ V +P+IDV +EKG+ +A+V KPEVKLG+ 120
RopA:	121 YKNLVVEVDASKEVSEDEVDAKIERERQNLAEI IKDGEAAQGD TVVIDFVGSVDGVEFD 180
TigBS:	121 YK L +E D + ++ + ++ ++G +G+TVV+DF G VDG F+ 180
RopA:	181 GKGDNFSLELGSGQFIPGFEDQLVGAKAGDEVEVNTFPESYQAE DLGAKA AKFMTTII 240
TigBS:	181 GGAENYSLEVSGSFIPGFEDQLVGLAEGA EKDV E VTFPEEYHAE DLGAKPAV FVKI H 240
RopA:	241 EVKTKEVPELDDELAKDIDEDVDLTKVKYRKELEAAQETAYDDAVEGA AIELAVANA 300
TigBS:	241 EIKAKELPELDDEFKADIDEVETLAELTEKTKRLEERAKENEADAKLREELVLKASENA 300
RopA:	301 EIVDLPEEMIHEEVNRSVNEFMGNMQRGISPEMYFQLTGTTQEDLHNQYSAEADKRVT 362
TigBS:	301 EI+D+P+ M+ E++R + EF +Q QG++ E+Y Q +G + L Q +A+KRVK+ 362
RopA:	363 HLVEIAIAKAEGFEATDSEIEQEINDLATEYNMPADQVRSLLSASADMLKHDIAMKAVEVI 420
TigBS:	363 NLLEIAIAKAENLEVSDEVDDELTKMAEAYNMPVENIKQAIGSSTDAMKEDLKVRKAIDFL 420

Fig. 3. RopA shows a high degree of homology to the Tig protein of *B. subtilis* (TigBS). The predicted amino acid sequence of the ORF disrupted by TnSpc (*ropA*, DDBJ/EMBL/GenBank accession No. AF073922) is compared to the TigBS protein (DDBJ/EMBL/GenBank accession No. Z99118). The two polypeptides are 49% identical (as indicated by identical amino acids shown between the two sequences), and 66% similar (indicated by a + sign between the sequences). The site of insertion of TnSpc (indicated by the triangle above the amino acid sequence) occurs within an arginine codon (R12). Conserved residues involved in binding of the inhibitor FK506 are marked with asterisks. The region of *ropA* which is missing in the in-frame deletion mutant (*ropA*_{Δ82–297}) is outlined in gray (amino acids 82–297).

HSC100. To address this issue, a deletion was introduced into the gene encoding the transposase of TnSpc in a copy of the insertionally inactivated allele of *ropA* (*ropA*1::TnSpc) which had been cloned in *E. coli*. Because the modified element (TnSpcΔ*tnp*) is not capable of

transposition, the resulting allele of *ropA* (*ropA*1::TnSpcΔ*tnp*) was used to replace the wild-type *ropA* allele in the unmutagenized parental strain. When this analysis was performed, 42 out of 42 progeny analyzed had a proteinase-defective phenotype identical to the

original mutant HSC100. The gene which encodes Trigger Factor is also highly conserved among a large number of diverse bacterial species and is often located adjacent to *clpX*, which encodes a regulator of the ClpP intracellular proteinase (Gottesman *et al.*, 1993). In contrast, RopA is located adjacent to a gene highly homologous to the delta subunit of the *B. subtilis* RNA polymerase (*rpoE*) (Lampe *et al.*, 1988). To rule out any possibility that the proteinase-deficient phenotype is the result of a polar effect on expression of *rpoE*, two additional constructs were generated. In the first, a strong polar element (Ω Km-2) was placed immediately downstream of *ropA*. In the resulting strain (HSC120), *ropA* itself is intact; however, Ω Km-2 is in a position to have a polar effect on expression of distal genes. This potential polar effect was ruled out by the observation that production of SCP proteolytic activity by HSC120 was indistinguishable from the wild-type strain on skimmed milk agar plates (data not shown). In the second construction, *ropA* in the wild-type strain was replaced with a non-polar in-frame deletion allele (*ropA* $_{\Delta 82-297}$) from which the region that encodes amino acids 82–297 was removed (Figure 3). Proteinase activity in the resulting strain (HSC130) appeared identical to the original mutant (HSC100) and was undetectable at a time point when the zone of clearance on skimmed milk agar plates was maximal in the wild-type strain (data not shown). Taken together, these data demonstrate a requirement for RopA in the expression of SCP proteolytic activity.

RopB, but not RopA, is required for transcription of *speB*

To investigate the mechanisms by which *ropA* or *ropB* contribute to expression of the SCP, the effect of a mutation in *ropA* or *ropB* on transcription of *speB* was examined. Total RNA that was isolated from wild-type and several mutant strains in early stationary phase was subjected to Northern blot analysis using a *speB*-specific probe. Two transcripts of 2.1 and 1.7 kb in size were observed to hybridize with the *speB* probe (Figure 4A). Because the coding region of *speB* is 1194 bp the sizes of the two transcripts suggests that multiple promoters are utilized in transcription of *speB*. This result was confirmed using a different *speB*-specific probe (data not shown). Significantly, when the hybridizing signal is normalized for the amount of RNA analyzed, the mutations in *ropA* (*ropA1::TnSpc* and *ropA* $_{\Delta 82-297}$) have no effect on the amount of the two *speB* transcripts (Figure 4B). In contrast, both *speB* transcripts were undetectable when examined in a *ropB* mutant background (Figure 4A, *ropB1::TnSpc*) even with prolonged exposure of the autoradiograph and with analysis of higher concentrations of total RNA (data not shown). Thus, consistent with its homology to *rgg*, *ropB* is a positive activator of *speB* transcription, while *ropA* contributes to expression of proteinase activity post-transcriptionally.

