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

William R.Lyon, Carmela M.Gibson and Michael G.Caparon

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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., 1995; Lantz, 1996; Kidd, 1996; Wong, 1998). Many pathogenic microorganisms also produce extracellular proteinases which contribute to the ability of these microorganisms to cause disease (Poppen et al., 1995; Lantz, 1996). 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 processing 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 effect 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 supplicative 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-1β (IL-1β)-converting enzymes by cleaving the inactive IL-1β 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 preprotein, which is secreted extracellularly as a 40 kDa proprotein (Hauser and Schlievert, 1990). Under reducing conditions, the proprotein can undergo autolytic 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 autolytic 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 function 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, depletion of Trigger Factor has not previously been shown to have any affect 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...
incapable of replication in Gram-positive hosts. Thus, when the chimeric plasmid (pMG57-spc) is introduced into \textit{S. pyogenes}, spectinomycin-resistant transformants arise as a result of transposition of TnSpc into the streptococcal chromosome. Transformants were obtained at frequencies between $10^3$ and $10^5$/μg of pMG57-spc DNA depending on the streptococcal host strain employed and the resulting libraries contained only insertions of the modified transposon, since the construction of TnSpc prevents independent transposition of IS256. Furthermore, integration of the entire pMG57-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 TnSpc 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 \textit{speB} itself, as was shown through the use of a \textit{speB}-specific probe (data not shown).

\textbf{Characterization of the insertions in HSC100 and HSC101}

Further analysis concentrated on two of the mutants which contained a single insertion of TnSpc (HSC100 and HSC101). A combination of direct cloning of chromosomal DNA with selection for the TnSpc spectinomycin-resistance determinant in \textit{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 \textit{ropA} and \textit{ropB} (regulator of proteinase) for HSC100 and HSC101, respectively.

\textbf{Characterization of ropB}

Comparison of the predicted 280 amino acid translation product of \textit{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 \textit{gftG} which encodes the extracellular glucosyltransferase of \textit{Streptococcus gordonii} (Vickerman et al., 1997). In \textit{S. gordonii}, \textit{rgg} is located immediately adjacent to \textit{gftG} (Sulavik et al., 1992). An examination of the sequences adjacent to \textit{ropB} in \textit{S. pyogenes} revealed that like \textit{rgg}, \textit{ropB} is also located immediately adjacent to its regulatory target (\textit{speB}). However, in contrast to \textit{rgg}, \textit{ropB} is transcribed in a divergent manner from its regulatory target (Figure 2). A prominent feature of the intergenic region between \textit{rgg} and \textit{gftG} is a large inverted repeat which overlaps the promoter for \textit{gftG}, 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 \textit{gftG} inverted repeat or each other, two distinct sets of inverted repeats are present in the \textit{ropB}–\textit{speB} intergenic region. Similar to \textit{gftG}, the −35 region of the putative promoter for \textit{speB} is included in an inverted repeat. The importance of \textit{ropB} for expression of SCP was confirmed by additional mutagenesis using an integrational plasmid strategy to insertionally inactivate \textit{ropB} through homologous recombination (see Materials and methods). For 20 out of 20 isolates examined, insertional inactivation of \textit{ropB} resulted in a complete loss of proteolytic activity (data not shown).

\textbf{Additional characterization of ropA}

Comparison of \textit{ropA} to sequences in DDBJ/EMBL/GenBank indicated that RopA was highly homologous to Trigger Factor from \textit{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 (Goethel 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 \textit{in vivo} (Guthrie and Wickner, 1990). Thus, it was important to confirm that disruption of \textit{ropA} was responsible for the proteinase-deficient phenotype of...
W.R.Lyon, C.M.Gibson and M.G.Caparon

**Streptococcus gordonii**

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) [IRL(bp 1063–1091) and IRR(bp 1131–1103)] and the putative promoter regions of *speB* (DDBJ/EMBL/GenBank accession No. U63134) [IR1L(bp 1034–1047), IR1R(bp 1074–1061), IR2L(bp 1490–1506) and IR2R(bp 1540–1524)] are indicated by the arrows.

**Streptococcus pyogenes**

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 (*ropA1::TnSpcΔtnp*) 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* (*ropA1::TnSpc*) which had been cloned in *E.coli*. Because the modified element (TnSpcΔmp) is not capable of transposition, the resulting allele of *ropA* (*ropA1::TnSpcΔmp*) 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 protease (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 protease-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 (ΩKm-2) was placed immediately downstream of ropA. In the resulting strain (HSC120), ropA itself is intact; however, ΩKm-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 (ropAΔ82–297) 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 ropAΔ82–297) 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 protease 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 protease activity (as determined with skimmed milk agar plates) at a time 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 (ropAΔ82–297) 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 (ropAΔ82–297, 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 (ropAΩSpBL22), they did possess detectable activity (Figure 5). Consistent with the behavior of the ropA mutants on skimmed milk agar plates, a strain with the
in-frame deletion exhibited greater SCP activity than a strain with the transposon insertion (Figure 5, compare ropAΔ82–297 with ropA1::TnSpc). The activity of a ropB mutant was undetectable over background values (Figure 5, ropBΔ::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 (ropAΩBL22) 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 ropAΔ82–297 with wild-type and ropAΩBL22). In contrast, neither the precursor nor processed form was readily detectable in supernatants from the strain with a transposon insertion in ropA (Figure 6A, ropA1::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, ropAΔ82–297). 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-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, ropA1::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; ropB1::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, ropAΔ82–297). 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 ropA1::TnSpc with wild-type and ropAΔ82–297). 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
Regulation and processing of SCP

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
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, TnSpe should prove a useful addition to the repertory of transposable elements available for Gram-positive bacteria.

