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Identification of *isp*, a Locus Encoding an Immunogenic Secreted Protein Conserved among Group A Streptococci

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The protein Mga (*mga*), which is required for transcription of several virulence genes of group A streptococci (GAS), including the antiphagocytic M protein, was suggested to act as the response regulator element of a bacterial two-component pathway. To investigate whether a gene encoding a cognate sensor protein is located upstream of *mga*, 3.1 kb of DNA 5′ of the *mga* translational start site was cloned from serotype M6 GAS strain JRS4. Sequence analysis of this region revealed two adjacent open reading frames, a previously described *orf* and a new locus, *isp* (immunogenic secreted protein), which could encode proteins of 9 and 59 kDa, respectively. Inactivation of either open reading frame had no significant effect on transcription of the gene encoding M protein (*emm*) under normal growth conditions, suggesting that neither *isp* nor *orf* is involved in the Mga regulatory circuit. A protein migrating at an apparent molecular weight of 65,000 was produced when *isp* was transcribed and translated in vitro. The predicted *isp* product (Isp) contains an amino-terminal signal sequence region homologous to that found in bacterial secreted proteins, and expression of *isp* in *Escherichia coli* resulted in the presence of Isp in the periplasmic fraction. Convalescent-phase serum from a patient with an active GAS infection recognized forms of Isp both from the periplasm of *E. coli* and the supernatant of a GAS strain. Both *isp* and *orf* are highly conserved among strains of GAS, as shown by hybridization analyses.

Members of the beta-hemolytic streptococci are differentiated into immunologic groups designated A through O based upon the presence of a specific carbohydrate antigen on their surface (17). Many of the streptococcal strains causing human infections belong to group A. The group A streptococci (GAS) are divided into more than 80 serotypes based on the immunologically variable M protein, an antiphagocytic molecule found on the bacterial surface. GAS produce a variety of suppurative diseases including acute pharyngitis, cellulitis of the skin, impetigo, and erysipelas. If these primary infections are not treated promptly, more serious sequelae such as glomerulonephritis and rheumatic fever can follow. In addition, GAS can cause severe invasive diseases such as streptococcal toxic shock syndrome, myositis, and necrotizing fasciitis (36). The ability of GAS to grow at different locations in the host suggests an environmentally responsive control of its virulence determinants.

The protein Mga is required for the expression of several known and potential virulence factors in GAS, including the antiphagocytic M protein (4, 6, 12, 26, 27). On the basis of homology, it has been postulated that Mga acts as the second element (response regulator) of a bacterial two-component signal-transducing pathway (22). This type of system (1) often involves a membrane-associated sensor protein that is autophosphorylated at a specific histidine residue in response to a change in the external environment. The signal is transduced by transferring the phosphate group to a conserved aspartate residue found within a cognate response-regulator protein. This activates the latter to bind to DNA and initiate the transcription of specific genes. The proposed role of Mga as a response regulator is supported by the environmental regulation of the transcription of *emm*, the gene encoding M protein (3, 19).

The genes encoding the sensor and regulator components of bacterial signal transduction pairs are often tightly linked and may form an operon (1). In all of the different strains of GAS analyzed, the region downstream of *mga* is always occupied by genes regulated by Mga (14, 25, 39). Very little is known about the region upstream of *mga* in the GAS chromosome. To investigate whether a gene encoding a cognate two-component sensor protein is located upstream of *mga*, we sequenced 3.1 kb of DNA from this region and began the characterization of its products.

MATERIALS AND METHODS

Bacterial strains and media. The *Escherichia coli* and GAS strains used in this study are listed in Table 1. *E. coli* was grown in Luria broth (31), and GAS were grown in Todd-Hewitt medium supplemented with 0.2% yeast extract (THY). Antibiotics were used at the following concentrations: ampicillin at 50 μg/ml for *E. coli*, erythromycin at 750 μg/ml for *E. coli* and 1 μg/ml for GAS, kanamycin at 30 μg/ml for *E. coli* and 500 μg/ml for GAS, spectinomycin at 100 μg/ml for *E. coli* and GAS, and chloramphenicol at 50 μg/ml for *E. coli* and 5 μg/ml for GAS.

DNA manipulations. Plasmid DNA was prepared from *E. coli* by alkaline lysis (2) followed by either phenol-chloroform extraction or equilibrium centrifugation through an ethidium bromide-cesium chloride gradient. Chromosomal DNA was prepared from GAS (8) following growth in THY supplemented with 20 mM glycine.

Nucleotide sequencing and analysis. Both strands of the approximately 3.1 kb of DNA upstream of *mga* in plasmid pJRS1020 (Table 1) were sequenced by the method of Sanger et al. (29), using Sequenase. Homology searches were performed on all available protein databases (Brookhaven protein database April 1994 Release, Swiss-Prot Release 31.0, PIR Release 45.0, and Genpept. Release 90) with both the FASTP (BLAST) and FASTA (GCG) algorithms, using the GCG Wisconsin package interface (WPI) software.

Construction of *orf-1*, *isp-1*, and *isp-2* mutant GAS strains. The *isp-1* mutation was constructed by insertion of the ΩKm-2 interposon (22) into the *Nco*I site of pJRS1020, resulting in plasmid pJRS2004Ω (Table 1). Disruption of *isp* in the GAS chromosome was done as follows. An 865-bp *Eae*I fragment from pJRS1020 (Fig. 1) was cloned into the temperature-sensitive shuttle vector pJRS233 (Table 1), resulting in plasmid pJRS2110 (Table 1). pJRS2110 was introduced into JRS145 by electroporation, and the cells were grown at a tem-

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TABLE 1. Bacterial strains and plasmids