Effects of mutations in RopA on secretion and processing of SCP

The observation that transcription of *speB* was not altered in *ropA* mutants helped to explain the observation that *ropA* mutants did not express detectable proteinase activity (as determined with skimmed milk agar plates) at a time

Fig. 4. RopB is required for transcription of *speB*.

(A) Northern blot analysis. Total RNA from strains HSC5 (wild-type), HSC100(*ropA1::TnSpc*), HSC130(*ropA* $_{\Delta 82-297}$) and HSC101(*ropB1::TnSpc*) were subjected to a Northern blot analysis using a *speB*-specific probe. RNA was harvested from cultures grown to early stationary phase. (B) Normalization of *speB*-specific transcription. The signal from the Northern blot shown in (A) was compared to the amount of total RNA analyzed for each sample based on the amount of 30S RNA present in the ethidium bromide-stained agarose gel prior to transfer to the nylon membrane. Band intensities were determined by densitometry and relative *speB* transcription is shown as the ratio of *speB* specific hybridization to 30S RNA. The asterisk (*) shown indicates that levels of *speB* transcription were indistinguishable above background values.

when the wild-type strain was expressing maximal activity (18 h), but upon extended incubation (36 h) some evidence of proteolytic activity was apparent around areas of heavy growth of the mutants. This effect was much more pronounced for strains containing the in-frame deletion allele (*ropA* $_{\Delta 82-297}$) compared with strains with the original transposon insertion (*ropA1::TnSpc*). Since there was no observable difference between the rate of growth of any *ropA* mutant and the wild-type strain (data not shown), the observation that the *ropA* mutants possessed some proteolytic activity suggested that they retained some partial ability to secrete and process SCP. This possibility was investigated using several more sensitive assays for SCP activity. Analysis of secreted proteolytic activity in culture supernatants using an assay which detects the altered fluorescence of the cleavage product of fluorescein isothiocyanate (FITC)-labeled casein demonstrated that while supernatants harvested from cultures of *ropA* mutants (*ropA* $_{\Delta 82-297}$, *ropA1::TnSpc*) at 7 and 9 h of growth had considerably reduced activity relative to wild-type or to a strain with polar insertion just downstream of *ropA* (*ropA* Ω pBL22), they did possess detectable activity (Figure 5). Consistent with the behavior of the *ropA* mutants on skimmed milk agar plates, a strain with the

Fig. 5. SCP activity is decreased in *ropA* and *ropB* mutants. Proteinase activity present in culture supernatants was measured at various time points during the growth of the cultures using an assay based on the cleavage of FITC-labeled casein. Strain HSC120 (*ropA*ΔpBL22) was tested in this assay along with the other strains described in Figure 4. Activity is presented as a percentile of the maximal proteinase activity produced by HSC5 (wild-type) at 9 h. Data represent the mean and standard error of the mean for at least two independent experiments.

in-frame deletion exhibited greater SCP activity than a strain with the transposon insertion (Figure 5, compare *ropA*Δ82-297 with *ropA*1::TnSpc). The activity of a *ropB* mutant was undetectable over background values (Figure 5, *ropB*1::TnSpc). When supernatants from these cultures were subjected to a Western blot analysis, it was observed that in both the wild-type and the strain with a downstream polar insertion (*ropA*ΔpBL22) that the 40 kDa proprotein form of SCP was readily detectable beginning between 5 and 7 h after inoculation of the cultures and that by 9 h, most of the SCP protein was present as the 28 kDa processed form (Figure 6A). Interestingly, while the strain with the *ropA* in-frame deletion did not produce high levels of proteolytic activity, the proprotein form of SCP was detected with a time course and in quantities that did not differ greatly from the wild-type strain (Figure 6A). However, the *ropA* in-frame deletion mutant did not process the precursor form to the active form as efficiently as strains with wild-type alleles of *ropA* (Figure 6A, compare *ropA*Δ82-297 with wild-type and *ropA*ΔpBL22). In contrast, neither the precursor nor processed form was readily detectable in supernatants from the strain with a transposon insertion in *ropA* (Figure 6A, *ropA*1::TnSpc). The *ropA* in-frame deletion mutant did demonstrate a delayed ability to process the proprotein to the processed form as was apparent in supernatants from cultures harvested much later in stationary phase (16 h incubation; Figure 6B, *ropA*Δ82-297). After 24 h of incubation, virtually all the proprotein form was converted to the processed form in the in-frame deletion mutant (Figure 6C). However, despite extensive processing by this time point, the proteinase activity detectable in supernatants of the in-frame deletion mutant was still <15% of wild-type activity as measured in the FITC-casein cleavage assay (Figure 6B and C). Thus, while the in-frame deletion mutant has a delayed ability to process the proteinase, much of the processed protein was not proteolytically active. This conclusion was further supported in mixing experiments between the supernatants of wild-type and mutant bacteria which indicated that *ropA* mutants do not produce an inhibitor of proteolytic activity (data not shown). Only

small amounts of precursor and processed SCP were detected from the strain with a transposon insertion in *ropA* (Figure 6A, *ropA*1::TnSpc) and the precursor and processed forms were not detected at any time point in supernatants harvested from a strain with a transposon insertion in the transcriptional regulator *ropB* (Figure 6A-C; *ropB*1::TnSpc).

