Spring 2002

Five Extracellular Proteins of Group A Streptococcus: Antigenicity and Gene Expression

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Five Extracellular Proteins of Group A *Streptococcus*: Antigenicity and Gene Expression

Submitted in partial fulfillment of the requirements for graduation with honors to the Department of Natural Sciences
Carroll College, Helena, Montana

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8 April 2002
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Acknowledgments

I would like to thank Sean D. Reid, Gerald F. Shields, Nicole M. Green, John Addis, Gail Sylva, Robert Cole, Samuel Alvey, James M. Musser and the National Institutes of Health for making this thesis possible.
Abstract

Five genes that encode novel extracellular proteins were previously identified through the genomic analysis of four Group A Streptococcus strains (serotypes M1, M3, M5, and M18) (Reid et al., 2001). Four of the five proteins have an LPXTG amino acid motif at the carboxyterminus, a motif that covalently links extracellular proteins to the cell surface in many gram-positive pathogens. All of the genes encode proteins with a secretion signal sequence at the aminoterminus. In vivo expression was demonstrated with Western blots using sera obtained from 80 patients with invasive infections, non-invasive skin infections, pharyngitis, and acute rheumatic fever. Real-time reverse transcriptase-PCR analysis of gene transcription showed that the amount and time of maximal gene transcript varies for the five genes among the M1, M3, and M18 serotypes. With the use of flow cytometry, surface expression was confirmed for proteins for which antibodies were available. These results indicate that further exploration of these genes as potential vaccine candidates is warranted.
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Introduction

Group A *Streptococcus* (GAS) is a gram-positive bacterial pathogen specific to the human population (Cue et al., 2000). It is the causative agent of several diseases, including pharyngitis, necrotizing fasciitis (commonly called the flesh-eating syndrome), and toxic shock syndrome. In addition, infection by GAS can lead to post-infectious sequelae such as acute rheumatic fever and rheumatic heart disease (Musser and Krause, 1998). Although GAS has been studied since the early 1930s, very little is known about GAS pathogenesis (Musser and Krause, 1998). Additionally, there has been a worldwide resurgence in GAS infection in the past 20 years (Cunningham, 2000). These two factors have led to a renewed interest in GAS in recent years.

Analysis of the genomes of four GAS strains (serotypes M1, M3, M5, and M18) recently led to the discovery of five genes (*spy0747, spy0843, spy0872, spy1361, and spy1972*) that encode novel extracellular proteins (Table 1), (Reid et al., 2001). The five proteins have conventional aminoterminal sequences that signal secretion, and four of the five proteins (*Spy0747, Spy0843, Spy0872, and Spy1972*) have a carboxyterminal LPXTG amino acid motif that covalently links many gram-positive virulence factors to the bacterial cell surface (Fischetti et al., 1990; Schneewind et al., 1992; Navarre and Schneewind, 1999; Reid et al., 2001). Sequencing and population genetic analysis of these genes in 37 GAS strains revealed restricted allelic variation, indicating that the proteins are highly conserved in the species (Reid et al., 2001). Western immunoblot analysis conducted with acute- and convalescent-phase sera obtained from four patients with invasive infections indicated that all five of the recombinant proteins were reactive with one or more of the sera, consistent with the hypothesis that these proteins are made during GAS infections (Reid et al., 2001). Taken together, these preliminary observations suggest that further analysis of these proteins is warranted.
The present study was undertaken to determine if these five proteins are expressed in multiple GAS disease types, and to assess if variation exists in the levels of gene transcript in different M serotypes. Evidence was sought for in vivo expression of these proteins in infected humans by Western immunoblot analysis of sera obtained from 80 patients with invasive infections, non-invasive soft tissue infections, pharyngitis, and rheumatic fever. Inasmuch as there is considerable genetic variation among GAS strains (Reid et al., 2001), transcript levels of the five genes at six points throughout the growth cycle were analyzed in three representative strains expressing serotype M1, M3, and M18 proteins. These strains were chosen for analysis because serotype M1 and M3 organisms commonly cause invasive infections worldwide (Musser and Krause, 1998; Cunningham, 2000), and M18 organisms have been implicated in outbreaks of pharyngitis and acute rheumatic fever (ARF) in the United States (Musser and Krause, 1998; Cunningham, 2000).