Plasmid or strain	Relevant characteristic(s)	Source or reference
Plasmids		
pJRS1020	4.6-kb *Hin*dIII-*Xba*I fragment (Fig. 1) of JRS4 chromosome in pUC18	5
pJRS2004Ω	*isp-1*, ΩKm-2 inserted into *Nco*I restriction site of pJRS1020 (Fig. 1)	This study
pJRS2005Ω	*orf-1*, ΩKm-2 inserted into *Afl*II restriction site of pJRS1020 (Fig. 1)	This study
pJRS233	Temperature-sensitive shuttle/suicide vector	23
pJRS2110	865-bp *Eae*I fragment from pJRS1020 in pJRS233	This study
pJRS2200	3.9-kb *Sac*I-*Sal*I fragment from pJRS2005Ω in pJRS233	This study
pSP73	T7 promoter expression vector	Promega
pJRS2003	3.1-kb *Spe*I fragment of pJRS1020 in T7 promoter vector pSP73, *isp* expression in *E. coli*	This study
pBRΩKm-2	ΩKm-2 interposon in pBR322	22
Strains		
S. pyogenes		
JRS4	*emm6*	32
JRS145	P*emm-cat86*	3
JRS2110	P*emm-cat86 isp-2*	This study
JRS2200	P*emm-cat86 orf-1*	This study
E. coli		
DH5α	*hsdR17 recA1 endA1 relA1*	BRL[a]
BL21(DE3)	*ompT hsdS gul* (λDE3 *lacI lacUV5 lacZ'* T7-*rnaP*)	37

[a] GIBCO Bethesda Research Laboratories, Inc.

perature (30°C) permissive for plasmid replication, with selection for erythromycin resistance. Chromosomal integration of the plasmid via homologous recombination was selected for at the nonpermissive temperature (37°C) to produce the *isp-2* strain JRS2110 (Table 1). The presence of the *isp-2* insertion mutation was verified by Southern analysis of *Hin*dIII-digested JRS2110 genomic DNA, using the *isp*-specific probe described under "Southern blot analysis" (data not shown).

Inactivation of *orf* was accomplished as follows. A 2.1-kb *Sma*I fragment containing the ΩKm-2 interposon from pBRΩKm-2 (Table 1) was cloned into the blunted *Afl*II site of pJRS1020 (Fig. 1), producing the *orf-1* allele in pJRS2005Ω. A 3.9-kb Klenow fragment-treated *Sac*I-*Sal*I fragment containing the *orf-1* allele from pJRS2005Ω was cloned into the *Sma*I site of pJRS233, resulting in pJRS2200. pJRS2200 was integrated into the JRS145 chromosome as described above for *isp-2*. The *orf-1* sequences were exchanged for the homologous wild-type sequences to produce the *orf-1* strain JRS2200. The *orf-1* mutation was verified by Southern analysis of *Asp*718-digested JRS2200 genomic DNA, using a radiolabeled 1.5-kb *Bam*HI fragment from plasmid pLZ12-21K (22) containing the *aphA3* gene of the ΩKm-2 interposon as a probe (data not shown).

In vitro transcription-translation. [^{35}S]methionine-labeled proteins were produced from DNA templates in vitro using the prokaryotic transcription/translation kit (Amersham) as described by the manufacturer. Products were separated on a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel and detected by autoradiography for 18 h.

GAS growth conditions and CAT assays. GAS strains were grown to stationary phase in liquid THY at 37°C without agitation as previously described (3). Cell extracts were prepared from a constant number of cells (determined by cell count). Activity of chloramphenicol acetyltransferase (CAT) in lysate supernatants was assayed by the method of Shaw (33) as previously described (3).

Expression and localization of Isp in *E. coli*. *E. coli* BL21(DE3) cells (Table 1) harboring either pSP72 or pJRS2003 (Table 1) were grown to early logarithmic phase (optical density at 600 nm of 0.2), and the production of T7 RNA polymerase was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM). Induction was continued at 30°C for 2 h. Samples for Western blot (immunoblot) analysis were processed as described below. Release of periplasmic proteins was accomplished as previously described (10).

Preparation of GAS whole-cell extracts. Cell extracts were prepared as outlined earlier (19). Briefly, cells were pelleted, washed once in 30 μl of 20 mM phosphate buffer, pH 7.0, resuspended in 30 μl of phosphate buffer, and lysed with 2.5 U of mutanolysin (Sigma) per μl for 1 h at 37°C. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min.

Western blot analysis. Whole-cell extracts of *E. coli* were obtained by boiling cells in SDS loading buffer (16) for 3 min. GAS supernatants were passed through a 0.45-μm-pore-size filter, and proteins were precipitated with 15% tricarboxylic acid on ice for 30 min. Following centrifugation, pellets were washed twice with ice-cold acetone to remove residual tricarboxylic acid and concentrated proteins were resuspended in SDS loading buffer. Samples were separated on a 12.5% polyacrylamide gel containing SDS, transferred to nitrocellulose membranes, and reacted with serum obtained from a patient with an active GAS throat infection. Prior to use with *E. coli* extracts, the serum was adsorbed twice with E. coli BL21(DE3) whole cells and once with sonicates of the same cells. Antigen-antibody complexes were visualized as described previously (24).

Southern blot analysis. Chromosomal DNAs from the different GAS strains were digested with *Hin*dIII, separated on a 0.8% agarose gel, and transferred to a nylon membrane. Hybridization was carried out at 42°C with no formamide, and the blot was washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 50°C. The *isp* probe was the 1.07-kb *Pvu*II fragment from pJRS1020 (Fig. 1) located internal to the *isp* coding region. The *orf* probe was a 241-bp *Ava*I-*Mbo*II fragment from pJRS1020 encompassing the 3′ portion of the *orf* coding region and including the *orf*-specific primer oyl6.

PCR assays. Crude cell lysates of streptococcal strains (15) were used for PCR assays. Primer pairs consisted of CAGTGCAGCGCCATTAGTTG (oyr20; 5′-*isp*) combined with either GGTGGTCACAAAGCCTG (oyl7; 3′-*isp*) or CCT AGTTTTAGGAGGTA (oyl6; *orf*) (Fig. 1).

Nucleotide sequence accession number. The sequence data have been assigned GenBank accession number U31811.

