INFECTION AND IMMUNITY, Dec. 2003, p. 7079–7086 Vol. 71, No. 12
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.12.7079–7086.2003

Prophage Induction and Expression of Prophage-Encoded Virulence Factors in Group A *Streptococcus* Serotype M3 Strain MGAS315

David J. Banks,[1] Benfang Lei,[1]† and James M. Musser[1,2]*

Laboratory of Human Bacterial Pathogenesis, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana 59840,[1] and Department of Pathology, Baylor College of Medicine, Houston, Texas 77030[2]

Received 21 May 2003/Returned for modification 22 July 2003/Accepted 27 August 2003

The genome of the highly virulent group A *Streptococcus* (GAS) serotype M3 strain MGAS315 has six prophages that encode six proven or putative virulence factors. We examined prophage induction and expression of prophage-encoded virulence factors by this strain under in vitro conditions inferred to approximate in vivo conditions. Coculture of strain MGAS315 with Detroit 562 (D562) human epithelial pharyngeal cells induced the prophage encoding streptococcal pyrogenic exotoxin K (SpeK) and extracellular phospholipase A_2 (Sla) and the prophage encoding streptodornase (Sdn). Increased gene copy numbers after induction correlated with increased *speK*, *sla*, and *sdn* transcript levels. Although *speK* and *sla* are located contiguously in prophage Φ315.4, these genes were transcribed independently. Whereas production of immunoreactive SpeK was either absent or minimal during coculture of GAS with D562 cells, production of immunoreactive Sla increased substantially. In contrast, despite a lack of induction of the prophage encoding speA during coculture of GAS with D562 cells, the *speA* transcript level and production of immunoreactive streptococcal pyrogenic exotoxin A (SpeA) increased. Exposure of strain MGAS315 to hydrogen peroxide, an oxidative stressor, induced the prophage encoding mitogenic factor 4 (MF4), and there was a concomitant increase in the *mf4* transcript. All prophages of strain MGAS315 that encode virulence factors were induced during culture with mitomycin C, a DNA-damaging agent. However, the virulence factor gene transcript levels and production of the encoded proteins decreased after mitomycin C treatment. Taken together, the results indicate that a complex relationship exists among environmental culture conditions, prophage induction, and production of prophage-encoded virulence factors.

Group A *Streptococcus* (GAS) has a wide range of disease presentation and tissue tropism, and strains of relatively few M protein serotypes account for the majority of particular clinical syndromes. For example, serotype M1 and M3 strains often cause deep tissue infections, such as necrotizing fasciitis, whereas serotype M18 strains have been repeatedly linked to contemporary cases of acute rheumatic fever in the United States (23, 25, 30). The genomes of one strain each of serotype M1, M3, and M18 GAS have been sequenced (3, 10, 30). Each strain is polylysogenic and has a unique array of prophage-encoded virulence factors that may increase bacterial fitness during host colonization, infection, and evasion of the immune system. Although prophages contribute substantially to the diversity in gene content in GAS and other pathogens (1, 27), the environmental signals and regulatory mechanisms that influence expression of prophage-encoded virulence factors are poorly understood for most bacterial pathogens.

Recent evidence from study of a GAS serotype M1 strain indicates that prophage induction is sensitive to environmental signals. For example, during coculture of GAS with a human pharyngeal epithelial cell line, increased gene copy numbers were associated with increased expression of Spd1 (a DNase also known as mitogenic factor 2 [MF2]) and streptococcal pyrogenic exotoxin C (SpeC) (4, 5). In addition, exposure of a GAS serotype M1 strain to human neutrophils stimulated transcriptional up-regulation of the prophage genes *spd3* and *speH*, encoding GAS DNase 3 and streptococcal pyrogenic exotoxin H (SpeH), respectively (34).

Inasmuch as serotype M3 strains of GAS are major causes of invasive episodes and pharyngitis cases (25, 29; http://www.cdc.gov/ncidod/dbmd/abcs/survreports/gas01_provis.pdf), we tested the hypothesis that prophage induction and expression of prophage-encoded virulence factors were influenced by distinct environmental signals. This hypothesis was tested with a highly virulent M3 serotype strain grown in vitro under conditions inferred to approximate in vivo conditions.

MATERIALS AND METHODS

Bacterial strain. Serotype M3 strain MGAS315 has been characterized extensively (16, 18, 20, 21). The genome of serotype M3 strain MGAS315 has been sequenced (3) and has six prophage-like elements that encode six putative or proven virulence factors (Table 1). For brevity, a prophage-like element will be referred to hereafter as a prophage; a proven or putative virulence gene will be referred to as a virulence gene, and the encoded protein will be referred to as a virulence factor. Prophage Φ315.1 does not encode a virulence factor, whereas prophage Φ315.4 encodes two virulence factors. Each of the other four prophages (Φ315.2, Φ315.3, Φ315.5, and Φ315.6) encodes one virulence factor (Table 1).

Culture of strain MGAS315 with mitomycin C or hydrogen peroxide. Strain MGAS315 was grown overnight at 37°C with 5% CO_2 in Todd-Hewitt (Difco) plus yeast extract (Difco) (THY) liquid medium lacking proteins with molecular masses of >10 kDa (protein-reduced THY [PR-THY]) (18). The overnight GAS culture was diluted 1:100 with prewarmed PR-THY and grown to an optical

* Corresponding author. Mailing address: Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-4198. Fax: (713)798-4595. E-mail: musser@bcm.tmc.edu.

† Present address: Department of Veterinary and Molecular Biology, Montana State University, Bozeman, MT 59717.

TABLE 1. Primers used for PCR amplification of prophage-specific genes[a]

Gene	Prophage	Primer		Product size (bp)
		Forward	Reverse	
ssa	Φ315.2	TGATCAAATATTGCTCCAAGGTG	TCCACAGGTCAGCTTTTACAG	502
mf4	Φ315.3	TGGCATTGCTTCATAGTAAAGG	ATCTACCTGAAGCTTTGTCGTG	494
speK	Φ315.4	GTGTGTCTAATGCCACCGTCT	GGAACATATATGCTCCTAGAT	564
sla	Φ315.4	CTCTAATAGCATCGGCTACGC	AATGGAAAATGGCACTGAAAG	440
speA3	Φ315.5	TGGAAAACAATAAAAAAG	TTACTTGGTTGTTAGGTAG	755
sdn	Φ315.6	AACGTTCAACAGGCGCTTAC	ACCCCATCGGAAGATAAAGC	489
speB[b]		ACCGTGTTATTGTCTATTACC	TGCCTACAACAGCACTTTGG	1,337

[a] Primers are listed in the 5′ → 3′ direction.
[b] SpeB is encoded by a chromosomal gene and was used as a negative control.

density at 600 nm of 0.2, divided into 50-ml aliquots, and treated with either hydrogen peroxide (0.5 mM; Fisher) or mitomycin C (0.2 μg/ml). The diluted bacteria were then grown for 3 h at 37°C with 5% CO_2.