***RopA* has multiple functions in secretion of SCP**

The distinct phenotypes of the different *ropA* mutants suggested that RopA may interact with multiple steps in the pathway for secretion of SCP. To investigate this a number of subcellular fractions were analyzed for the presence of the proteinase, including cell wall, cell membrane and cytoplasm. The 40 kDa proprotein form of SCP was detected in a Western blot analysis of the cell-wall fractions derived from the wild-type, transposon insertion and in-frame deletion *ropA* mutants (Figure 7). When the cell-membrane fractions from early stationary phase were examined, the proprotein form of SCP was detected in membrane preparations from the wild-type and in-frame deletion mutants, but not from the transposon insertion mutant (data not shown). In contrast, in later stationary phase cultures, the SCP proprotein was detected only in the membrane fractions of the in-frame deletion mutant (Figure 7, *ropA*Δ82-297). When the cytoplasmic fractions were examined from early stationary phase, bands reacting with the SCP antiserum were present in fractions prepared from the wild-type and in both *ropA* mutants (data not shown). However, in later stationary phase only the fraction derived from the transposon insertion mutant showed detectable amounts of SCP (Figure 7, compare *ropA*1::TnSpc with wild-type and *ropA*Δ82-297). The significance of the two bands reactive with the SCP antiserum in the cytoplasmic fraction is unknown and may reflect degradation of the unsecreted polypeptide or may indicate that some cleavage within the signal sequence can occur in the cytoplasmic compartment as has been described for other proteins from Gram-positive bacteria (Aono, 1992). Regardless, the different *ropA* mutations have a differential effect on the localization of SCP between distinct subcellular fractions, supporting the hypothesis that RopA has multiple functions in secretion of SCP.

***Mutations in ropA and ropB* have a minimal effect on secretion of several other proteins**

It was of interest to determine if *ropA* and *ropB* have a more general effect on secretion of other proteins by *S.pyogenes* or whether their function is restricted to expression and secretion of the SCP proteinase. Expression of protein F, a cell-wall-associated fibronectin-binding protein, is minimal in environments where the proteinase is most active (e.g. low oxygen tension). However, when examined under low oxygen conditions favorable for expression of proteolytic activity, mutation of either *ropA* or *ropB* resulted in a modest 2- to 3-fold increase in protein F-dependent fibronectin-binding activity (Figure 8). Since SCP is known to cleave other proteins away from the streptococcal cell wall, this increase in fibronectin-binding activity in *ropA* and *ropB* mutants probably results from the absence of proteolytic activity, rather than a direct effect on expression of protein F. Similarly, analysis of secreted protein profiles by SDS-PAGE revealed numerous

A 3-9 hours incubation **B 16h incubation** **C 24 h incubation**

Fig. 6. RopA mutants vary in their ability to secrete and process SCP. The proteolytic activity of SCP in culture supernatant was determined at: (A) 3–9 h, (B) 16 h and (C) 24 h of incubation of the cultures. SCP was detected by a Western blot analysis using an SCP-specific antiserum. The secreted SCP zymogen is 40 kDa, while the processed form is 28 kDa. Proteinase activity, as determined by a fluorescence assay (see Materials and methods), is shown as a percentile of wild-type activity determined for HSC5 at 16 and 24 h, and is shown below the respective panels. Activity listed as <0.2% are essentially undetectable above background values.

Fig. 7. Subcellular localization of SCP is affected by mutations in *ropA*. Cytoplasmic, membrane and cell-wall fractions from the wild-type and mutant strains of *S. pyogenes* indicated were prepared following 24 h of culture (late stationary phase). The distribution of SCP was analyzed by Western blotting using a SCP-specific antiserum.

differences between wild-type and proteinase deficient mutants, which could also reflect an indirect effect of the lack of proteolytic activity. Therefore, to discount for the influence of proteolytic activity, the expression of several known proteinase-resistant proteins was examined in more detail. The *S. pyogenes* strain used in these studies secretes

three distinct DNases whose activities can be individually quantitated following electrophoresis through a DNA-impregnated polyacrylamide gel. Neither of the mutations in *ropA* or mutation of *ropB* had any observable effect on expression of any of the DNases (data not shown). Similarly, no mutation of *ropA* or *ropB* had any effect on

Fig. 8. Mutations in *ropA* or *ropB* cause an increase in fibronectin binding. Strains examined for protein F-derived binding activities are the same as those examined in Figure 6. The results describe the percentage of fibronectin binding compared to the total amount of [125 I]fibronectin added to the assay. Results represent the mean and standard error of the mean of at least two independent experiments.

the levels of secretion or activity of streptolysin O, a hemolysin secreted by *S.pyogenes* (data not shown). Taken together, these data suggest that mutation of *ropA* or *ropB* does not have a direct global effect on streptococcal protein secretion.