RESULTS

Sequence and analysis of the region upstream of *mga* reveals two open reading frames. Plasmid pJRS1020 (Table 1) contains a 4.6-kb *Hin*dIII-*Xba*I fragment of the chromosome from the serotype M6 strain JRS4, including most of *mga* and approximately 3.1 kb of DNA upstream of its start of translation (Fig. 1). The 482 nucleotides immediately 5′ to *mga* are required for its transcriptional regulation and do not contain an open reading frame (21, 22). The sequence of the DNA

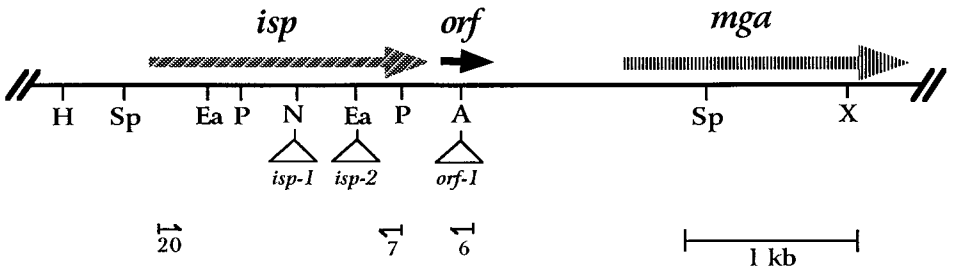


FIG. 1. Location of open reading frames, insertion mutations, and PCR primers on GAS DNA. Shown is a 5-kb region of serotype M6 GAS strain JRS4 chromosomal DNA including *mga* (22) and upstream DNA sequences derived from this study. Arrows above the line indicate open reading frames. Triangles represent sites of insertion that create the *isp-1*, *isp-2*, and *orf-1* mutant alleles. The *isp-1* allele is carried on plasmid pJRS2004Ω, while *isp-2* and *orf-1* were exchanged for wild-type GAS chromosomal sequences in strains JRS2110 and JRS2200, respectively. Locations of primers (20, oyr20; 6, oyl6; 7, oyl7) used for PCR assays are shown below the line. Restriction sites shown are as follows: A, *Afl*II; Ea, *Eae*I; H, *Hin*dIII; N, *Nco*I; P, *Pvu*II; Sp, *Spe*I; and X, *Xba*I.

further upstream of this region was determined and revealed two open reading frames of 252 (*orf*) and 1,605 (*isp*) nucleotides.

The smaller ORF was proximal to *mga*, and its translation predicts a protein of 83 amino acids with a molecular mass of 9.7 kDa. This coding sequence showed 98% DNA homology to an open reading frame of unknown function upstream of the *mga* gene from a serotype M49 GAS (26) which was called *orf* (Fig. 1). *orf* has a potential Shine-Dalgarno box (GAGGT) (34) 8 nucleotides upstream of its start of translation. A 23-bp inverted repeat followed by a T-rich region is located immediately downstream of the stop codon, which is a characteristic of factor-independent transcriptional termination sites. No consensus −10 or −35 element was apparent upstream of *orf*.

The larger open reading frame, located 5′ to *orf* (Fig. 1), predicts a protein of 534 residues with a molecular mass of 58.9 kDa and was termed *isp* for reasons described below. A Shine-Dalgarno box (AGGAG) was found 12 bp upstream of the *isp* start codon and is within the optimal range of spacing for gram-positive organisms (13). Since there are no significant inverted repeats characteristic of transcriptional terminators in the 76-bp *isp-orf* intergenic region, it is possible that *isp* and *orf* are in the same operon. Several potential −10 and −35 elements are present in the region upstream of *isp*, although determination of promoters in GAS by sequence alone is unreliable due to the high A+T content of the organism.

Characterization of the potential Isp and Orf proteins. The predicted amino acid sequences of both *isp* and *orf* were used to search the protein databases (see Materials and Methods), using both the FASTA (GCG) and FASTP (BLAST) algorithms. While Orf did not exhibit significant homology to any proteins in the database, Isp could be divided into four discrete regions based on homology (Fig. 2).

Domain A (amino acids 1 to 23) closely resembles amino-terminal signal sequences for secreted proteins from both gram-positive and gram-negative organisms (Fig. 2A). It contains positively charged amino acids followed by a core of hydrophobic residues. The highest homology in this domain (50% identity across the 23 residues) was to Usp45 (unknown secreted protein) of *Lactococcus lactis*.

Domain B (amino acids 23 to 178) showed limited homology to repeat regions within several different proteins from a number of eukaryotic organisms. However, no direct or inverted repeat sequences were identified in the domain B region of Isp when compared with itself by dot plot analysis (data not shown). The majority of homologous proteins were surface antigens rich in proline, serine, aspartic acid, and glutamic acid. The bacterial protein which matched most closely was the M protein of *Streptococcus equi* (Fig. 2B).

Domain C (amino acids 178 to 416) showed no significant homology with proteins in the database.

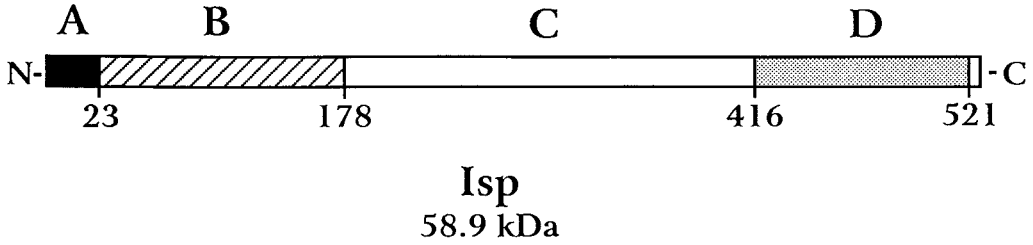
Domain D (amino acids 416 to 521) is found at the C terminus of Isp. The highest homology both within this region and throughout the entire protein (56% identity across 105 residues) is to TrsG/TraG (9, 20). This protein is encoded by several conjugative plasmids in *Staphylococcus aureus* and is required for transfer of these plasmids (Fig. 2D). In addition, Usp45 from *L. lactis* and an unnamed open reading frame upstream of the *lsp* gene in *Staphylococcus carnosus* showed 50 and 33% homology, respectively (Fig. 2D). No consensus gram-positive cell wall binding domain (LPXTG) (30) was detected in the C-terminal portion of Isp.