Tissue culture and media. Immortalized Detroit 562 (D562) pharyngeal epithelial cells were purchased from the American Type Culture Collection (CCL-138). The cells were grown to confluence in minimal essential medium (MEM; Invitrogen) supplemented with 5% fetal bovine serum (BD Biosciences) in 150-cm^2 screw cap tissue culture flasks (Corning) at 37°C with 5% CO_2.

Culture of strain MGAS315 with D562 epithelial cells. The serum-containing MEM was removed from confluent D562 cells, and the cells were washed twice with prewarmed serum-free MEM (10 ml). Prewarmed serum-free MEM (25 ml) was added, and the D562 cells were incubated for 3 to 4 h at 37°C with 5% CO_2. A 1:100 dilution of bacteria (100 ml) was grown to an optical density at 600 nm of 0.2, centrifuged at 3,000 × *g* for 15 min, washed in phosphate-buffered saline (PBS), suspended in prewarmed serum-free MEM (10 ml), and divided into two 5-ml aliquots. One aliquot was transferred to a flask containing only MEM (control), and the other aliquot was added to a flask containing confluent D562 cells. These flasks were incubated for 3 h at 37°C with 5% CO_2.

Phage particle purification, electron microscopy, and phage DNA purification. After 3 h of incubation with mitomycin C, hydrogen peroxide, or D562 cells, bacteria were centrifuged at 8,000 × *g* for 15 min, and the supernatant was sterilized with a 0.45-μm-pore-size filter (Millipore). The filter-sterilized liquid was centrifuged at 141,000 × *g* for 4 h at 10°C, and the pellet was suspended in 1 ml of phage suspension buffer. Phage suspension buffer contains 0.15 M NaCl, 10 mM Tris HCl (pH 7.5), 5 mM $MgCl_2$, and 1 mM $CaCl_2$ (24). Intact phage particles present in the suspension buffer will be referred to as a phage suspension. Electron microscopy was conducted at ×300,000 magnification on negatively stained (uranyl acetate) phage suspensions.

To purify phage DNA, the phage suspension (0.5 ml) was treated with Benzonase (25 U) (Novagen) for 1 h at 37°C. The phage particles were lysed with 0.5% sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories), 10 mM EDTA (Sigma), and 500 μg of proteinase K (Sigma)/ml for 1 h at 37°C. Phage DNA was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma), followed by an equal volume of chloroform-isoamyl alcohol (24:1) (Sigma). Phage DNA was precipitated with a 20% volume of 3 M NaOAc (pH 4.2) (Sigma) and a twofold volume of ethanol at −70°C for 2 h, washed with 70% ethanol, and suspended in distilled H_2O.

PCR with purified phage DNA template. Prophage induction was monitored by PCR with forward and reverse primers (Table 1) specific for virulence factor genes encoded by prophages present in the genome of strain MGAS315. PCR was conducted with purified phage DNA template in 50 mM KCl, 10 mM Tris-HCl, 15 mM $MgCl_2$, 0.25 mM (each) deoxynucleoside triphosphate (pH 8.3), and 0.5 U of *Taq* polymerase (Promega) for 30 cycles of 1 min at 95°C, 0.5 min at 55°C, and 1 min at 72°C.

Western immunoblot analysis. Supernatants from ultracentrifuged (141,000 × *g*) cultures were concentrated and examined for prophage-encoded virulence factors by Western immunoblot analysis. Supernatants (10 ml) prepared from strain MGAS315 cultured in PR-THY medium (control) and PR-THY medium plus mitomycin C or hydrogen peroxide were treated with trichloroacetic acid (2 ml) (Sigma) on ice for 10 min, and the precipitates were collected by centrifugation at 8,000 × *g* for 15 min at 10°C. The pellets were washed twice with ice-cold acetone (Sigma), dried at 100°C for 2 min, and suspended in 0.5 ml of 10 mM Tris, pH 8.0. Supernatants (10 ml) from strain MGAS315 cultured in MEM (control) and MEM containing D562 cells were concentrated with Centricon Plus-20 filters (Amicon) to 0.5 ml. Proteins present in the concentrated supernatants were separated with precast SDS–15% polyacrylamide gel electrophoresis (PAGE) (Bio-Rad Laboratories) and transferred (TransBlot; Bio-Rad Laboratories) to nitrocellulose membranes (Protran; Schleicher & Schuell) in Towbin's buffer at 15 V for 1 h. Additionally, previously described recombinant proteins were included in Western immunoblot analyses as positive controls (3, 22). The membranes were incubated for 30 min in PBS containing 0.1% Tween 20 (Bio-Rad Laboratories) (PBST) and 3% bovine serum albumin (BSA; Sigma) and incubated for 1 h with primary antibody (3, 22) (1:10,000) in PBST plus 0.1% BSA. The membranes were washed three times in PBST plus 0.1% BSA, incubated for 1 h with secondary antibody (1:3,000; goat anti-rabbit-conjugated horseradish peroxidase; Santa Cruz), and washed three times in PBST plus 0.1% BSA. The membranes were incubated with Super Signal West Pico chemiluminescent substrate reagent (Pierce) according to directions suggested by the manufacturer, exposed to HyperFilm (Amersham Pharmacia), and developed.

RNA isolation and TaqMan real-time PCR analysis. Bacteria cultured in PR-THY medium, PR-THY medium plus either mitomycin C or hydrogen peroxide, MEM, or MEM plus D562 cells were centrifuged at 8,000 × *g* and washed once in RNAprotect (10 ml; Qiagen), and total RNA was isolated as described by Smoot et al. (31). The RNA quality was assessed with a model 2100 Bioanalyzer instrument (Agilent Technologies). The absence of contaminating DNA was confirmed by PCR and TaqMan real-time PCR. RNA purified from strain MGAS315 cocultured with MEM (control), MEM plus D562 cells, PR-THY medium (control), or PR-THY medium plus mitomycin C was analyzed by TaqMan real-time PCR for relative *speA*, *speK*, *sla*, and *sdn* transcript levels. RNA purified from strain MGAS315 cultured with PR-THY medium (control) or PR-THY medium plus hydrogen peroxide was analyzed by TaqMan real-time PCR for the relative *mf4* transcript level. Triplicate assays were performed with RNA isolated from three independent cocultures. Two-step multiplex reverse transcription (RT)-PCR with TaqMan Universal PCR Master Mix (Applied Biosystems) was conducted for each target gene with a 7900HT instrument (Applied Biosystems). The level of *proS* (prolyl-tRNA synthetase; *spy*1962) transcript was used to normalize TaqMan threshold cycle (Ct) values obtained for target genes, as described previously (12, 31, 33). Target gene transcript levels under experimental conditions were expressed as the difference (*n*-fold) relative to target gene transcript levels under control conditions. The differences were determined by dividing the larger normalized target gene transcript level (control or experimental) by the smaller normalized target gene transcript level (control or experimental). A positive value was ascribed to the difference in target gene transcript levels when experimental values exceeded control values. A negative value was ascribed to the difference in target gene transcript levels when control values exceeded experimental values. Primers and probes designed by Primer Express software (Applied Biosystems) were synthesized by Applied Biosystems and labeled with 6-carboxy-4′,5′-dichloro-2′,7′-dimethylfluorescein (see Table 3).