Materials and Methods

Genes analyzed. The genes analyzed (spy0747, spy0843, spy0872, spy1361, and spy1972; Table 1) were identified in a recent study of the genomes of four GAS strains (serotypes M1, M3, M5, and M18; Reid et al., 2001). Prior to that publication, none of the genes had been described.

DNA sequence analysis. Chromosomal DNA was isolated with the Puregene DNA Isolation kit (Gentra Systems). DNA sequencing primers were designed on the basis of M1, M3, and M18 genome data (Ferretti et al., 2001; Laboratory of Human Bacterial Pathogenesis, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health). Sequence data obtained from both DNA strands with an Applied Biosystems 3700 automated sequencer were assembled with DNAStar (Madison, Wisc.). Multiple-sequence alignment of the inferred amino acid sequences was performed with CLUSTALW (version 1.8)
(Thompson et al., 1994), and analysis of sequence polymorphisms was performed with MEGA II (Kumar et al., 2001).

Gene cloning and expression of recombinant glutathione-S-transferase (GST) fusion proteins. Cloning primers were designed on the basis of M1 genome data (Ferretti et al., 2001), and MGAS5005 (serotype M1) was used as the source strain for DNA. Full-length genes, minus the region encoding the putative signal secretion sequence, were cloned with the Univector Plasmid-fusion System (UPS) (Liu et al., 1998). The method employs bacteriophage P1 cre-lox site-specific recombination to catalyze in vitro plasmid fusion between the Univector containing the gene of interest, and a host vector (pHB2-GST) containing a GST tag. The method is described in detail elsewhere (Liu et al., 1998). Briefly, each gene was cloned into the pUNI-D vector in the same frame as the loxP site using vaccinia virus topoisomerase I-based cloning (Shuman, 1994). For these experiments, the pUNI-D vector adapted with topoisomerase I was obtained from Invitrogen, Inc., and PCR products of the genes of interest were inserted into the plasmid according to the manufacturer’s instructions. The pUNI-D clones were converted to glutathione-S-transferase fusions by combining 0.4 μg of the pUNI-D clone with pHB2-GST DNA in 20 μl of 1X Buffer S (50 mM Tris-Cl pH 7.5, 10 mM MgCl2, 30 mM NaCl, and 0.1 mg/ml BSA) on ice. Immediately after addition of 0.1 to 0.2 μg GST-Cre enzyme, the reaction mixture was incubated at 37°C for 20 min followed by 5 min at 65°C to inactivate the GST-Cre enzyme. Recombination products of the UPS were selected on Luria Bertani agar containing 50 μg/ml kanamycin after transformation of Escherichia coli (DH5α) by standard methods. Clones were sequenced to rule out the possibility of spurious mutations. To assess protein production, recombinant E. coli DH5α strains were grown at 37°C in 10 ml of Luria-Bertani broth supplemented with 50 μg/ml kanamycin. Cultures were induced at A600 = 0.5 with 0.2 mM – 0.5 mM IPTG followed by growth overnight at 25°C. Cells were pelleted by centrifugation, lysed, and analyzed by SDS-PAGE.
Western immunoblot analysis. A 1:20 dilution of each *E. coli* lysate containing recombinant protein was analyzed by SDS-PAGE, transferred to a nitrocellulose membrane (Millipore, Bedford, Ma.), and probed with patient sera. The sera studied included: convalescent-phase sera collected from nine patients with pharyngitis, paired acute- and convalescent-phase sera obtained from 27 patients with invasive GAS infections, paired acute- and convalescent-phase sera collected from 4 patients with superficial skin infections, and convalescent-phase sera obtained from 40 patients with a history of acute rheumatic fever (ARF). Convalescent-phase sera were collected approximately three weeks post-infection. In some cases, sera obtained from patients with a history of ARF were collected several years after the last documented presentation with ARF symptoms.