In vitro transcription-translation of *isp* and *orf*. To learn whether *isp* and *orf* produce protein products, an *E. coli* extract was used to transcribe and translate these genes in vitro. Plasmid pJRS1020 (Table 1), which contains both genes, generated a product that migrated with an apparent molecular weight of 65,000 on SDS-PAGE (Fig. 3, lane D). Inactivation of *isp* was accomplished by insertion of the ΩKm-2 interposon (22), which prevents transcriptional and translational readthrough (28), into the *isp* coding sequence in pJRS1020 to generate the *isp-1* allele (see Materials and Methods). The 65-kDa product was absent from reactions programmed with either the *isp-1* plasmid pJRS2004Ω (Table 1) or the pUC18 vector control (Fig. 3, lanes A and C). The predicted molecular mass for Isp is 59 kDa, and the slower mobility of the observed band may result from the high proline content of the protein (11). The ca. 49-kDa product observed only in *isp-1* extracts (Fig. 3, lane C) may represent a truncated Isp resulting from translation of the N-terminal portion of the protein. Two additional protein bands migrating with apparent molecular weights of 40,000 and 43,000 were observed in the isp^+ extracts (Fig. 3, lanes B and D) but were absent from the *isp-1* extracts (Fig. 3, lanes A and C). These and other lower-molecular-weight bands may be breakdown products of Isp. Disruption of *orf* was accomplished by inserting the ΩKm-2 interposon into the *orf* coding sequence of pJRS1020 to produce the *orf-1* allele (see Materials and Methods). All proteins produced from pJRS1020 in vitro were also made from the *orf-1* (see Materials and Methods) plasmid pJRS2005Ω (Table 1; Fig. 3, lanes B and D).

Neither *isp* nor *orf* is involved in environmental activation of *emm* transcription. To investigate the function of *isp* and *orf* in GAS virulence, chromosomal insertions within the respective coding sequences were constructed in JRS145 (Table 1), a strain that has the *cat86* gene fused to the promoter of the structural gene for the M protein (*emm*) in the chromosome (P*emm-cat86*). In order to disrupt *isp*, an internal *Eae*I fragment (Fig. 1) was cloned into the temperature-sensitive shuttle vector pJRS233, resulting in plasmid pJRS2110 (Table 1). Integration of this plasmid into the JRS145 chromosome inactivated *isp*, producing the *isp-2* strain JRS2110 (Table 1; see Materials and Methods). A fragment containing *orf-1* was cloned into pJRS233 and exchanged for the homologous wild-type sequences in the JRS145 chromosome, producing the *orf-1* strain JRS2200 (Table 1). Southern blot analysis was used to verify the insertion mutations (data not shown; see Materials and Methods).

If phosphorylation of Mga is required to activate P*emm* transcriptional activity and either *isp* or *orf* encodes a sensor protein, we would expect their mutant phenotypes to resemble that of an *mga* mutant. Insertional mutation of *mga* in JRS145 results in a decrease in P*emm*-specific CAT activity to levels that are not detectable (3). Western blot analysis indicates a reduction of at least 50-fold in *emm* expression (4). Therefore, to determine if either *isp* or *orf* is involved in the Mga-dependent activation of P*emm*, CAT assays were performed on the two mutant strains JRS2110 (*isp-2*) and JRS2200 (*orf-1*), and results were compared with those for JRS145 (P*emm-cat86*). The average CAT activities (units of CAT per milligram of total protein) were 31.5 ± 2.4 for JRS145, 47.8 ± 8.0 for JRS2110, and 56.6 ± 6.7 for JRS2200. Thus, no decrease in CAT activity was observed. It is not clear if the small increase observed (1.5-fold and 1.8-fold for *isp-2* and *orf-1*, respectively) is significant.

Isp is immunogenic and antibodies to Isp are present during an active streptococcal infection. Because Isp contains a potential signal sequence and is homologous to numerous surface and secreted proteins, we hypothesized that it might interact with the human host during the infectious process. Serum from a patient with an active GAS throat infection was used to probe whole-cell extracts made from *E. coli* BL21(DE3)/pJRS2003, which expresses *isp* from the T7 promoter. Following induction



A.

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Isp S. pyogenes:   (1) MKKRKLLAVTLLSTILLNSAAPL (23)
Usp45 L. lactis:   (1)  m:K:IISai:M::VI:SA:::: (22)
Prt L. lactis:     (2) qRKK:g:SIl:ag:Va:gAl:v: (24)
TraT E. coli:      (2) ::t:::Mm:a:V:St:al:gcga (24)
Cbf2 C. jejuni:    (1) :::fs:V:a::IAgVV::vn:at (23)
SacC B. subtilis:  (1)  m:KR:Iq:mIMf:L::tm:fsa (22)
FimA E. coli:      (1)  m:i:t::IvV::aLs:S:t:a: (22)
LukS S. aureus:    (1) :l:n:I::t::svSL:apl:n:: (23)
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B.

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Isp  S. pyogenes:     (129) ESQNKDGRPTPSPDQQKDQTPDKTPEKGPEKAAEKTPEPNRDAPKPIQPP (178)
Ssp T. parva:         (254) Dg:Dsq:t:e:q:Ep:pEpq:Epq::pq::pqp:pq:::qpEpqpep::e (400)
Parp T. brucei:       (203) DTngt:pd:e:e:EpEpEpe:Epe::pe::pep:pe:::epEpepepE:e (349)
Vsp1 C. reinhardtii:  (666) --------:S:::EpEvEdS:spS::pev:dSpspS:::mdES:a:eps: (803)
M prot. S. equi:      (263) peakpepK:e:k:EpKpEpk:Epk::pk::pkp:pk:::kpEpkpkp::- (315)
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D.