RT-PCR analysis of *speK* and *sla* transcription. RNA was purified from strain MGAS315 cocultured with D562 epithelial cells, and cDNA was synthesized as described above. RT-PCR was conducted with primers specific for *speK* and *sla* and primers that generate an amplicon that includes part of *speK* and *sla* and their intergenic region. The PCR conditions and primers described above were used.

RESULTS

Electron microscopy of phage suspension prepared from strain MGAS315 treated with mitomycin C. The genome of strain MGAS315 has six prophage-like elements, but it is un-

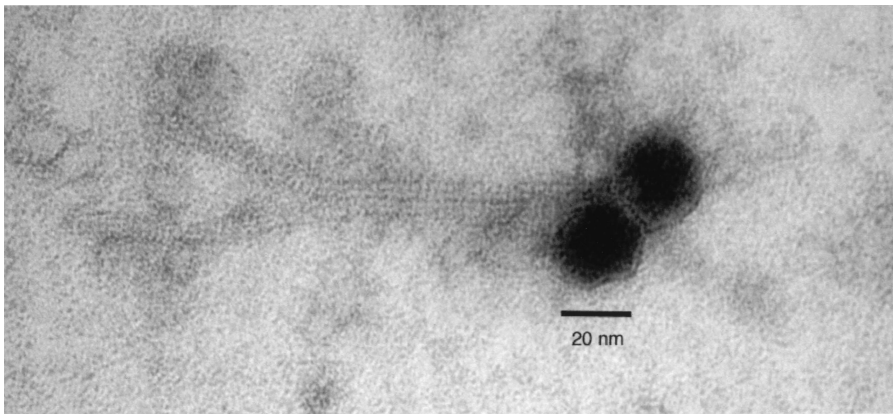

FIG. 1. Electron microscopy of phage particles purified from strain MGAS315. The bacteria were treated with mitomycin C and centrifuged at 141,000 × *g*. Phage particles were suspended in buffer and negatively stained with uranyl acetate. Magnification = ×300,000.

known if they can be induced. Mitomycin C is a DNA-damaging agent and is known to activate *recA*-dependent bacteriophage induction in gram-negative and gram-positive bacteria, including GAS (10). To test the hypothesis that one or more of these prophage-like elements was inducible, we treated strain MGAS315 with mitomycin C. Bacteriophage induction was confirmed by electron microscopy (Fig. 1). The phage particles had long noncontractile tails and isoscahedral heads, a morphology typical of *Siphoviridae* (7).

Identification of prophages induced by mitomycin C treatment. To determine which of the prophages were induced by treatment of strain MGAS315 with mitomycin C, we conducted PCR with purified phage DNA template and primers specific for each of the six virulence factor genes carried by the prophages in this strain (Table 1). Prophages Φ315.4 and Φ315.6 were induced spontaneously during the growth of strain MGAS315 in PR-THY medium (Table 2 and Fig. 2). In contrast, prophages Φ315.2, Φ315.3, and Φ315.5 were induced by mitomycin C treatment but were not spontaneously induced during growth in PR-THY medium (Table 2 and Fig. 2). There were clear qualitative differences in the degree of induction of each phage. For example, prophage Φ315.2, which encodes streptococcal superantigen (SSA), was induced at a very low level by mitomycin C treatment, whereas the levels of induction of prophage Φ315.5 (encoding SpeA3) and prophage Φ315.3 (encoding MF4) were demonstrably higher (Fig. 2). Taken together, these results indicated that each of the five prophages in strain MGAS315 that encode virulence factors can be induced and hence are not simply defective prophage-like elements.

Prophage induction by treatment of strain MGAS315 with hydrogen peroxide. Hydrogen peroxide exposes bacteria to oxidative stress and, similar to mitomycin C, is a DNA-damaging agent. Hydrogen peroxide has been reported to induce prophage λ in *Escherichia coli* (6). To determine if hydrogen peroxide treatment induced the prophages present in strain MGAS315, we conducted experiments analogous to those described above for mitomycin C treatment. Hydrogen peroxide treatment induced prophage Φ315.3, encoding MF4, whereas the prophage was not induced during culture in PR-THY medium alone (Table 2 and Fig. 2).

Prophages induced during exposure of strain MGAS315 to D562 pharyngeal epithelial cells. Coculture of a serotype M1 GAS strain with immortalized eukaryotic epithelial cells has been reported to induce prophages (4, 5). We assessed whether prophages were induced by coculture of strain MGAS315 with D562 human pharyngeal epithelial cells. Prophage Φ315.6, encoding Sdn, was induced spontaneously at a very low level during culture of strain MGAS315 in MEM alone (Table 2 and Fig. 3). Induction of this prophage was enhanced substantially during coculture with D562 cells (Table 2 and Fig. 3). Similarly, induction of prophage Φ315.4 (encoding SpeK and Sla) was increased greatly when strain MGAS315 was cultured with D562 pharyngeal cells (Table 2 and Fig. 3).

Western immunoblot analysis of prophage-encoded virulence factors present in culture supernatants during culture of

TABLE 2. Induction of prophages in strain MGAS315

Prophage	Gene	Induction[a]				
		PR-THY			MEM	
		Spontaneous	Mitomycin C	Hydrogen peroxide	Spontaneous	D562 cells
Φ315.2	*ssa*		±			
Φ315.3	*mf4*		++	++		
Φ315.4	*speK*, *sla*	++	+++	+++		+++
Φ315.5	*speA3*		++			
Φ315.6	*sdn*	++	+++	+++	±	+++

[a] Relative degree of prophage induction is indicated as follows: ±, variable and weak; ++, intermediate; +++, strong.