Discussion

Through the use of a novel derivative of Tn4001, we have identified a role for an Rgg-like transcriptional regulator and a homologue to Trigger Factor in the transcription, secretion and processing of the cysteine proteinase of *S.pyogenes*. The Rgg-like regulator (RopB) is an activator of transcription of the gene which encodes the proteinase (*speB*). In contrast, Trigger Factor (RopA) contributes to at least two post-transcriptional steps in expression of the cysteine proteinase. First, Trigger Factor is required for targeting the primary translation product of the proteinase to the secretory pathway. This role is supported by the observation that while a *ropA* null mutant was proficient for transcription of the gene which encodes the cysteine proteinase (*speB*) and while the unprocessed proteinase could be detected in the cytoplasmic compartment of the mutant, the mutant was defective in its ability to secrete the proteinase across the single cellular membrane of this Gram-positive bacterium. A second role for Trigger Factor is in promoting the ability of the secreted proprotein to become processed into the mature, active proteinase. This role is supported by the observation that while the *ropA* in-frame deletion mutant was proficient for secretion of the proteinase, the kinetics of processing the proprotein to the mature form was delayed, and much of the processed proteinase remained proteolytically inactive.

These latter observations are of particular interest, since depletion of Trigger Factor has not previously been associated with a defect of the secretion or processing of any known polypeptide *in vivo*.

A number of transposons, including Tn4001, have been utilized for transposon mutagenesis in a selection of different Gram-positive bacteria. However, all have had limitations, including low frequency of insertion, insertion into only a limited number of sites, and the use of

temperature-sensitive delivery vectors which can be difficult to transform and/or have limited host ranges. As a composite-type transposon, Tn4001 has the potential to overcome many of these problems. First, because transposition does not require replication, it can easily be introduced into any host that can be transformed using a 'suicide-type' delivery vector. Secondly, it appears to transpose with a high degree of randomness (Lunsford, 1995). Thirdly, as a composite-type transposon, it is small and has a simple structure that essentially only requires the short terminal repeats and transposase in order to transpose. Since various reporter genes can be included on the element immediately adjacent to one of the short terminal repeats, it will probably also be possible to develop Tn4001 for the identification of specific classes of genes, as has been achieved with other composite-type transposons in Gram-negative bacteria (Manoil and Beckwith, 1985). However, as was discussed above, it was necessary to modify Tn4001 in order to overcome many of the problems that have restricted its use in the past. One limitation which was not solved by the present derivative was a tendency to generate chromosomes with multiple insertions. However, in several subsequent screens for mutations of other phenotypes, multiple insertions have been observed only rarely (<1%) (C.Gibson and M.Caparon, manuscript in preparation). Thus, TnSpc should prove a useful addition to the repertory of transposable elements available for Gram-positive bacteria.

Mutagenesis with TnSpc was used to identify *ropB*, a transcriptional regulator with homology to *rgg*. In *S.gordonii*, *rgg* regulates the gene which encodes glucosyl-transferase (*gftG*), an enzyme that polymerizes glucose into extracellular glucan (Vickerman *et al.*, 1997). An additional gene (*gadR*) with homology to *rgg* has recently been described as a regulator of genes involved in acid resistance in *Lactococcus lactis* (Sanders *et al.*, 1998). In addition to sequence homology, comparisons among *ropB*, *rgg* and *gadR* reveal a number of other similarities. Each of these regulators acts as a positive regulator of transcription and each is involved in regulation of a function that involves secretion; an extracellular polymer for *rgg*, a transmembrane antiporter for *gadR*, and a secreted proteinase in the case of *ropB*. Each is located immediately adjacent to their regulatory target, although unlike *rgg* and *gadR*, *ropB* is oriented divergently relative to its target. Finally, the promoters controlled by these regulators overlap prominent inverted repeats which may represent the binding site for the regulators. These similarities suggest that these genes are the prototype members of what may be a much larger family of transcriptional regulators in Gram-positive bacteria that may share a common regulatory mechanism. In this light, it is interesting to note that analysis of the available genome information for *S.pyogenes* revealed two additional ORFs highly homologous to *rgg* (W.Lyon and M.Caparon, unpublished observation). One additional similarity may be found in the observation that *S.gordonii* gives frequent rise to variants which express low levels of extracellular glucosyl-transferase activity, apparently as the result of a frameshift mutation in *rgg* (Sulavik *et al.*, 1992). In *S.pyogenes*, while all isolates possess *speB*, not all isolates express the proteinase *in vitro* (Talkington *et al.*, 1993). This suggests that some mechanism for variation of SCP expression

exists in *S.pyogenes* and it will be of interest to determine if this involves *ropB*.

An exciting result of this study is the finding that the secretion and processing of SCP is assisted by a homologue of Trigger Factor. This molecular chaperone has remarkable properties including a peptidyl-prolyl isomerase activity, an association with nascent polypeptides on ribosomes, and that it binds to chaperonin 60 (GroEL) and enhances the affinity of GroEL for certain unfolded proteins and promotes degradation of these proteins (Lill *et al.*, 1988; Kandror *et al.*, 1995). Equally remarkable is the fact that depletion studies have not demonstrated a role for Trigger Factor in the secretion and processing of any polypeptide *in vivo* (Guthrie and Wickner, 1990), despite the fact that it was originally identified as a chaperone essential for the translocation of a protein into isolated membrane vesicles *in vitro* (Crooke and Wickner, 1987; Crooke *et al.*, 1988). Depletion of Trigger Factor does cause *E.coli* to lose viability at a faster rate at 4°C (Kandror *et al.*, 1997), and overexpression causes an increase in filamentation in growing cultures of *E.coli* (Guthrie and Wickner, 1990; Tai *et al.*, 1992). However, while these latter studies have suggested that Trigger Factor is important, the physiological function of Trigger Factor has remained an enigma.