Recombinant proteins were transferred with a Bio-Rad semi-dry transfer chamber (Bio-Rad Laboratories) for 60 min at 15 V. Following transfer, the membrane was treated with a 5% w/v solution of dehydrated milk in blocking buffer (100 mM Tris-HCl, pH 7.4 and 150 mM NaCl) for 1 h. Primary antibody (patient serum) was added to the blocking reagent and the membrane was incubated for 1 h. The patient sera were used at a dilution of either 1:500 or 1:1000, depending on serologic reactivity. Goat anti-human affinity purified IgG (Bio-Rad, Hercules, Ca.) was used as the secondary antibody. Signal detection was conducted with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, Ill.)

**TaqMan real-time reverse transcriptase PCR analysis.** Cultures of representative GAS serotype M1 (MGAS5005), serotype M3 (MGAS315), and serotype M18 (MGAS8232) strains were grown in THY medium overnight at 37°C (5% CO₂). A 100 μl aliquot of each culture was added to 50 ml of THY medium, incubated at 37°C (5% CO₂), and harvested at six points ($A_{600} = 0.05, 0.1, 0.2, 0.4, 0.6, 0.8$) throughout the growth cycle. Total RNA was isolated at each time point as previously described (Chaussee et al., 2001).
The primers and probes used for each gene were designed on the basis of gene regions that were identical in all three strains. TaqMan assays were performed with an ABI 7700 instrument (PE Applied Biosystems, Framingham, Mass.) as described by Chaussee et al., (2001). Briefly, RT-PCR was performed with the TaqMan One-Step RT-PCR Master Mix Reagents kit (PE Applied Biosystems) as described by the manufacturer. The amplification profile used was as follows: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. The critical threshold cycle (Ct) is defined as the cycle at which fluorescence becomes detectable above background and is inversely proportional to the logarithm of the initial concentration of template. A standard curve was plotted for each reaction with Ct values obtained from amplification of known quantities of genomic DNA isolated from strain MGAS5005. The standard curves were used to transform Ct values of the experimental samples to the relative number of DNA molecules. The quantity of cDNA for each experimental gene was normalized to the quantity of the constitutively transcribed control gene (gyrA) in each sample. Specific transcript levels were expressed as fold-difference compared to gyrA, or the fold-difference between the conditions compared (Chaussee et al., 2001).

Purification of recombinant Spy0843 and Spy1972. Purified recombinant Spy0843 and Spy1972 were obtained with the B-PER GST Fusion Protein Purification Kit (Pierce, Rockford, Ill.) with slight modifications. One milliliter of resin was used for every 100 ml of bacterial culture. Benzonuclease (1 μl) (Novagen, Madison, Wisc.) was added for every ml of B-PER Reagent used to resuspend the cell pellet and phenylmethylsulfonyl fluoride was added to a final concentration of 25 mM.

Expression of Spy0843 and Spy1972 on the GAS cell surface. Surface expression of Spy0843 and Spy1972 was analyzed by flow cytometry (FACScaliber, Becton Dickinson, Mountain View, Cal.). Antigen-specific immune sera from rabbits immunized with purified Spy0843 and Spy1972 was obtained by affinity chromatography (Bethyl Laboratories, Montgomery, Texas). Flow
cytometry was performed with these purified antibodies. Purified rabbit IgG raised against an irrelevant protein antigen was used as a control for non-specific antibody binding. Briefly, GAS strain MGAS5005 (serotype M1) was grown to late-exponential phase ($A_{600} = 0.7$) in THY medium, harvested by centrifugation, washed twice in DPBS pH 7.2, and resuspended in DPBS at $1\times10^8$ cfu/ml. Anti-Spy1972 and anti-Spy0843 antibodies were added to 100 μl of bacterial suspension at a final concentration of 2 μg and incubated for 30 min on ice. DPBS containing 1% goat serum was added, the preparation was centrifuged, and phycoerythrin-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, Pennsylvania) was added to the bacterial cell pellet at a 1:400 dilution, and incubated on ice for 30 min prior to flow cytometry.