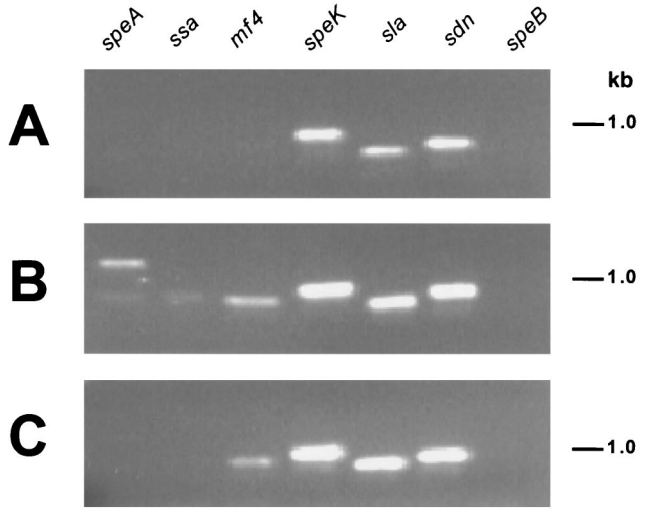

FIG. 2. PCR amplification of virulence factor genes present in culture supernatants of GAS. PCR was conducted with purified phage DNA templates obtained from strain MGAS315 cultured with PR-THY medium (control) (A), PR-THY medium plus mitomycin C (B), or PR-THY medium plus hydrogen peroxide (0.5 mM) (C). The genes that were amplified are listed above the lanes. The *speB* gene was used as a negative control to confirm that there was no contaminating GAS chromosomal DNA in the phage preparations. *speA*, streptococcal pyrogenic exotoxin A; *ssa*, streptococcal superantigen; *mf4*, mitogenic factor 4; *speK*, streptococcal pyrogenic exotoxin K; *sla*, phospholipase A_2; *sdn*, streptodornase; *speB*, streptococcal pyrogenic exotoxin B.

strain MGAS315 with mitomycin C or D562 epithelial cells. To determine if prophage induction was accompanied by increased production of prophage-encoded extracellular virulence factors, Western immunoblot analysis was used to analyze culture supernatant proteins. Specific antibodies are available for SpeK, Sla, and SpeA but not for SSA, MF4, and Sdn (3, 28). Hence, we examined the culture supernatant proteins prepared from strain MGAS315 treated with mitomycin

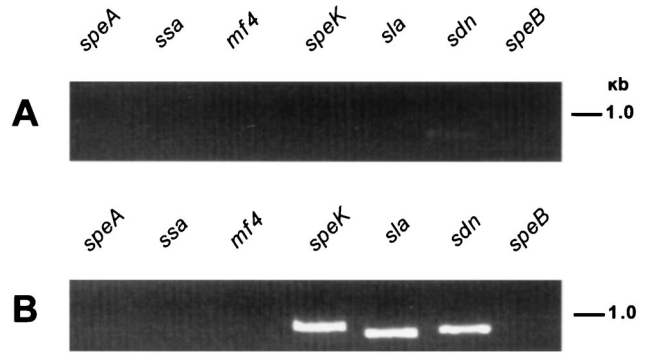

FIG. 3. Induction of phages during strain MGAS315 coculture with pharyngeal epithelial cells. PCR was conducted with purified phage DNA templates obtained from strain MGAS315 cultured in MEM (A) or MEM plus D562 cells (B). The genes that were amplified are listed above the lanes. The *speB* gene was used as a negative control to confirm that there was no contaminating GAS chromosomal DNA in the phage preparations. *speA*, streptococcal pyrogenic exotoxin A; *ssa*, streptococcal superantigen; *mf4*, mitogenic factor 4; *speK*, streptococcal pyrogenic exotoxin K; *sla*, phospholipase A_2; *sdn*, streptodornase; *speB*, streptococcal pyrogenic exotoxin B.

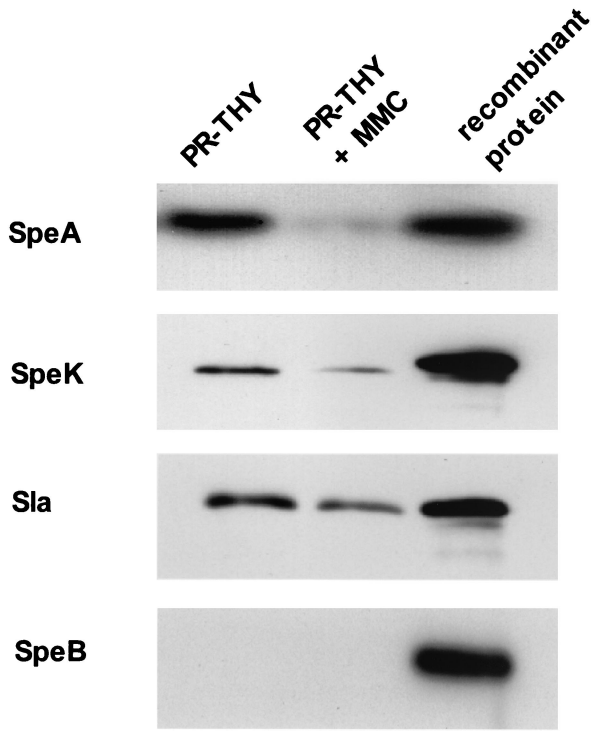

FIG. 4. Western immunoblot analysis of prophage-encoded secreted proteins made by MGAS315 during culture with mitomycin C. Proteins present in the culture supernatant were precipitated, separated by SDS–12% PAGE, and transferred to nitrocellulose. The membranes were probed with primary rabbit antibody (1:10,000) specific for each protein and secondary antibody (1:3,000) conjugated to horseradish peroxidase, and reactivity was visualized with chemiluminescent reagents. The treatments were as follows: PR-THY medium and mitomycin C (MMC).

C for immunoreactive SpeK, Sla, and SpeA. Compared to the culture supernatant of strain MGAS315 grown in PR-THY medium alone, the levels of immunoreactive SpeK, Sla, and SpeA in the culture supernatant decreased during culture of strain MGAS315 in PR-THY medium plus mitomycin C (Fig. 4). This decrease occurred despite the induction of prophages Φ315.4 and Φ315.5 carrying these genes (Fig. 2).

We next examined culture supernatant proteins prepared from strain MGAS315 cocultured with D562 cells for production of immunoreactive SpeK, Sla, and SpeA. As assessed by Western immunoblot analysis, neither SpeK nor Sla was expressed during culture of strain MGAS315 in MEM alone (Fig. 5). However, immunoreactive Sla and SpeK were present in the supernatant prepared from coculture of strain MGAS315 with D562 cells. The amount of immunoreactive Sla was considerably greater than the amount of SpeK (Fig. 5 and data not shown). Since prophage Φ315.5 (encoding SpeA3) was not induced by culture of strain MGAS315 in MEM alone, or coculture with D562 cells, we did not necessarily expect that the amounts of immunoreactive SpeA in culture supernatants would differ for these two treatments. However, SpeA was present in the culture supernatant prepared from strain MGAS315 grown in MEM alone, and the level of immunoreactive SpeA protein in the culture supernatant increased during coculture with D562 cells (Fig. 5). These data indicated