In the present study, data which indicated that the various *ropA* mutations have different effects on expression of SCP suggest that Trigger Factor makes contributions at several steps in the secretion of SCP. A working model based on the behavior of the *ropA* mutants and the known *in vitro* properties of Trigger Factor would propose that the first contribution RopA makes is to assist in the targeting of the SCP precursor polypeptide to the secretory pathway. In this model, the very low amounts of SCP produced by a null mutation in *ropA* (e.g. *ropA1::TnSpc*) are due to the loss of this Trigger Factor-promoted targeting activity. This would also explain why these mutants accumulate the precursor in the cytoplasmic compartment. The second contribution would be at the level of assisting in the folding of the precursor after it has been targeted for secretion. In this scenario, the in-frame deletion mutation (*ropA*_{Δ82-297}) retains targeting activity, but lacks the domain which participates in folding. In this case, the presence of the precursor in the membrane fraction and the delayed kinetics of processing of the secreted protein are due to an alteration in the normal pathway of folding. Since the in-frame deletion removes the putative peptidyl-prolyl isomerase domain (Hesterkamp and Bukau, 1996; Stoller *et al.*, 1996; Göthel *et al.*, 1997) and because peptidyl-prolyl isomerases assist in folding, it is attractive to speculate that it is the loss of this activity which is responsible for the observed effects. Consistent with this hypothesis, is the general observation that peptidyl-prolyl isomerases are not essential for folding, but rather accelerate the rate of folding (Stoller *et al.*, 1995). An alteration in the rate of folding could explain why the mutants still can process the proprotein, although with a delayed time course. Furthermore, once some molecules have become processed to the active form through spontaneous isomerization, they may rapidly process the rest of the molecules in the population, even though these molecules may not have completed the folding process. This then could

explain why most of the processed protein is apparently not proteolytically active.

The processing of extracellular proteinases from other Gram-positive bacteria also requires the contributions of accessory gene products. For example, the processing of the cell-wall-associated PrtP proteinase of *L.lactis* requires PrtM (maturation protein) (Haandrikman *et al.*, 1991). In *prtM* mutants, PrtP is secreted but processing of the proprotein to the active form is completely blocked and it appears that this defect is due to the misfolding of the proprotein (Haandrikman *et al.*, 1991). It is interesting to note that it has recently been observed that PrtM has some homology to peptidyl-prolyl isomerases of the parvulin family (Rahfeld *et al.*, 1994). Thus, a common mechanism may underlay the processing pathways of multiple secreted proteinases. However, unlike Trigger Factor, PrtM is not required for the efficient secretion of PrtP and is itself a secreted lipoprotein.

The model presented above postulates that the Trigger Factor homologue, RopA, has at least two functional domains, one which participates in targeting the polypeptide for secretion and a second domain which participates in folding. Consistent with this model, previous studies have shown that large truncations can be introduced into both the C- and N-terminus of Trigger Factor with minimal effect on proline isomerase activity (Stoller *et al.*, 1996). Furthermore, studies on the folding of Trigger Factor itself have suggested that it has a modular structure composed of at least three distinct domains each of which is capable of folding independently (Zarnt *et al.*, 1997). The N- and C-terminal domains which flank the central peptidyl-prolyl domain appear to interact with each other and are important for binding to substrate polypeptides (Zarnt *et al.*, 1997). Since the in-frame deletion mutant constructed in this study lacks the central domain and probably does not have peptidyl-prolyl isomerase activity and since this mutant was competent for secretion of SCP, the two functional domain model implies that proline isomerase activity is not an essential component of the targeting activity. Previous studies have also shown that disruption of specific proline isomerases do not have a direct effect on the targeting and translocation of other proteins (Pissavin and Hugovieux-Cotte-Pattat, 1997; Warth *et al.*, 1997). It is also interesting that mutations of *ropA* did not influence secretion of several other proteins, suggesting that the targeting function of RopA is required to stabilize a specific and unusual conformation of the nascent SCP polypeptide in a secretion competent state. Furthermore, if the proline isomerase activity is involved in influencing the subsequent translocation, folding and processing events, it also must be required in response to a specific and unusual feature of SCP, since streptolysin O contains 21 prolines and secretion of this protein was not affected by mutation in *ropA*. This could imply that the isomerization state of only certain proline residues are rate limiting for translocation and folding. In comparing the distribution of prolines in streptolysin O and SCP, there are several candidate residues which may have an influence on translocation and folding and represent the targets of Trigger Factor. For example, SCP contains a single proline in its signal sequence and a single proline is contained in the pro fragment which is cleaved during processing to the active proteinase. In contrast, streptolysin O has no proline

in its signal sequence and is not secreted as a zymogen. Interestingly, *in vivo* experiments have shown that Trigger Factor is not required for secretion of proOmpA (Guthrie and Wickner, 1990), and that this polypeptide also lacks a proline in its signal sequence (Beck and Bremer, 1980).

Further analysis of the interaction of Trigger Factor and SCP should provide a useful model system to probe the contribution of Trigger Factor to secretion and folding. In addition, studies will provide valuable insight into the process of protein export in Gram-positive bacteria. Finally, further analysis of *ropA*, *ropB* and additional *rop* genes will be essential for understanding how a microorganism regulates the temporal and spatial activity of a proteinase, which in turn, will be valuable for understanding the role of the proteinase in the pathogenesis of infection.