Results

DNA sequence analysis. All five genes (spy0747, spy0843, spy0872, spy1361, and spy1972) were sequenced in a serotype M1, M3, and M5 GAS strain (MGAS5005, MGAS315, and MGAS8232, respectively). Each serotype was found to contain a unique allele of each gene. The spy1972 gene was found to have the highest level of nucleotide divergence, averaging 34.7 nucleotide substitutions for every 3496 base pairs. There were low amounts of variation found in each of the five proteins at the amino acid level. The low end of this variation occurred in Spy0872, with an average of 4.0 amino acid replacements per 670 sites, while the high end of the variation was observed in Spy1972, averaging 17.3 amino acid replacements per 1165 sites. These levels of variation are similar to those found in seven GAS housekeeping genes (Enright et al., 2001). The spy0747, spy0843, spy0872, and spy1361 genes all had an average adenosine/thymidine (AT) content of 63%, a level consistent with a genome-wide AT content calculation of 61.5% (Ferretti et al., 2001). On the other hand, the AT content of spy1972 was lower than expected, at 56.9%.
Western immunoblot analysis. Western immunoblot analysis was conducted with paired acute- and convalescent-phase sera of 27 patients with invasive GAS infections and from four patients with superficial skin infections (Table 2). GAS infections were identified as invasive when positive bacterial culture results were obtained using tissue samples taken from a normally sterile site. Superficial skin infections are classified as non-invasive. The infecting strains represented 19 distinct M-protein serotypes (Table 2).

Spy0747, Spy0843, and Spy1972 reacted with 81% of the convalescent sera obtained from patients with invasive disease. These three proteins also reacted with 100% of the convalescent sera of patients with superficial skin infections (non-invasive disease). Antibodies in 67% of sera from convalescent-phase invasive disease reacted with Spy1361, a protein that also reacted to 50% of convalescent-phase sera of patients with superficial skin infections. Contrastingly, Spy0872 reacted with only 41% of invasive episode convalescent-phase sera and 50% of sera isolated from non-invasive episodes. TaqMan results show that recombinant Spy0872 was expressed at the same level as the other four recombinant proteins, thus levels in protein synthesis did not cause reduced reactivity. Also, very little sera from patients in the acute phase of disease reacted with the recombinant proteins. In those cases where acute-phase sera were reactive, the convalescent-phase sera were far more reactive.

Western immunoblot analysis was also conducted using convalescent-phase sera obtained from nine patients with recent pharyngitis and 40 patients with a history of acute rheumatic fever (ARF, Table 2). Reactivity of the recombinant proteins with antibodies in the 49 patient sera tested here was similar to the reactivity of the previous 31 patients with invasive disease or non-invasive superficial skin infections (Table 2). When tested with sera from pharyngitis patients, recombinant proteins Spy0747, Spy0843, and Spy1972 reacted with 89%, 78%, and 78% of those sera, respectively. Similarly, Spy0747, Spy0843, and Spy1972 reacted with ≥ 85% of the sera from patients with ARF. The reactivity of Spy1361 was less than that of the
previous three proteins, with 56% reactivity for pharyngitis sera and 70% reactivity observed in ARF sera, while Spy0872 was the least reactive of the five recombinant proteins. Less than 25% of the pharyngitis or ARF sera reacted with Spy0872.

*TagMan* real-time reverse transcriptase PCR analysis. The variation observed in the Western blot analysis led to an investigation in transcriptional variation for each of the five genes. Representative strains of GAS serotypes M1, M3, and M5 (MGAS5005, MGAS315, and MGAS8232, respectively) were selected for study, as serotypes M1 and M3 are found to be the most common causes of invasive GAS infections worldwide through the use of population genetic analysis (Musser and Krause, 1998) and serotype M18 has been identified in many pharyngitis cases and ARF outbreaks in the United States (Musser and Krause, 1998). RNA isolated at six optical density (OD) points throughout the growth curve ($A_{600} = 0.05, 0.1, 0.2, 0.4, 0.6, 0.8$) was used in equal amounts for this analysis. Two important observations were made.

First, the OD point of maximal gene transcript level differed among the three strains studied (Figure 1). For example, the maximal transcript level of the M18 *spy0747* allele occurred at an OD ($A_{600}$) of 0.4, in mid-exponential phase. On the other hand, the transcript level of the M1 and M3 alleles was highest in early stationary phase ($A_{600} = 0.8$).