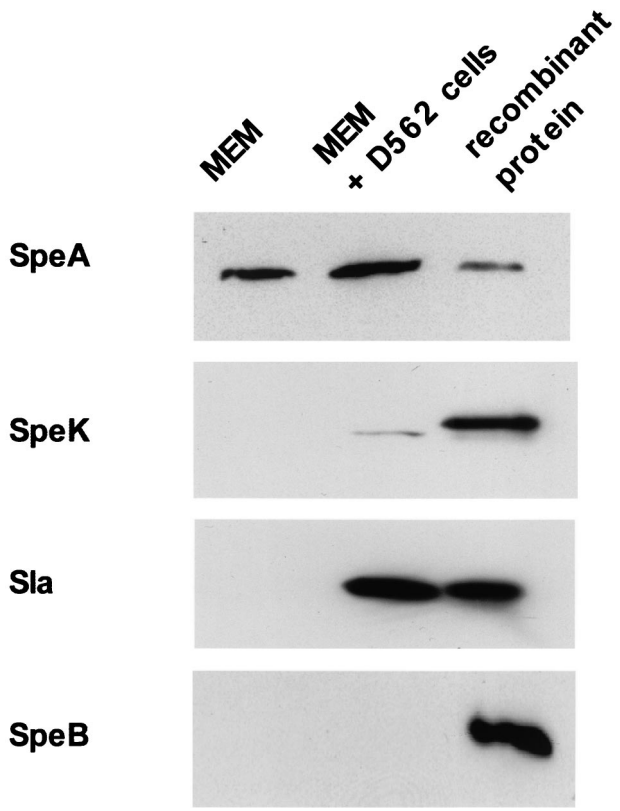

FIG. 5. Western immunoblot analysis of prophage-encoded secreted proteins made by MGAS315 during coculture with D562 epithelial cells. Proteins present in the culture supernatant were concentrated, separated by SDS–12% PAGE, and transferred to nitrocellulose. The membranes were probed with primary rabbit antibody (1:10,000) specific for each protein and secondary antibody (1:3,000) conjugated to horseradish peroxidase, and reactivity was visualized with chemiluminescent reagents.

that in vitro production of SpeA by strain MGAS315 occurred independently of prophage induction.

The chromosomally encoded extracellular cysteine protease SpeB has been reported to degrade SpeA and other GAS culture supernatant proteins (15). It is possible that the failure to detect immunoreactivity of some of the virulence factors was due to the presence of this or other proteases in culture supernatants. However, as assessed by Western immunoblotting, SpeB was not present in culture supernatants of strain MGAS315 cultured with MEM alone or with D562 cells (Fig. 5). Inasmuch as SpeB is expressed mainly in the stationary phase (11, 26), the absence of this protein in culture supernatants may be related to the failure of GAS to reach stationary phase after transfer to the tissue culture media.

TaqMan real-time PCR analysis of prophage-encoded virulence factor transcript levels during culture of strain MGAS315 with mitomycin C, hydrogen peroxide, and human D562 pharyngeal epithelial cells. The results described above indicated that increased virulence factor gene copy numbers during prophage induction did not necessarily correlate with an increased level of immunoreactive protein in culture supernatants. TaqMan real-time PCR analysis was used to test the hypothesis that the level of transcripts of prophage-encoded

TABLE 3. TaqMan primers and probes[a]

Gene[b]	Forward primer	Reverse primer	Probe
speK	GATAAAGACGAAATTTTGGATAATAGAAGA	TTCCAAACTGTAGTATTTTCATCCGTAT	AAATGCCAAACAAGGAACGCAATTGATTT
sla	CGTTGCCAGTAGTAGATGTTTTGG	CAATACCGGCACCCACTTA	CAAGGTTGCCAAAACCACGATAGTTGCT
speA	TTTTTGTTTTAGTGACATTTCTTGGACT	GGAGTTGGCTTGGATCGGG	ACAATCTCGCAAGAGGTATTTGCTCAACAAGA
mf4	GGAGATGCCCTTAGAGTAAATGCT	TCATCCCTCCATTGCCAGA	GACCTCCAACATTTTGTGTTCTTGTTCCTC
sdn	GAAACTCTCTCGCCAGAATAGTTGT	TGTACTGTGATGATAAGTTGGAATCG	CAGCATTCGCATCATTTGCTGTGATG
ssa	GTGCACAATTATTATCGATTAGTGTTTTT	CACCAGTAAATTGGCTAGATTTGTTTA	CAAGTAGTCAGCCTGACCCTACTCCAGAAACAA

[a] Primers are listed in the 5′ → 3′ direction.
[b] Gene abbreviations: *speK*, streptococcal pyrogenic exotoxin K; *sla*, phospholipase A_2; *speA*, streptococcal pyrogenic exotoxin A; *mf4*, mitogenic factor 4; *sdn*, streptodomase; *ssa*, streptococcal superantigen.

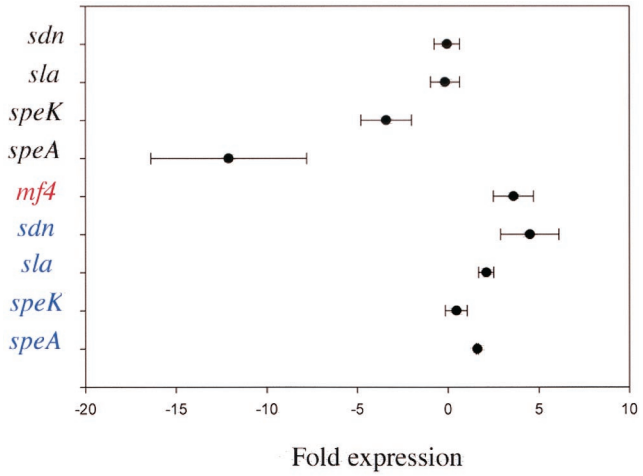

FIG. 6. Transcript levels of prophage-encoded genes in strain MGAS315 during culture with mitomycin C, hydrogen peroxide, and D562 epithelial cells relative to transcript levels under control conditions. Gene names are listed on the *y* axis. Relative transcript levels of genes after exposure to mitomycin C (black), hydrogen peroxide (red), and D562 epithelial cell coculture (blue) were normalized to the transcript level of *proS*, a constitutively expressed endogenous gene (10, 27). The mean transcript levels (± standard errors of the mean) of the prophage-encoded genes under experimental conditions are expressed as differences (*n*-fold) relative to control conditions.

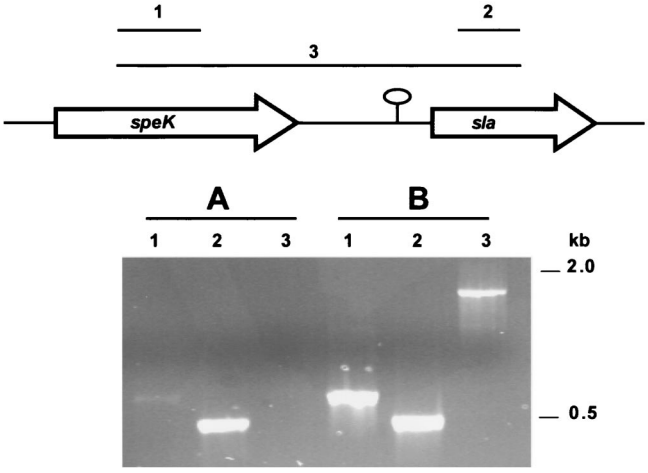

FIG. 7. RT-PCR analysis of *speK* and *sla* transcription. RNA purified from strain MGAS315 cocultured with D562 epithelial cells was reverse transcribed into cDNA, and PCR was conducted with primers specific for *speK* and *sla*. The amplicons generated were *speK* (1), *sla* (2), and *speK*, *sla*, and the intergenic region (3). The template used was cDNA synthesized from RNA purified from strain MGAS315 cocultured with D562 epithelial cells (A) or purified chromosomal DNA (control) (B). The stem-loop structure (not to scale) is located in the intergenic region (472 bp), 116 nucleotides upstream of the *sla* start codon.