Materials and methods

Bacterial strains

The bacterial strains used in this study are described in Table I. *Escherichia coli* DH5 α was used for molecular cloning experiments, and *E. coli* HB101 was used in fibronectin-binding assays. *Escherichia coli* strains were cultured in Luria-Bertani broth (Scott, 1972) at 37°C. *Streptococcus pyogenes* was grown in Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (Difco) (THY media) or in C medium which consists of 0.5% Proteose Peptone #3 (Difco), 1.5% yeast extract (Difco), 10 mM K₂HPO₄, 0.4 mM MgSO₄, 17 mM NaCl, adjusted to pH 7.5. To produce solid medium, Bacto agar (Difco) was added to a final concentration of 1.4% (w/v). For analysis of SCP proteolytic activity in supernatants, *S. pyogenes* was cultured in THY medium at 37°C in sealed tubes without agitation. Solid media for the examination of proteolytic activity employed C medium supplemented with 1.5% skimmed milk (skimmed milk agar medium) with culture at 37°C in an anaerobic environment (<0.03% O₂, 10% CO₂) generated by a commercial gas generator (GasPak, BBL). When appropriate, antibiotics were added to the medium at the following concentrations: kanamycin, 50 µg/ml for *E. coli* and 500 µg/ml for *S. pyogenes*; spectinomycin 100 µg/ml for *E. coli* and *S. pyogenes*; erythromycin 750 µg/ml for *E. coli* and 1 µg/ml for *S. pyogenes*. All mutations in *S. pyogenes* were stably maintained to the extent that culture for all functional assays did not require the addition of any antibiotics.

Manipulation of DNA

Plasmid DNA was isolated by standard techniques and transformed into *E. coli* by the method of Kushner (1978). *Streptococcus pyogenes* was transformed by electroporation as described previously (Caparon and Scott, 1991). Restriction endonucleases, ligases, and polymerases were used according to the recommendations of the manufacturers. Incompatible restriction fragment ends were subjected to ligation following treatment with T4 DNA polymerase to produce blunt fragment ends. Chromosomal DNA was purified from *S. pyogenes* as described previously (Caparon and Scott, 1991). Hybridization analyses utilized the method of Southern (1975) and appropriate DNA probes labeled with ³²P by a random priming method (Rediprime™, Amersham).

Construction of TnSpC

The insertion sequence IS256 derived from Tn4001 was modified as follows: PCR was used to amplify a 1.3 kb fragment from pISM2062 (Knudtson and Minion, 1993) using primers IS256Ev (AGTCA AGTCC AGACT CCTGT GTAAA ATGAT ATCCA ATGTT T) and M13 Reverse (GGACA CACTT TAACA ATAGG CGA). This modified IS256 by the introduction of a unique site for *EcoRV* (embedded in primer IS256Ev, underlined) immediately adjacent to the inside end of IS256 (replacing bp 1292–1297 of the IS256 sequence, DDBJ/EMBL/GenBank accession No. M18086). The resulting PCR product was introduced into the *HincII* site of pHSG396 by a TA-tail procedure (Douglas *et al.*, 1991) to generate pMGC57. A 1.2 kb *EcoRI*–*BamHI* fragment from pGEM7-spc containing the spectinomycin resistance determinant *aad9* (LeBlanc *et al.*, 1991) was inserted into the *EcoRV* site of pMGC57 to produce pMGC57-spc. The resulting version of IS256 is referred to as TnSpC (Figure 1).

Mutagenesis strategy

Mutagenesis with TnSpC was accomplished through the use of electroporation to introduce pMGC57-Spc into HSC16. Since the origin of replication of pMGC57-Spc does not function in *S. pyogenes*, all spectinomycin resistant transformants will have arisen from transposition of TnSpC into the streptococcal chromosome. Isolated colonies of transformants were patched onto skimmed milk agar medium and cultured as described above. Following an overnight incubation, colonies were examined for a reduction in SCP proteinase activity, which was apparent as a lack of clear zone, caused by the proteolytic cleavage of casein, surrounding the colony in the opaque medium.

Cloning of TnSpC insertions

Chromosomal DNA from HSC100 was isolated, digested with *Bgl*II and inserted into the *Bam*HI site of the low copy cloning vector pHSG575. Selection for the spectinomycin-resistance determinant of TnSpC in *E. coli* resulted in the identification of pBL11, which contains ~5.5 kb of streptococcal DNA sequence including *ropA*::TnSpC. Cloning of *ropB*::TnSpC utilized an inverse PCR technique with primers which anneal to sites in TnSpC on a chromosomal DNA template which had been digested with *Sph*I, size selected and subjected to ligation. The initial cloning utilized left arm primer SpcR (CTCTT GCCAG TCACG TTACG TTATT AG), right arm primer TnpL (CGCAT CTTCC CCAAT CAAAC ATCAG CC) and the long-range, high-fidelity polymerase KlentaqLA (Sigma) to obtain one chromosomal flanking region of *ropB*::TnSpC. Sequence data from this clone was used to identify the ORF found in the streptococcal genome database. Primers 3'ropB whole (CGCCC TGGAT CCGTT GAGAT AAA) and ropB5' whole (ATCGT GATAG CATGC ACAAC ACC) were constructed based on this predicted sequence and used to amplify the full-length *ropB*. The amplification product was inserted into a commercial TA cloning vector (pCRII, Invitrogen) to generate pBL42. Additional clones of chromosomal sequences were generated by PCR using probes designated according to the sequence data as described below.