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Isp S. pyogenes:   (416) YKSETYSFGQCTWYAYNRVKELGYQVDRYMGNGGDWQRKPGFVTTHKPKVGYVVSFA (472)
TrsG S. aureus:    (242) :ngQS:pW::::::vHq:r::I:kp:pltw::::::aKaq:Wevgs:::A:RGA:VK (298)
TraG S. aureus:    (242) :ngQS:pW::::::vHq:r::I:kp:pltw::::::aKaq:Wevgs:::A:RGA:VK (298)
Orf S. carnosus:   (152) -----:ek::::Y::FDK::Kd:nmIsnrw:DaeN:aKRd:Y:vn:::a:haLMqtt (208)


Isp  S. pyogenes:  (463) PGQAGADATYGHVAVVEQIKEDGSILISESNVMGLGTISYRTFTAEQA (521)
TrsG  S. aureus:   (289) ::NF::ppp:::Imf::KV:K::g:VV::A::k:::v::s:e:SkaEt (351)
TraG  S. aureus:   (289) ::nf::ppp:::Imf::KV:K::g:VV::A::k:::v::s:e:SkaEt (351)
Orf S. carnosus:   (209) -:N-------::::YI:RV:K::SFDV::M:YIKPYKV:S:PI:::E: (248)
Usp45 L. Lactis:   (413) dfvgy:nSp:::::I:KsVns::T:T:K:ggYgtTwwGhe::vS:sgV (452)
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FIG. 2. Domains of amino acid homology within the predicted Isp protein. A linear map of the 58.9-kDa protein derived from the translated *isp* coding sequence is shown at the top. Numbers represent amino acid residues beginning from the N terminus. The areas shown as A (filled box), B (striped box), C (open box), and D (shaded box) represent separate regions of amino acid homology obtained from database search analyses as described in the text. Direct comparison of homologous sequences for domains A, B, and D, are shown at the bottom. The gene designation and organism are indicated at the left of each sequence. Numbers in parentheses reflect the locations of the first (left) and last (right) residues within their respective proteins. Homologous amino acids are shown as colons, conserved residues appear as uppercase letters, and nonconserved residues appear as lowercase letters.

of T7 polymerase with IPTG, samples were taken at 1, 3, and 4 h and subjected to Western blot analysis using the convalescent-phase serum adsorbed with *E. coli* BL21(DE3) cells. A protein product that migrated with an apparent molecular weight of 65,000 was recognized at 1 h after induction (Fig. 4A, lane 6) that was not observed in the vector control experiment (Fig. 4A, lanes 1 to 4). This 65-kDa protein was localized to the soluble periplasmic fraction (data not shown), demonstrating that Isp can be translocated across the inner membrane of *E. coli*. The decrease in the amount of the 65-kDa Isp species over time may indicate that it is unstable in *E. coli* (Fig. 4A, lanes 6 to 8).

The same serum was used to probe both cell extracts and concentrated supernatant proteins from GAS strains JRS4 (*isp*$^+$), JRS145 (*isp*$^+$), and JRS2110 (*isp-2*). No significant differences were noted in cell extracts from the three different strains (Fig. 4B, lanes 1 to 3). However, concentrated supernatants from the *isp-2* strain JRS2110 lacked a protein with an apparent molecular weight of 43,000 (Fig. 4B, lane 5) that was present in the *isp*$^+$ strains JRS145 and JRS4 (Fig. 4B, lanes 4 and 6). The presence of several heavy protein bands migrating at ca. 65 kDa prevented visualization of an Isp species of this apparent molecular mass (Fig. 4B, lanes 4 to 6). Identical results were seen with a second serum sample from a patient

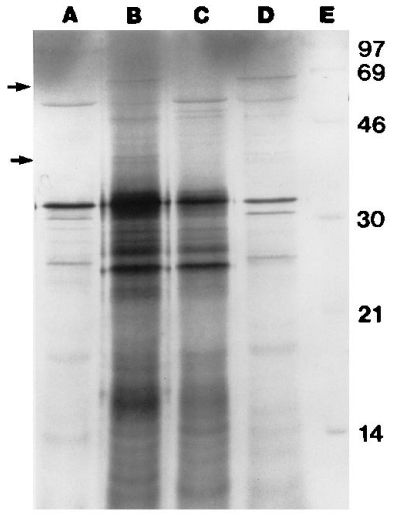

FIG. 3. In vitro transcription-translation of *isp* and *orf* DNA in an *E. coli* extract. The [^{35}S]methionine-labeled products of in vitro transcription and translation were separated on a 12.5% polyacrylamide gel and subjected to autoradiography. Templates for the reactions were as follows: lane A, pUC18 (vector); lane B, pJRS2005Ω (*orf-1*); lane C, pJRS2004Ω (*isp-1*); lane D, pJRS1020 (*isp*$^+$ *orf*$^+$). The molecular sizes (in kilodaltons) of the rainbow molecular weight markers in lane E are indicated on the right. The arrows indicate a protein with an apparent molecular weight of 65,000 (upper), which appears to be full-length Isp, and potential breakdown products at 43,000 (lower).