Mutagenesis with TnSpe was used to identify ropB, a transcriptional regulator with homology to rgg. In S.gordonii, rgg regulates the gene which encodes glucosyltransferase (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 glucosyltransferase 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 vivo (Talkington et al., 1993). This suggests that some mechanism for variation of SCP expression

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**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 [125I]fibronectin added to the assay. Results represent the mean and standard error of the mean of at least two independent experiments.
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.*, ropA::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 (ropA382–297) 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 (Zartn *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 (Zartn *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 Hugouvieux-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 insights 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 DH5α 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 K_{2}HPO_{4}, 0.4 mM MgSO_{4}, 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 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 K_{2}HPO_{4}, 0.4 mM MgSO_{4}, 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).

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 (Knudtzon and Minion, 1993) using primers IS256Ev (AGTCA AGTCC kanamycin, 50 μg/ml for E. coli and 500 μg/ml for S. pyogenes; spectinomycin 100 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.

Mutagenesis strategy

Mutagenesis with TnSpc was accomplished through the use of electroporation to introduce pMG57-Spc into HSC16. Since the origin of replication of pMG57-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 BglII and inserted into the BamHI site of the low copy cloning vector pHSG57. 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 ropA1::TnSpc. Cloning of ropB1::TnSpc utilized an inverse PCR technique with primers which anneal to sites in TnSpc on a chromosomal DNA template which had been digested with SpeI, size selected and subjected to ligation. The initial cloning utilized left arm primer SpeR (CTCTT GCCAG TCAG TTACG TTATG AG), right arm primer TnpL (CGCAT CTTCG CCAAT ATCAC ATCCG CC) and the long-range, high-fidelity polymerase KlenTaqLA (Sigma) to obtain one chromosomal flanking region of ropB1::TnSpc. Sequence data from this clone was used to identify the ORF found in the streptococcal genome database. Primers 3′ropB whole (CGCAT CTTCG CGTTG CCGTT AAAAA) and ropB5′whole (ATCGT TATGG 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 ropA1::TnSpc) was modified using an ‘inside-out’ PCR technique with primers TnpL (CGCAT CTTCG CCAAT ATCAC ATCCG CC) and Tn4001-out (GGAGC TGCGA TTGAA TTAGC AGT) which flank the ropA gene which encodes the transposase of TnSpc. The plasmid (pBL12) containing the modified transposon (TnSpcΔopt) 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 ropA1::TnSpcΔopt. 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 (GGCAT AGTCA TCCGT TTAGA AATTG) and RopA3′PstI (CTCAT ATCAC AGTCA TCCGT TTAGA CCAAT) were used to amplify a fragment containing ropA which was inserted into the XbaI and PstI 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-IDFup (GCTGA CAGCT GCTTC ATAA GCTT) and RopA-IDFdown (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 (ropAΔ352-297) and the plasmid generated (pBL41) was used to replace ropA with ropAΔ352-297 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 verified that all progeny which contained ropAΔ352-297 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 ropA5/XbaI (GGCAT GTCGA CTCGT CTAAG TAATG C) and ropA3/PstI (CTCAT ATACG TGCGA CTGTA CAAAT C). The resulting amplification product was inserted between the XbaI and PstI sites of pHSG575 using the XbaI and PstI sites embedded in the primers (underlined) to generate pBL19. Digestion of pBL19 with HindIII (which is within ropA) and PstI generated a fragment containing the 1050 terminal bases of ropA and 150 bp of downstream sequence. The resulting fragment was inserted between the HindIII and PstI sites of pHSG575. A 2.2 kb fragment containing the polar interposon element ŒKm-2 (Perez-Casal et al., 1991) was then inserted into the EcoRI 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 polar KanⅡelement 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 EcoRI fragment of pBL42 was inserted into the EcoRI 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 prophage 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 to have a similar absorbance at OD600 of the culture prior to removal of the SDS–PAGE gel. Proteins were visualized by exposure to film (Classic Blue Sensitive, Molecular Technologies). The proteolytic activity 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 presence of the proprotein and processed forms of SCP was determined as described previously (Ruiz et al., 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.

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References

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-DH5α. Total RNA was isolated by the method of Chiang 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+) according to 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 speB1850for (CAAGG CAGGT CGACG AAGCG CAG) and speB2400rev (ATACG TGGCA ACA GGCT TCTCT AGG). These results were confirmed in separate experiments using a second speB-specific probe which was amplified from chromosome of HSC5 with primers speB3606B1 (CTTCT CGCGT TCCGG ATTCG CCTGT G) and speB-35PI (AAAATA CTGCA GTTTG TCAGT GTCA CTAAAC 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 eubiotic 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 muregalytic 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 diithiothreitol, 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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