Sequence analysis

DNA sequences were determined using a modified T7 DNA polymerase (Sequenase 2.0, Amersham) as recommended by the manufacturer using appropriate oligonucleotide primers. Analysis of these sequences was conducted using the Genetics Computer Group sequence analysis package (University of Wisconsin, Madison, WI) and sequences were compared to the Oklahoma group A streptococcal genome sequencing project (<http://www.genome.ou.edu/strep.html>). Homologues of the ORFs disrupted by TnSpC insertion were identified using a Gapped Blast algorithm (Altschul *et al.*, 1997) and the non-repetitive sequence database at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Disruptions of ropA

For additional disruption of *ropA*, pBL11 (which contains *ropA*::TnSpC) was modified using an 'inside-out' PCR technique with primers TnpL (CGCAT CTTCC CCAAT CAAAC ATCAG CC) and Tn4001R-out (GTACC GTAAA AGGAC TGTTA TATGG CC) in order to delete the gene which encodes the transposase of TnSpC. The plasmid (pBL12) containing the modified transposon (TnSpCΔ*tnp*) was converted to a linear molecule by digestion with *EcoRI* and used to transform wild-type strain HSC5. Deletion of transposase prevents further transposition of TnSpC; thus, all spectinomycin-resistant transformants arise via a double recombination event between homologous sequences shared by the linear fragment and the chromosome and results in the allelic replacement of *ropA* for *ropA*::TnSpCΔ*tnp*. Successful allelic replacement was confirmed using PCR and primers of the appropriate sequences and one strain (HSC110) was chosen for further analysis.

Construction of HSC130

An in-frame deletion allele of *ropA* was created by the method of Ji *et al.* (1996). Briefly, primers RopA5'XbaI (GCCAT AGTCA TCCGT CTAGA AATGC) and RopA3'PstI (CTCAT ATCAC TGCAG CTGCA CAAAT C) were used to amplify a fragment containing *ropA* which was inserted into the *Xba*I and *Pst*I sites of the streptococcal/*E. coli* shuttle vector pJRS233 using the *Xba*I and *Pst*I restriction sites embedded into the primer sequences (underlined). The resulting plasmid (pBL40) was used as template in an 'inside-out' PCR reaction with primers RopA-IFDup (CGTCA CAGCT GCTTC ATAA GCTT) and RopA-IFDown (GGAGC TGCGA TTGAA TTAGC AGT). Kinase treatment of this amplification product followed by subsequent re-ligation results in an in-frame deletion of the region of *ropA* which encodes amino acids

82–287. The sequence of the deletion was confirmed for the resulting in-frame deletion allele (*ropA*_{Δ82–297}) and the plasmid generated (pBL41) was used to replace *ropA* with *ropA*_{Δ82–297} in wild-type strain HSC5 by a method which makes use of the temperature sensitivity of the plasmid origin of replication as was described previously (Ji *et al.*, 1996; Ruiz *et al.*, 1998). Conformation of the *ropA* allelic replacement through PCR analysis and assays on skimmed milk medium verified that all progeny which contained *ropA*_{Δ82–297} had an identical phenotype as was described in the text above. One isolate was chosen for additional analyses and was designated HSC130.

Construction of HSC120

To control for any potential polar effects on transcription of genes distal to *ropA*, a strain was constructed to contain a polar insertion immediately downstream of an unaltered copy of *ropA*. To accomplish this, the full-length *ropA* was amplified using primers *ropA*5'*Xba*I (GCCAT AGTCA TCCGT CTAGA TAATG C) and *ropA*3'*Pst*I (CTCAT ATCAC TGCAG CTTGA CAAAT C). The resulting amplification product was inserted between the *Xba*I and *Pst*I sites of pHSG575 using the *Xba*I and *Pst*I sites embedded in the primers (underlined) to generate pBL19. Digestion of pBL19 with *Hind*III (which is within *ropA*) and *Pst*I generated a fragment containing the 1050 terminal bases of *ropA* and 150 bp of downstream sequence. The resulting fragment was inserted between the *Hind*III and *Pst*I sites of pHSG575. A 2.2 kb fragment containing the polar interposon element ΩKm-2 (Perez-Casal *et al.*, 1991) was then inserted into the *Eco*RI site of the resulting plasmid to generate pBL22. Integration of pBL22 into the chromosome of a wild-type strain (HSC5) by homologous recombination generates a strain (HSC120) which contains the strong polar ΩKm-2 element immediately downstream of an unaltered copy of *ropA*. This chromosomal structure was verified through PCR with appropriate primers.

Disruptions of *ropB*

For a directed disruption of *ropB*, a region internal to *ropB* contained on a 600 bp *Eco*RI fragment of pBL42 was inserted into the *Eco*RI site of the integration vector pCIV2 (Okada *et al.*, 1993). Integration of the resulting plasmid, pBL43, into the *ropB* chromosomal locus by homologous recombination generates an insertional inactivation of *ropB*. Verification of the chromosomal structure was accomplished with PCR using primers of the appropriate sequences.