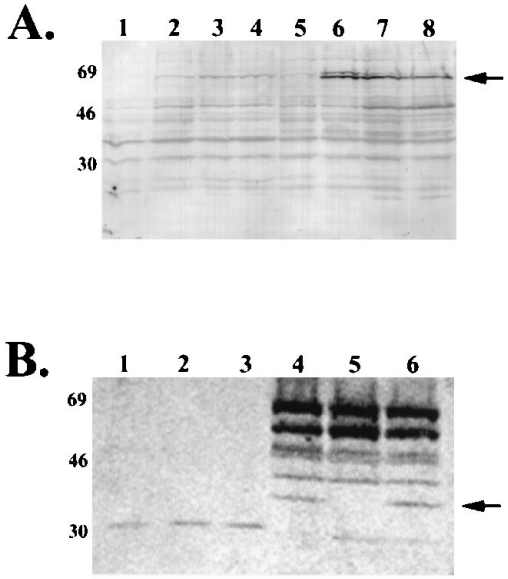

FIG. 4. Immunoblot analyses using convalescent-phase serum from a patient with an active GAS infection. (A) Expression of *isp* from the T7 promoter in *E. coli*. Cell extracts of BL21(DE3) harboring either the vector control (pSP73) or the PT7 *isp* plasmid (pJRS2003) were obtained at several times following induction of T7 polymerase with IPTG (1 mM) at 30°C. Lanes correspond to samples taken at 0, 60, 180, and 240 min postinduction for both BL21(DE3)/pSP72 (lanes 1 to 4) and BL21(DE3)/pJRS2004 (lanes 5 to 8), respectively. Following separation by SDS–12.5% PAGE and transfer to nitrocellulose, the blot was probed with the convalescent-phase serum (see the text). The arrow indicates the location of the 65-kDa Isp protein, and the locations of relevant molecular weight markers are shown at the left. (B) Protein profiles of cell lysates and supernatants from *isp*$^+$ and *isp-2* GAS strains. Mutanolysin cell extracts (lanes 1 to 3) and tricarboxylic acid-precipitated supernatant proteins (lanes 4 to 6) were obtained from JRS4 (lanes 1 and 4), JRS145 (lanes 2 and 6), and JRS2110 (*isp-2*) (lanes 3 and 5) cultures grown overnight in THY broth at 37°C. Following separation by SDS–12.5% PAGE and transfer to nitrocellulose, the blot was probed with the same convalescent-phase serum as for panel A. The arrow indicates the presumptive Isp protein in lanes 4 and 6. The locations of relevant molecular weight markers are shown at the left.

with a positive blood culture for GAS (data not shown). However, sera from 13 control subjects without an active GAS infection were also able to recognize the 43-kDa supernatant protein to various degrees (data not shown). The significance of these results will be discussed later.

To determine if the 43-kDa *isp*-associated band observed in the GAS supernatant results from proteolytic cleavage of Isp, *E. coli* periplasmic fractions containing the 65-kDa Isp protein were incubated for 0, 24, and 48 h at 37°C. Western analysis of these samples using the convalescent-phase serum showed a stable 43-kDa product (Fig. 5A, lanes 2 to 4) identical in size to the *isp*-associated protein in the supernatant of GAS (Fig. 4B, lanes 4 and 6). These two products were shown to migrate to the same position when compared by SDS-PAGE (data not shown). The 43-kDa *E. coli* product was not seen in periplasmic samples derived from the vector control strain (Fig. 5A, lane 1). Appearance of this product also coincided with degradation of the larger Isp over the same period of time (Fig. 5A, lanes 2 to 4). Incubation of the periplasmic fraction containing the 65-kDa *isp*-associated band in the presence of the insoluble cell fraction at 37°C resulted in a decrease in the amount of the 65-kDa protein and an increase of the 43-kDa form (Fig. 5B, lanes 6 to 8). These results strongly suggest that the 43-kDa protein in the supernatant of GAS, as well as that found following Isp synthesis in vitro, is a processed form of the full-length Isp.

The *isp* gene is conserved among the members of GAS. To determine if *isp* is conserved among GAS strains of different M protein serotypes, Southern blot analysis was used. A radiolabeled 1.1-kb *Pvu*II fragment from pJRS1020 (Fig. 1), located internal to the *isp* coding sequence, was used to probe *Hin*dIII-digested chromosomal DNA derived from several GAS strains chosen to represent a genetically diverse selection as determined by multilocus enzyme electrophoretic analysis (38). This *isp*-specific probe hybridized to a chromosomal DNA fragment of the same size from five of the six strains tested (Fig. 6, lanes B, C, E, F, G, and H) and to a smaller fragment from the M22 strain (Fig. 6, lane D). Identical results were seen when the blot was probed with an internal *orf*-specific fragment (see Materials and Methods; data not shown), as expected since both probes should hybridize to the same *Hin*dIII fragment.

***isp* sequences are present in other streptococcal groups.** To investigate whether *isp* and *orf* sequences are found in streptococcal groups other than group A, PCR assays were performed using primer pairs specific for *isp* (oyr20 and oyl7) and for *isp* plus *orf* (oyr20 and oyl6) (Fig. 1). Crude cell lysates were prepared from strains representing different Lancefield streptococcal groups. With the *isp*-specific primer pair, four of the eight strains tested, including those in groups B, C, G, and N, produced a product of the same size (1.3 kb) as that produced by strain JRS4 (Fig. 7, lane A2 compared with lanes B2, C2, G2, and N2). The remaining strains produced smaller *isp*-specific products (Fig. 7, lanes D2, F2, H2, and L2). In addition, a full-length *isp*-specific product was produced from cell lysates of *Streptococcus sanguis* (Challis) and *Streptococcus mutans* (262RF), both members of the viridans group of oral streptococci (data not shown). A product specific for *isp* plus *orf* of the size predicted from the sequence of the group A strain (1.7 kb) was observed with DNA only from the strains of groups A and G (Fig. 7, lanes A1 and G1). This suggests that the *orf* sequence corresponding to the oyl6 primer is divergent among other streptococcal species.

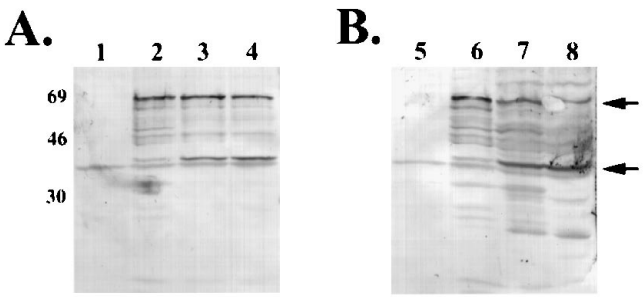

FIG. 5. In vitro time course analysis of *E. coli* periplasmic fractions containing Isp. Periplasmic samples (18 μg) either devoid of cell material (A) or in the presence of the insoluble cell fraction (B) were incubated for various amounts of time at 37°C. Western analysis was performed using the convalescent-phase serum adsorbed with *E. coli* as described in Materials and Methods. Lanes 1 and 5 correspond to periplasmic samples from the vector control strain BL21(DE3)/pSP72 without incubation. Periplasmic samples from the *isp*-expressing BL21(DE3)/pJRS2003 strain were incubated at 37°C for 0, 24, and 48 h either in the absence (A, lanes 2 to 4) or presence (B, lanes 6 to 8) of the insoluble cell fraction. The arrows indicate the positions of the Isp species that migrate at 43 and 65 kDa. The locations of relevant molecular weight markers are shown at the left.