virulence factors correlated with the level of immunoreactive protein present in culture supernatants (Table 3). The results showed that the level of immunoreactive protein in the culture supernatant was related to the transcript levels of the prophage-encoded virulence factor genes (Fig. 6). For example, mitomycin C treatment resulted in decreased levels of *speA*, *speK*, and *sla* transcripts and immunoreactive SpeA, SpeK, and Sla present in the culture supernatant (Fig. 6). In contrast, coculture of strain MGAS315 with D562 cells resulted in increased levels of *speA*, *speK*, and *sla* transcripts and immunoreactive SpeA, SpeK, and Sla in the culture supernatant (Fig. 6). Due to a lack of specific antibodies, we were unable to examine the level of immunoreactive Sdn and MF4 present in culture supernatants. However, we note that the level of *sdn* transcript decreased during mitomycin C treatment and increased during induction of the prophage containing this gene during coculture with D562 cells (Fig. 6). In addition, hydrogen peroxide treatment resulted in an increased level of *mf4* transcript concomitant with induction of the prophage containing this gene (Fig. 6).

RT-PCR analysis of *speK* and *sla* transcription. The *speK* and *sla* genes are adjacent in prophage Φ315.4. The levels of immunoreactive SpeK and Sla in culture supernatants from strain MGAS315 cocultured with D562 cells differed significantly, suggesting that the two genes may be transcribed independently. Consistent with this possibility, with a neural network program (http://www.fruitfly.org/seq_tools/promoter.html), we identified canonical promoter sequences located immediately upstream of the start codon for each gene. In addition, a predicted rho-independent transcriptional terminator sequence was identified 116 nucleotides upstream of the *sla* start methionine (9; http://www.tigr.org/software/transterm.html). Taken together, these findings led us to hypothesize that *speK* and *sla* are each expressed as a single transcript. We used RT-PCR and RNA purified from strain MGAS315 cocultured with D562 cells to test this hypothesis. Transcripts specific for each gene were identified, whereas a polygenic transcript was not identified (Fig. 7). Furthermore, the amounts of *speK* and *sla* RT-PCR products were qualitatively similar to the SpeK and Sla protein levels in supernatants obtained from the GAS-D562 cell coculture experiments (Fig. 5).

DISCUSSION

Serotype M3 strains and disease association. GAS pathogenesis represents the outcome of a complex interplay between the strain genotype, in vivo expression of virulence factors, host genetics (17), and underlying host conditions (25). For unknown reasons, in certain patient populations serotype M3 strains are significantly more likely to cause necrotizing fasciitis and death than GAS strains expressing other M protein serotypes (29). Evidence has been presented that an unusually high-virulence phenotype associated with contemporary serotype M3 strains is linked to the emergence of an M3 subclone (represented by strain MGAS315) that contains a unique array of phage-encoded virulence factors (3). Importantly, nothing is known about host and environmental signals or genetic regulatory features that control the expression of serotype M3 prophage-encoded virulence factors. The availability of the genome sequence of strain MGAS315 facilitated our analysis of prophage induction, transcription of prophage-encoded virulence factor genes, and levels of immunoreactive prophage-encoded virulence factors present in culture supernatants under in vitro conditions predicted to approximate infection in vivo.

Environmental conditions influence GAS prophage induction, prophage-encoded virulence factor transcription, and levels of immunoreactive protein in culture supernatants. Exposure of strain MGAS315 to D562 epithelial cells induced prophage Φ315.4 (encoding SpeK and Sla) and prophage Φ315.6 (encoding Sdn). Importantly, the induction of prophage Φ315.4 was linked to increased transcription of *sla* and Sla expression. Prophage Φ315.4 has been closely associated with the recent rapid dissemination of a distinct subclone of serotype M3 in many regions of the world (3, 13). Our observation of substantial up-regulation of Sla production during epithelial cell interaction supports the hypothesis (3) that this extracellular phospholipase A_2 is important in host-pathogen interactions. Additionally, it is possible that the increase in Sla production during MGAS315 coculture with D562 epithelial cells is related to the previously described secreted factor made by these immortalized human cells (4). Alternatively, it is possible that other as yet unidentified secreted host factors contribute to the condition-dependent up-regulation of Sla production.

One additional point is of particular interest. We found that *speK* and *sla* were transcribed independently of one another. Hence, it is possible that transcription of these genes occurs differentially in vivo in response to distinct environmental or host signals.

Prophage induction is neither necessary nor sufficient for increased production of prophage-encoded virulence factors. Mitomycin C treatment induced all of the prophage-like elements present in strain MGAS315 that encode virulence factors, resulting in an increase in the copy numbers of the prophage-encoded virulence genes. However, the transcript level and amount of immunoreactive virulence factor protein decreased during mitomycin C treatment relative to the control culture. We speculate that this phenomenon occurs because of a toxic effect of mitomycin C on GAS. An alternative hypothesis is that after phage induction, phage-encoded virulence factors were depleted from the cultured supernatants due to association with phage particles. Since hyaluronidase is known to associate with phage tail proteins in GAS (2), it is possible that phage-encoded DNases and superantigens behave similarly.

The SpeK-encoding prophage Φ315.4 was induced during coculture with D562 cells, but immunogenic SpeK was often absent in culture supernatants. Conversely, the levels of *speA* transcript and immunoreactive SpeA increased during coculture of GAS with D562 cells, but prophage Φ315.5, encoding *speA*, was not induced. Taken together, these results indicate that an increase in the gene copy numbers of some prophage-encoded virulence factors in strain MGAS315 is neither necessary nor sufficient to achieve enhanced production of extracellular proteins.

Is induction of strain MGAS315 prophages by oxidative stress a common theme among DNase-encoding prophages? Culturing strain MGAS315 with hydrogen peroxide induced prophage Φ315.3, encoding *mf4*, an extracellular DNase. Of note, the prophage in serotype M1 strain MGAS5005 that encodes *mf3* (Φ370.3-like), the prophage in serotype M18 strain MGAS8232 that encodes *mf3* (Φ8232.4), and Φ315.3 in strain MGAS315 are integrated at the analogous chromosomal attachment site (3, 30; A. Madrigal and J. M. Musser, unpublished data). All of these prophages have reduced genome sizes, and they are very closely related in overall sequence similarity, suggesting that they have shared a recent common ancestor (3). Voyich et al. (34) reported that coculture of the serotype M1 strain MGAS5005 with human polymorphonuclear leukocytes (PMNs) resulted in transcriptional up-regulation of *mf3*. PMNs kill phagocytized bacteria by producing hydrogen peroxide and other toxic metabolites (32). Hence, it is possible that a common response of GAS to oxidative stress involves induction of a DNase-encoding prophage and increased DNase production. Consistent with this idea, the level of *mf4* transcript increased during the culture of strain MGAS315 with hydrogen peroxide. In addition, we note that Virtaneva et al. (33) reported that the level of transcript of another DNase (Sda) was nearly 62-fold higher than that of the control transcript (from *proS*) during acute pharyngitis infection in humans. These observations lead us to speculate that DNases contribute to the ability of GAS to withstand phagocytosis and killing by human PMNs.