SCP assays

The presence of the proprotein and processed forms of SCP was determined in a Western blot analysis of culture supernatants which had been subjected to SDS–PAGE followed by transfer to a PVDF filter. The amount of supernatant fluid analyzed for each culture was adjusted for cell number as determined by OD₆₀₀ of the culture prior to removal of the cells by filtration (0.45 µm Sterile Acrodisc, Gelman Sciences) and cultures were initiated from overnight growth in THY media which had been washed in phosphate-buffered saline to remove any residual proteinase. The initial OD₆₀₀ of cultures was adjusted to 0.01 and samples were removed at the indicated time points during incubation at 37°C. Western blots were developed with a rabbit SCP antiserum (a generous gift of Dr Lars Björck, University of Lund, Sweden) using an anti-rabbit horseradish peroxidase conjugate (Sigma) and a chemiluminescent substrate (SuperSignal, Pierce Chemicals). Reacting bands were visualized by exposure to film (Classic Blue Sensitive, Molecular Technologies). The proteolytic activity of SCP was quantitated as follows: cell-free culture supernatants were obtained by filtration (0.45 µm Sterile Acrodisc, Gelman Scientific) following overnight growth of the relevant *S. pyogenes* strains in THY medium. The resulting supernatants were then diluted with fresh THY medium to normalize for any differences in growth between the strains based on the OD₆₀₀ of the untreated overnight cultures. Proteinase activity in culture supernatants was then assayed by the method of Hauser and Schlievert (1990) which measures the increase in relative fluorescence generated by the proteolytic cleavage of FITC–casein (Sigma). The activity of uninoculated THY medium was used to derive background values and typically was undetectable under the conditions of this assay. Control for the specificity of the reaction for SCP included the routine addition of the cysteine proteinase-specific inhibitor E-64 (final concentration, 10 µM; Sigma) to selected samples. The treatment typically reduced activity by >95%.

RNA isolation and analysis

Overnight cultures of the relevant strains of *S. pyogenes* were diluted 1:100 in 10 ml of fresh THY medium and grown to early stationary phase at 37°C (~6 h). Streptococcal cells were harvested by centrifugation

(2500 g, 10 min, 4°C) and resuspended in 200 µl of diethyl pyrocarbonate treated-dH₂O. Total RNA was isolated by the method of Cheung *et al.* (1994) using a commercial reagent (FastPrep™ Blue, Bio 101) and a high speed reciprocating shaking device (FP-120, Savant Instruments). RNA concentrations were determined by absorbance at 260 nm and 10 µg samples from each strain were subjected to electrophoresis on a 1.2% agarose–0.66 M formaldehyde gel in a buffer consisting of 40 mM 3-[N-Morpholino]propanesulfonic acid (MOPS) (pH 7.0), 10 mM sodium acetate and 0.2 mM EDTA. Following electrophoresis, RNA was transferred to a nylon membrane (Hybond™-N⁺) as suggested by the manufacturer (Amersham) and hybridized with DNA probes labeled with ³²P as described above. The probe used for the data presented in Figure 4A consisted of a 550 bp DNA fragment which was amplified by PCR from HSC5 chromosomal DNA using primers *speB*1850for (CAAAG CAGGT GCAGC AAGCG CAG) and *speB*2400rev (ATAGC TGCAA ACAAG TTCTT AGG). These results were confirmed in separate experiments using a second *speB*-specific probe which was amplified from chromosome of HSC5 with primers *speB*360BI (CTTCT GCGCT TCGGG ATCCT GCTTT G) and *speB*-35PI (AAAAA CTGCA GGTGT TCACT GTCAA CTAAC CGT). Levels of *speB* transcription were normalized against input RNA using densitometry (NIH-Image; <http://rsb.info.nih.gov/nih-image/>) to compare the level of hybridization of each sample with the amount of 30S RNA present in the ethidium bromide-stained agarose gel prior to transfer to the membrane.

Cell fractionation

Protoplasts of *S. pyogenes* were prepared as described by Pancholi and Fischetti (1989) with the exceptions that THY medium for overnight culture was supplemented with 20 mM glycine and mutanolysin (1000 U/ml, Sigma) was used as the murelytic agent. Protoplasts were separated from the soluble cell-wall fraction by centrifugation (13 000 g, 10 min), resuspended in TE buffer (10 mM Tris–HCl, 1.0 mM EDTA pH 8.0) and then lysed following three cycles of freezing (–80°C) and thawing (37°C) by the addition of 10% SDS and 2% Tween-20 to final concentrations of 1.4% (w/v) and 0.34% (v/v), respectively. Incubation of the samples at 65°C for 1 h served to facilitate the lysis of the protoplasts, and to reduce the viscosity of the lysate. Separation of the insoluble membrane fraction from the cytoplasmic fraction was accomplished by centrifugation. It should be noted that in general, streptococci are difficult to lyse and require harsh conditions in order to develop fractions. Furthermore, the distribution of enzymes which are used to distinguish cellular fractions in other organisms may be distributed differently in *S. pyogenes* (Pancholi and Fischetti, 1992, 1998). Protein concentrations of the cell wall and cytoplasmic fractions were determined using the Bradford assay (Bradford, 1976) (Sigma) to allow for equal loadings on respective gels. Pelleted membranes were resuspended by boiling for 10 min in sample buffer (2% SDS, 100 mM dithiothreitol, 10% glycerol, 0.1% bromophenol blue, 50 mM Tris–HCl pH 6.8) and adjusted to equivalent concentrations based on the intensity of the staining pattern following SDS–PAGE and staining with Coomassie Blue.

Analysis of expression of other streptococcal proteins

Streptolysin O-dependent hemolytic activity for rabbit erythrocytes was determined as described previously (Ruiz *et al.*, 1998). The activity of various DNases was determined by the method of Rosenthal and Lacks (1977) which employs electrophoresis of culture supernatants through DNA-impregnated polyacrylamide gels. Protein F-dependent fibronectin-binding was quantitated using ¹²⁵I-labeled fibronectin as described elsewhere (Hanski and Caparon, 1992).

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