Second, the level of gene transcript also varied among the three strains (Figure 1). For example, the *spy0843* transcript levels exceeded those of either the M1 or the M18 strains at all but the earliest OD point ($A_{600} = 0.05$). Similarly, the transcript levels of *spy0872* were fairly steady throughout the OD points until stationary phase ($A_{600} = 0.8$), when the M1 transcript levels increased more than 2.5-fold and the M3 and M18 transcript levels decreased. In general, the expression of the *spy1361* transcript increased throughout the growth curve, with the highest level of transcript recorded at $A_{600} = 0.6$ for the alleles present in the M1 and M3 strains. The transcript level of the allele of *spy1361* in the M18 strain was greatest at $A_{600} = 0.8$. Low transcript levels of all three alleles of *spy1972* were observed until $A_{600} = 0.6$ when the transcript level
increased ≥35-fold. Notably, the transcript level of \textit{spy1972} in the M1 strain remained greatly elevated at $A_{600} = 0.8$, whereas the transcript levels of the M3 and M18 alleles decreased to near-original levels.

\textit{Expression of Spy0843 and Spy1972 on the GAS cell surface.} Flow cytometry was used to confirm the presence of Spy0843 and Spy1972 on the surface of live GAS cells. The binding of purified anti-Spy0843 and anti-Spy1972 antibodies to GAS was demonstrated by the observation of increased fluorescence as compared with the fluorescence of the control antibody. Although each antibody recognized its respective antigen on the GAS surface, higher levels of anti-Spy0843 bound to the cell surface, as demonstrated by a substantial shift in fluorescence from the control antibody fluorescence.

\textbf{Discussion}

Group A \textit{streptococcus} was first classified in the early 1900s by Rebecca Lancefield (Lancefield, 1928; Lancefield and Todd, 1928; Dochez \textit{et al.}, 1919, and Hirst and Lancefield, 1939). Despite this early identification of GAS as a human pathogen, relatively little is known about GAS pathogenesis. This lack of knowledge is due to the considerable chromosomal, allelic, and serological diversity of GAS, as well as the complexity of the GAS-host interaction. Several studies have shown that GAS produces several extracellular proteins (Halbert \textit{et al.}, 1955a; 1955b; 1958; Halbert and Auerbach, 1960; Halbert and Keatinge, 1961, and Lei \textit{et al.}, 2000), though most of these proteins have not been identified. Potential antigens can now be identified through a genomic analysis as well as other post-genomic techniques (Grandi, 2001; Janulczyk and Rasmussen, 2001; Musser and Kaplan, 2001; Pizza \textit{et al.}, 2000; Reid \textit{et al.}, 2001; Lei \textit{et al.}, 2000, and Chakravarti \textit{et al.}, 2000a and 2000b). This present study was inspired by previous work in which five proteins were identified through a genomic analysis of three GAS strains (Reid \textit{et al.}, 2001). The gene sequence encoding these proteins included signal sequences that are associated with the
secretion of extracellular proteins. Due to their putative extracellular domain, these five proteins may be antigenic in nature and thus are potential vaccine candidates.

**DNA Sequence Analysis.** Although each strain studied contained a unique allele of the five genes in question, the variation at the amino acid level was comparable to the levels of variation identified in seven GAS housekeeping genes. This similarity indicates that these genes may be acted upon by selective pressure to maintain protein structure and function. Therefore, if the proteins elicit a protective immune response, the response may be effective against a wide range of GAS serotypes.

**Western Immunoblot Analysis.** The next step in characterizing these proteins was the determination of the proteins' expression during the course of GAS infection. Given that the specific array of genes expressed during the course of various GAS disease types may differ, the reactivity of human sera to the five proteins was investigated using sera obtained from patients with distinct infection types (pharyngitis, necrotizing fasciitis, acute rheumatic fever, etc.). Importantly, these Western immunoblot analyses demonstrated that all five proteins (Spy0747, Spy0843, Spy0872, Spy1361, and Spy1972) are produced during multiple GAS disease types. Thus, antibodies directed toward any one of these proteins may protect against multiple forms of GAS disease.