Conclusion. The interactions among prophages, their bacterial hosts, and the environment that regulate the genetic switch between the lytic and lysogenic life cycle stages are complex and varied. Bacterial genes and environmental signals that contribute to maintaining lysogeny, participate in development of a lytic state, and alter transcription of phage-encoded virulence factors are poorly understood in GAS. We discovered that prophage induction and expression of prophage-encoded virulence factors occurred in vitro under conditions inferred to approximate some of the environmental signals encountered in vivo during host-pathogen interactions. Importantly, specific external signals induced some prophages but not others. Moreover, prophage induction, transcription of prophage-encoded virulence factors, and virulence factor production were not always linked. Taken together, our results suggest that GAS can respond to variable environments with condition-dependent prophage induction and the expression of prophage-encoded virulence factors. It is worth noting that our results regarding the effects of mitomycin C and hydrogen peroxide on phage induction in GAS might have important implications for antibiotic treatment of GAS infections. Mitomycin C, hydrogen peroxide, and fluoroquinolone antibiotics, which are potent inducers of prophage lambda and the SOS response, promote DNA damage (6, 8). Although we did not examine the effects of DNA-damaging antibiotics on phage induction and the expression of phage-encoded virulence factors in GAS, it is possible that antibiotics that disrupt prokaryote DNA replication could lead to phage induction and the expression of phage-encoded virulence factors in GAS. Indeed, fluoroquinolones that poison DNA topoisomerase II induce Shiga toxin-encoding bacteriophages and toxin production in *E. coli* (35) and induce bacteriophages in *Streptococcus canis* (14). Given the critical importance of phages in mediating strain diversity and evolution in GAS, and the contribution of prophage-encoded virulence factors to host-pathogen interactions, it is crucial to unravel the molecular events underlying our observations.

ACKNOWLEDGMENTS

We thank S. F. Hayes for performing the electron microscopy of phage particles and S. Beres for assistance with data analysis. We also

thank P. Rosa and R. Rebeil for helpful comments during the manuscript preparation