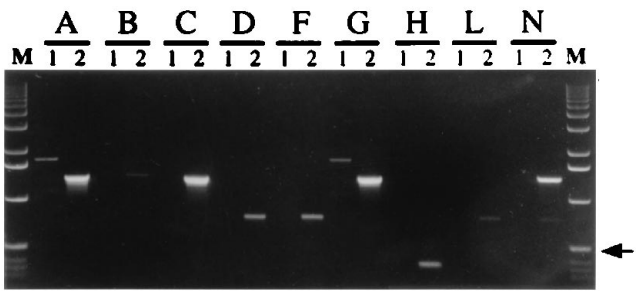

FIG. 7. PCR analysis of chromosomal DNA from streptococcal strains of various Lancefield groups, using *isp*- and *isp*-plus-*orf*-specific primers. Template DNA was obtained from crude cell lysates as described in the text. Primer pairs used for the *isp* plus *orf* (oyr20 and oyl7) and *isp* (oyr20 and oyl6) regions are shown in Fig. 1. PCR reactions for each lysate with *isp* plus *orf* (lanes 1) and *isp* (lanes 2) primer pairs are indicated. Letters refer to Lancefield streptococcal groups: A, strain JRS4; B, strain 090R; C, strain C74; D, strain D76; F, strain F68C; G, strain D166B; H, strain F90A; L, strain D167A; and N, strain C559. The arrow indicates the molecular size marker (lanes M) corresponding to 0.5 kb; bands above this point represent fragments of 1.0, 1.6, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 kb, respectively.

DISCUSSION

No two-component sensor protein gene is found upstream of *mga*. The GAS transcriptional activator Mga controls expression of virulence genes, including that encoding the M protein, in response to environmental conditions. It was proposed that Mga may be a second component (response regulator) of the two-component signal transducing system protein family (22). However, the location and organization of response regulator domains within Mga are unique.

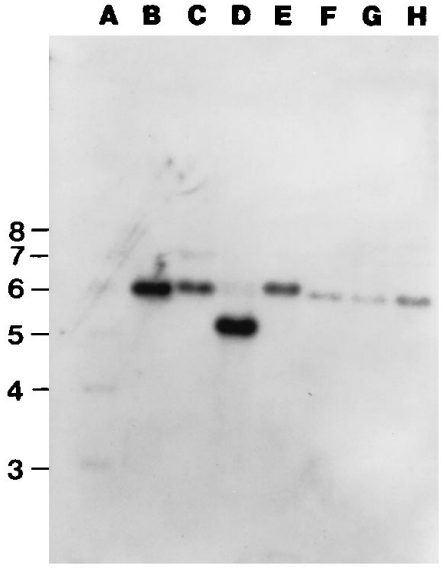

FIG. 6. Southern blot of an *isp*-specific probe hybridized to chromosomal DNA from GAS strains with different M protein serotypes. Chromosomal DNA was digested with *Hin*dIII, separated on a 1.0% agarose gel, and transferred to nitrocellulose. The blot was probed with a radiolabeled 1.1-kb *Pvu*II fragment from pJRS1020 containing only sequences internal to the *isp* coding sequence. Lane A, 1-kb DNA molecular weight markers (sizes in kilobases) are indicated at the left. DNA from strains of different M types are as follows: lane B, M6 strain JRS4; lane C, M2 strain T2/44; lane D, M22 strain D734; lane E, M19 strain SS400; lane F, M49 strain M49B737/137/2; lane G, M1 strain JRS301; lane H, M3 strain JRS333. Hybridization temperature was 42°C with no formamide. Washes were performed at 50°C with 0.1% SDS–0.1× SSC.

To demonstrate that Mga is actually a two-component response regulator, we sequenced the region upstream of *mga* to look for a gene encoding its cognate sensor kinase. Two open reading frames were found in this region, *isp* and *orf*. However, no decrease in activation of *emm* transcription occurred when either *isp* or *orf* was insertionally inactivated in the GAS chromosome. These data suggest that *isp* and *orf* are not required for the Mga-dependent activation of M protein expression. However, a gene for a cognate sensor kinase may be located elsewhere in the genome. It is also plausible that the specificity of Mga for a sensor is not very rigorous and a sensor from another two-component system might activate Mga in the *orf-1* and *isp-2* insertional mutants. Consistent with this is our recent finding that the Mga protein purified from *E. coli* extracts binds to target promoters without the need for further phosphorylation (18). Alternatively, it is possible that Mga does not require direct activation via phosphate transfer from a sensor protein. Studies are currently under way to determine whether the Mga protein can be phosphorylated in vitro.

Conservation of *isp* and *orf* among GAS strains. To begin to assess how commonly *isp* and *orf* are found in unrelated GAS strains, hybridization to both an *isp*-specific probe and an *orf*-specific probe was used. Strains of different M types which are distantly related, as deduced from multilocus enzyme electrophoresis (38), were among those analyzed. We found that both the *isp* probe and *orf* probe hybridized to the DNA from all of the strains tested. The size of the *Hin*dIII fragment (ca. 6 kb) was identical in all but one of these strains. (The exceptional strain [M22] appears to have an additional *Hin*dIII site in this fragment since the probe reacted weakly with a band of 6 kb, which probably results from a partial digestion, as well as with a smaller fragment [Fig. 6, lane D, and data not shown]). Thus, not only are *isp* and *orf* commonly found in GAS strains, but the size of the restriction fragment including both genes is conserved as well. This fragment includes *mga*, the DNA between *isp* and *mga*, which contains *orf*, and the 5′ end of the first *emm* or "*emm*-related" gene. Since these *emm*-related genes encode proteins with different functions in some of the strains studied (7) and since the N-terminal parts of the M proteins in different strains are variable in size as well as sequence, it is difficult to understand the reasons for the constancy of the size of the *Hin*dIII fragment. Because it was previously noted that the *Hin*dIII restriction pattern of GAS

strains can be used to distinguish among strains that are phylogenetically disparate (35), it seems likely that there is selective pressure to conserve this particular 6-kb *Hin*dIII fragment.