The protein Spy0872 had a lower percent reactivity with patient sera when compared to that of the other proteins analyzed. Despite the low overall reactivity to this protein, reactivity was observed with all serum samples obtained from patients infected with an M1 GAS organism. Notably, only the serum samples obtained from patients with serotype M1-caused infections were consistently reactive with all five of the recombinant proteins. Worldwide, GAS disease caused by serotype M1 organisms outnumbers disease caused by other GAS serotypes (Musser and Krause, 1998). The very low levels of amino acid variation in each of the five proteins, as found above, discount the hypothesis that this phenomenon of high M1 percent reactivity is due
to strain-specific variation in amino acid sequence. One possible explanation for the low reactivity of Spy0872 with patient sera is a lower level of transcription in many GAS serotypes leading to a reduction in protein synthesis and subsequent antibody production. Consequently, the high incidence of M1-caused GAS infection may be a result of comparatively higher levels of gene transcription and protein production, on average, in M1 serotypes than in other GAS serotypes.

*TaqMan* Real-Time Reverse Transcriptase PCR Analysis. Transcription levels of the five proteins in three GAS serotypes (M1, M3, and M18) were quantified with the use of TaqMan Real-Time Reverse Transcriptase PCR (TaqMan). Two important observations were made from this analysis – the transcript levels of the five genes in question differed among the three strains studied, as did the point of maximal gene expression. This suggests that, despite the high level of amino acid conservation, the role of each protein in infection may vary in different serotypes. This variation in the point of and amount of gene transcript levels may fundamentally alter the proteins' functions in host-pathogen interactions.

In tandem with the low percent reactivity of the Spy0872 protein with patient sera, relatively low levels of the *spy0872* gene were observed in the TaqMan analysis. Only the serotype M1 strain exhibited an increase in *spy0872* gene transcript levels throughout the growth cycle. Therefore, the decreased level of serological reactivity to this protein is possibly a result of relatively minimal transcription of the gene by non-M1 serotypes.

*Expression of Spy0843 and Spy1972 on the GAS Cell Surface.* Four of the five genes in question include an LPXTG amino acid motif on the carboxy-terminal. More than 50 extracellular proteins with this LPXTG motif have been described in gram-positive bacteria and many of these proteins are virulence factors (Fischetti, 2000). For example, in GAS this amino acid motif is present in M protein, M-like proteins, C5a peptidase, GRAB protein, serum opacity factor, a fibronectin-binding protein, streptococcal protective antigen, and two collagen-like proteins.
(Lukomski et al., 2000; Fischetti, 2000; Courtney et al., 1999; Dale et al., 1999; Rasmussen et al., 1999, and Lukomski et al., 2001). All of these described proteins are known to be accessible on the GAS cell surface. The M protein, C5a peptidase, serum opacity factor, a fibronectin-binding protein, and streptococcal protective antigen (the five GAS cell-surface proteins that have been extensively studied) are all virulence factors and contribute to protective immunity in mouse models (Courtney et al., 1999; Dale et al., 1999; Dale, 1999; Guzman et al., 1999, and Ji et al., 1997). These studies, combined with the present study's confirmation of the presence of the proteins Spy0843 and Spy1972 (both of which contain the LPXTG motif) on the GAS cell surface, indicate that the proteins Spy0843 and Spy1972 may be important virulence factors. Hence, it will be important to determine if immunization with Spy0843 and Spy1972 can stimulate a protective immune response in animal infection models.

In summary, the five novel proteins in question, Spy0747, Spy0843, Spy0872, Spy1361, and Spy1972, were found to be produced in human hosts during the course of GAS infection and each of the GAS serotypes studied exhibited differential expression of the genes encoding these five proteins. Furthermore, direct evidence for the location of two of the proteins, Spy0843 and Spy1972, on the GAS cell surface was obtained. These results indicate that further investigation into these proteins as potential vaccine candidates is warranted.
References


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Table 1. Chromosomal location and putative function of 5 Group A *Streptococcus* genes.