REFERENCES

1. **Banks, D. J., S. B. Beres, and J. M. Musser.** 2002. The fundamental contribution of phages to GAS evolution, genome diversification and strain emergence. Trends Microbiol. **11:**515–521.
2. **Benchetrit, L. C., E. D. Gray, and L. W. Wannamaker.** 1977. Hyaluronidase activity of bacteriophages of group A streptococci. Infect. Immun. **15:**527–532.
3. **Beres, S. B., G. L. Sylva, K. D. Barbian, B. Lei, J. S. Hoff, N. D. Mammarella, M. Y. Liu, J. C. Smoot, S. F. Porcella, L. D. Parkins, D. S. Campbell, T. M. Smith, J. K. McCormick, D. Y. M. Leung, P. M. Schlievert, and J. M. Musser.** 2002. Genome sequence of a serotype M3 strain of group A *Streptococcus*: phage-encoded toxins, the high-virulence phenotype, and clone emergence. Proc. Natl. Acad. Sci. USA **99:**10078–10083.
4. **Broudy, T. B., V. Pancholi, and V. A. Fischetti.** 2001. Induction of lysogenic bacteriophage and phage-associated toxin from group A streptococci during coculture with human pharyngeal cells. Infect. Immun. **69:**1440–1443.
5. **Broudy, T. B., V. Pancholi, and V. A. Fischetti.** 2002. The in vitro interaction of *Streptococcus pyogenes* with human pharyngeal cells induces a phage-encoded extracellular DNase. Infect. Immun. **70:**2805–2811.
6. **DeMarini, D. M., and B. K. Lawrence.** 1992. Prophage induction by DNA topoisomerase II poisons and reactive-oxygen species: role of DNA breaks. Mutat. Res. **267:**1–17.
7. **Desiere, F., W. M. McShan, D. van Sinderen, J. J. Ferretti, and H. Brussow.** 2001. Comparative genomics reveals close genetic relationships between phages from dairy bacteria and pathogenic streptococci: evolutionary implications for prophage-host interactions. Virology **288:**325–341.
8. **Elespuru, R. K.** 1987. Inducible responses to DNA damage in bacteria and mammalian cells. Environ. Mol. Mutagen. **10:**97–116.
9. **Ermolaeva, M. D., H. G. Khalak, O. White, H. O. Smith, and S. L. Salzberg.** 2002. Prediction of transcription terminators in bacterial genomes. J. Mol. Biol. **301:**27–33.
10. **Ferretti, J. J., W. M. McShan, D. Ajdic, D. J. Savic, G. Savic, K. Lyon, C. Primeaux, S. Sezate, A. N. Suvorov, S. Kenton, H. S. Lai, S. P. Lin, Y. Qian, H. G. Jia, F. Z. Najar, Q. Ren, H. Zhu, L. Song, J. White, X. Yuan, S. W. Clifton, B. A. Roe, and R. McLaughlin.** 2001. Complete genome sequence of an M1 strain of *Streptococcus pyogenes.* Proc. Natl. Acad. Sci. USA **98:**4658–4663.
11. **Gerlach, D., H. Knoll, W. Kohler, J. H. Ozegowski, and V. Hribalova.** 1983. Isolation and characterization of erythrogenic toxins. V. Communication: identity of erythrogenic toxin type B and streptococcal proteinase precursor. Zentbl. Bakteriol. Mikrobiol. Hyg. 1 Orig. A **255:**221–223.
12. **Graham, M. R., L. M. Smoot, C. A. Migliaccio, K. Virtaneva, D. E. Sturdevant, S. F. Porcella, M. J. Federle, G. J. Adams, J. R. Scott, and J. M. Musser.** 2002. Virulence control in group A *Streptococcus* by a two component gene regulatory system: global expression profiling and in vivo modeling. Proc. Natl. Acad. Sci. USA **99:**13855–13860.
13. **Ikebe, T., A. Wada, Y. Inagaki, K. Sugama, R. Suzuki, D. Tanaka, A. Tamaru, Y. Fuginaga, Y. Abe, Y. Shimizu, and H. Watanabe.** 2002. Dissemination of the phage-associated novel superantigen SpeL in recent invasive and non-invasive M3/T3 *Streptococcus pyogenes* isolates in Japan. Infect. Immun. **70:**3227–3233.
14. **Ingrey, K. T., J. Ren, and J. F. Prescott.** 2003. A fluoroquinolone induces a novel mitogen-encoding bacteriophage in *Streptococcus canis*. Infect. Immun. **71:**3028–3033.
15. **Kansal, R. G., V. Nizet, A. Jeng, W. J. Chuang, and M. Kotb.** 2003. Selective modulation of superantigen-induced responses by streptococcal cysteine protease. J. Infect. Dis. **187:** 398–407.
16. **Kapur, V., J. T. Maffei, R. S. Greer, L. L. Li, G. J. Adams, and J. M. Musser.** 1994. Vaccination with streptococcal extracellular cysteine protease (interleukin-1β convertase) protects mice against challenge with heterologous group A streptococci. Microb. Pathog. **16:**443–450.
17. **Kotb, M., A. Norrby-Teglund, A. McGeer, H. El-Sherbini, M. T. Dorak, A. Khurshid, K. Green, J. Peeples, J. Wade, G. Thomson, B. Schwartz, and D. E. Low.** 2002. An immunogenetic and molecular basis for differences in outcomes of invasive group A streptococcal infections. Nat. Med. **12:**1398–1404.
18. **Lei, B., S. Mackie, S. Lukomski, and J. M. Musser.** 2000. Identification and immunogenicity of group A *Streptococcus* culture supernatant proteins. Infect. Immun. **68:**6807–6818.
19. **Llewelyn, M., and J. Cohen.** 2002. Superantigens: microbial agents that corrupt immunity. Lancet Infect. Dis. **2:**156–162.
20. **Musser, J. M., A. R. Hauser, M. H. Kim, P. M. Schlievert, K. Nelson, and R. K. Selander.** 1991. *Streptococcus pyogenes* causing toxic-shock-like syndrome and other invasive diseases: clonal diversity and pyrogenic exotoxin expression. Proc. Natl. Acad. Sci. USA **88:**2668–2672.
21. **Musser, J. M., V. Kapur, S. Kanjilal, U. Shah, D. M. Musher, N. L. Barg, K. H. Johnston, P. M. Schlievert, J. Henrichsen, D. Gerlach, R. M. Rakita, A. Tanna, B. D. Cookson, and J. C. Huang.** 1993. Geographic and temporal distribution and molecular characterization of two highly pathogenic clones of *Streptococcus pyogenes* expressing allelic variants of pyrogenic exotoxin A (scarlet fever toxin). J. Infect. Dis. **167:** 337–346.
22. **Musser, J. M., K. Stockbauer, V. Kapur, and G. W. Rudgers.** 1996. Substitution of cysteine 192 in a highly conserved *Streptococcus pyogenes* extracellular cysteine protease (interleukin 1β convertase) alters proteolytic activity and ablates zymogen processing. Infect. Immun. **64:**1913–1917.
23. **Musser, J. M., and R. M. Krause.** 1998. The revival of group A streptococcal diseases, with a commentary on staphylococcal toxic shock syndrome, p. 185–218. *In* R. M. Krause (ed.), Emerging infections. Academic Press, Washington, D. C.
24. **Nida, S. K., and J. J. Ferretti.** 1982. Phage influence on the synthesis of extracellular toxins in group A streptococci. Infect. Immun. **36:**745–750.
25. **O'Brien, K. L., B. Beall, N. L. Barrett, P. R. Ceislak, A. Reingold, M. M. Farley, R. Danila, E. R. Zell, R. Facklam, B. Schwartz, and A. Schuchat.** 2002. Epidemiology of invasive group A *Streptococcus* disease in the United States, 1995–1999. Clin. Infect. Dis. **35:**268–276.
26. **Ogburn, C. A., T. N. Harris, and S. Harris.** 1958. Extracellular antigens in steady-state cultures of the hemolytic streptococcus: production of proteinase at low pH. J. Bacteriol. **76:**142–151.
27. **Ohnishi, M., K. Kurokawa, and T. Hayashi.** 2001. Diversification of *Escherichia coli* genomes: are bacteriophages the major contributors? Trends Microbiol. **9:**481–485.
28. **Reda, K. B., V. Kapur, J. A. Mollick, J. G. Lamphear, J. M. Musser, and R. R. Rich.** 1994. Molecular characterization and phylogenetic distribution of the streptococcal superantigen gene (*ssa*) from *Streptococcus pyogenes.* Infect. Immun. **62:**1867–1874.
29. **Sharkawy, A., D. E. Low, R. Saginur, D. Gregson, B. Schwartz, P. Jessamine, K. Green, and A. McGeer.** 2002. Severe group A streptococcal soft-tissue infections in Ontario: 1992–1996. Clin. Infect. Dis. **34:**454–460.
30. **Smoot, J. C., K. D. Barbian, J. J. Van Gompel, L. M. Smoot, M. S. Chaussee, G. L. Sylva, D. E. Sturdevant, S. M. Ricklefs, S. F. Porcella, L. D. Parkins, S. B. Beres, D. S. Campbell, T. M. Smith, Q. Zhang, V. Kapur, J. A. Daly, L. G. Veasy, and J. M. Musser.** 2002. Genome sequence and comparative microarray analysis of serotype M18 group A *Streptococcus* strains associated with acute rheumatic fever outbreaks. Proc. Natl. Acad. Sci. USA **99:**4668–4673.
31. **Smoot, L. M., J. C. Smoot, M. R. Graham, G. A. Somerville, D. E. Sturdevant, C. A. Lux Migliaccio, G. L. Sylva, and J. M. Musser.** 2002. Global differential gene expression in response to growth temperature alteration in group A *Streptococcus.* Proc. Natl. Acad. Sci. USA **98:**10416–10421.
32. **Storz, G., and J. A. Imlay.** 1999. Oxidative stress. Curr. Opin. Microbiol. **2:**188–194.
33. **Virtaneva, K., M. R. Graham, S. F. Porcella, N. P. Hoe, H. Su, E. A. Graviss, T. J. Gardner, J. E. Allison, W. J. Lemon, J. R. Bailey, M. J. Parnell, and J. M. Musser.** 2003. Group A *Streptococcus* gene expression in humans and *Cynomolgus* macaques with acute pharyngitis. Infect. Immun. **71:**2199–2207.
34. **Voyich, J. M., D. E. Sturdevant, K. R. Braughton, S. D. Kobayashi, B. Lei, K. Virtaneva, D. W. Dorward, J. M. Musser, and F. R. DeLeo.** 2003. Bacterial pathogen genome-wide protective response to human innate immunity: molecular strategies used by group A *Streptococcus* to evade destruction by human polymorphonuclear leukocytes. Proc. Natl. Acad. Sci. USA **100:**1996–2001.
35. **Zhang, X., A. D. McDaniel, L. E. Wolf, G. T. Keusch, M. K. Waldor, and D. W. K. Acheson.** 2000. Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. J. Infect. Dis. **181:**664–670.

Editor: D. L. Burns