Because of the conservation of *isp* among different GAS strains, we investigated whether this gene could be found in streptococci of other Lancefield groups. A PCR assay of genomic DNA from strains belonging to eight different Lancefield groups, using *isp*-specific primer pairs, was used to assess this. DNA products of identical size were generated from strains representing four different groups (B, C, G, and N). Members of group B represent serious human pathogens, and groups C and G are sometimes associated with human disease. A full-length *isp*-specific product was also generated by two members of the alpha-hemolytic viridans group of streptococci, *S. sanguis* and *S. mutans*. Strains of both of these species are found among the normal human oral flora and can cause dental caries (*S. mutans*) and infective endocarditis (*S. sanguis*). The remaining strains tested generated *isp*-specific products that were smaller, and it is possible that these products are from strains containing a truncated *isp* that is incapable of producing a full-length protein.

A protein is produced from *isp*. Since we found no phenotype for mutants in which *isp* and *orf* were insertionally inactivated, the question of whether these open reading frames actually produce proteins needed to be answered. Both are preceded by potential ribosome binding sites within 8 to 12 nucleotides of their respective start codons. Transcription and translation in *E. coli* extracts of *isp* produced a protein close to the size expected; however, no product of the expected size (9 to 11 kDa) was detectable when the extract was programmed with *orf*. Thus, it is not clear whether *orf* produces a protein product.

DNA from all of six unrelated GAS strains tested hybridized with an *orf*-specific probe, indicating that the region containing *orf* is highly conserved among GAS. This suggests that *orf* has a function. It is likely that *orf* is transcribed since there is no obvious transcriptional terminator between *isp* and *orf*. If it does not produce a protein, it is possible that *orf* produces an RNA which has a direct function in the streptococcal cell.

Nature of the Isp protein. Analysis of homologies divides the predicted Isp protein into four domains. The carboxy-terminal domain, D, showed significant homology with several gram-positive proteins, including the major secreted product of *L. lactis*, Usp45. Unfortunately, the function of this protein is not known. More intriguing is the strong homology between Isp and a protein (TraG/TrsG) encoded by conjugative plasmids of *S. aureus*. Although the exact role of this protein remains undetermined, it is known to be required for conjugation. Although TraG/TrsG appears to contain neither an obvious signal sequence nor a cell-wall-anchoring domain, based on its hydrophobicity, it has been suggested that this protein is localized to the membrane of *S. aureus* (9). The similarity of the hydrophobicity profile for Isp, Usp45, and TraG/TrsG over this homologous C-terminal region suggests a possible conserved conformation that may contribute to the function of these homologs.

Just N-terminal of the D domain is a region with no strong homology to any proteins currently in the database (domain C). The next region, domain B, shows similarity to repeat domains found in surface antigens from several different eukaryotic organisms. These repeat domains are rich in proline, serine, aspartic acid, and glutamic acid residues. Although domain B of Isp contains many of the same four residues, Isp does not appear to contain a repeated sequence. The significance of this homology is therefore not clear.

The *isp* gene encodes a secreted protein of GAS. The amino terminus (domain A) of the predicted Isp protein is typical of bacterial signal peptides of both gram-negative and gram-positive proteins that are recognized by the Sec pathway and secreted through the cytoplasmic membrane. Therefore, it seemed likely that after GAS growth, Isp would be secreted and found either attached to the cell wall on the bacterial surface or present in the medium. Surface proteins of gram-positive bacteria are usually anchored to the cell wall using a specific sorting motif consisting of a conserved LPXTG sequence followed by a hydrophobic domain and a charged carboxy-terminal tail (30). Although Isp contains two positively charged amino acids in the last 10 carboxy-terminal residues, there is no potential LPXTG motif or hydrophobic domain to suggest that Isp is anchored to the cell wall of GAS. Therefore, we anticipated that it would be found in the cell supernatant. This is also suggested by the 24% identity between the entire Isp protein sequence and that of the major secreted product of *L. lactis*, Usp45. It seems possible that these two proteins play similar roles.

Direct evidence for the secretion of Isp was sought by Western blot analysis using serum from a patient with an active GAS infection (see below). A protein that is absent from the insertionally inactivated *isp-1* mutant was identified in the supernatant, but not the cell extract, of a wild-type GAS strain. This protein migrated on SDS-PAGE with an apparent molecular weight of 43,000, which is smaller than the predicted size for Isp (59 kDa).

Confirmation of the identity of this protein as Isp was sought by cloning *isp* and expressing it both in vitro and in *E. coli*. In vitro transcription-translation produced an *isp*-specific band with an apparent molecular mass of 65 kDa, as well as two smaller bands that migrated at ca. 40 to 43 kDa. In *E. coli*, an *isp*-specific protein that migrated at 65 kDa was found in the periplasm. Incubation at 37°C of a fraction containing the 65-kDa Isp resulted in the time-dependent appearance of a 43-kDa protein coinciding with a decrease in the amount of the larger species. Incubation of the same samples in the presence of the insoluble *E. coli* cell fraction resulted in an increase both in the degradation of the 65-kDa Isp and in the appearance of the 43-kDa protein. This result was also observed when *isp* was expressed in vivo, suggesting that a membrane-associated protein may be required for efficient production of the 43-kDa form. Therefore, it is likely that the 43-kDa protein identified as Isp in supernatants of GAS cultures results from the processing of the full-length Isp molecule.

Presence of anti-Isp antibodies in humans. Because it seemed likely that Isp is secreted from GAS, to assess whether it is produced during infections, we looked for the presence of antibody in the human host. Convalescent-phase serum from a patient with an active GAS throat infection recognized a protein in the supernatant of a wild-type GAS strain that was absent from the supernatant of the *isp-2* strain JRS2110, as mentioned above. Serum from a second patient with a positive GAS blood culture also reacted strongly with a protein of the same size. However, control sera from 13 individuals not known to possess an active GAS infection were also able to recognize the 43-kDa protein in GAS. Furthermore, other streptococcal species, including the normal oral residents *S. mutans* and *S. sanguis*, possess homologous *isp* sequences and thus potentially produce and secrete an Isp protein. Thus, the presence of anti-Isp antibodies within the human host may result from production of Isp by streptococci of groups other than A. Our studies demonstrate that Isp is expressed by GAS in the human host and generates an antibody response. Fur-

ther investigations will be required to determine if Isp is involved in the pathogenesis of human streptococcal infections.

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