<table>
<thead>
<tr>
<th>Gene (spy No.)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Putative function of encoded protein&lt;sup&gt;b&lt;/sup&gt;</th>
<th>LPXTG&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>spy0747</em></td>
<td>Extracellular nuclease</td>
<td>LPKTS</td>
</tr>
<tr>
<td><em>spy0843</em></td>
<td>Cell surface protein</td>
<td>LPRTG</td>
</tr>
<tr>
<td><em>spy0872</em></td>
<td>2',3'-cyclic-nucleotide 2'-phosphodiesterase</td>
<td>LPITG</td>
</tr>
<tr>
<td><em>spy1361</em></td>
<td>Internalin homolog</td>
<td>No</td>
</tr>
<tr>
<td><em>spy1972</em></td>
<td>Pullulanase</td>
<td>LPKTS</td>
</tr>
</tbody>
</table>

<sup>a</sup> spy No. refers to the corresponding numerical identifier of each ORF as listed in GenBank.

<sup>b</sup> Putative function as determined by BLAST analysis.

<sup>c</sup> Amino acid motif used to anchor proteins to the cell wall in gram-positive organisms.

Single letter amino acid abbreviations are used (L, leucine; P, proline; K, lysine; R, arginine; I, isoleucine; T, threonine; G, glycine)
Table 2. Percent immunoreactivity of recombinant GAS proteins with human sera obtained from patients with GAS infections.

<table>
<thead>
<tr>
<th>Protein (Spy No.)</th>
<th>Invasive (n=27)a</th>
<th>Non-Invasive (n=4)b</th>
<th>Pharyngitis (n=9)c</th>
<th>ARF (n=40)d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spy0747</td>
<td>85%</td>
<td>100%</td>
<td>89 %</td>
<td>85 %</td>
</tr>
<tr>
<td>Spy0843</td>
<td>78</td>
<td>100</td>
<td>78</td>
<td>90</td>
</tr>
<tr>
<td>Spy0872</td>
<td>41</td>
<td>50</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>Spy1361</td>
<td>67</td>
<td>50</td>
<td>56</td>
<td>70</td>
</tr>
<tr>
<td>Spy1972</td>
<td>81</td>
<td>100</td>
<td>78</td>
<td>88</td>
</tr>
</tbody>
</table>

a Convalescent-phase sera isolated from 27 individuals with invasive GAS disease. An infection was classified as invasive if a positive GAS culture was obtained from a normally sterile site.

b Convalescent-phase sera isolated from 4 individuals with non-invasive GAS disease (superficial skin infection).

c Convalescent-phase sera isolated from 9 individuals with recent episodes of GAS pharyngitis.

d Convalescent-phase sera isolated from 40 individuals with a history of ARF.
Figure 1. Fold increase in gene transcript levels assessed by TaqMan assays at 6 optical density (OD) points throughout the growth cycle. Overnight cultures of representative GAS serotype M1 (MGAS5005), serotype M3 (MGAS315), and serotype M18 (MGAS8232) strains were diluted 1:500 in 50 mL of THY medium, incubated at 37°C (5% CO₂), and harvested at 6 OD points (A₆₀₀ = 0.05, 0.1, 0.2, 0.4, 0.6, 0.8) throughout the growth cycle. cDNA prepared from representative strains of serotype M1 (MGAS5005), M3 (MGAS315), and M18 (MGAS8232) was quantified for spy0747 (putative extracellular nuclease), spy0843 (putative cell surface protein), spy0872 (putative 2',3'-cyclic-nucleotide 2'-phosphodiesterase), spy1361 (Listeria monocytogenes internalin homolog), and spy1972 (putative pullulanase). All measurements were normalized to gyrA as described in Materials and Methods. Values are expressed as the fold increase in transcript compared to the transcript level at A₆₀₀ = 0.05. The data represent values obtained with at least two independently isolated RNA samples analyzed in triplicate. Note that spy0747, spy0872, and spy1361 have the same Y-axis scale, whereas spy0843 and spy1972 have different Y-axis scales.
Fold increase in transcript

**Foid increase in transcript**

**spy0747**
(Extracellular nuclease)

**spy0872**
(phosphodiesterase)

**spy1361**
(internalin homolog)

**spy0843**
(cell surface protein)

**spy1972**
(pululianase)