Genetic Diversity among Type *emm28* Group A *Streptococcus* Strains Causing Invasive Infections and Pharyngitis

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Received 12 January 2005/Returned for modification 1 March 2005/Accepted 29 April 2005

Genome sequencing of group A *Streptococcus* (GAS) has revealed that prophages account for the vast majority of gene content differences between strains. Serotype M28 strains are a leading cause of pharyngitis and invasive infections, but little is known about genetic diversity present in natural populations of these organisms. To study this issue, population-based samples of 568 strains from Ontario, Canada; Finland; and Houston, Texas, were analyzed. Special attention was given to analysis of variation in prophage-encoded virulence gene content by a PCR-based method. Thirty and 29 distinct prophage-encoded virulence gene profiles were identified among pharyngitis and invasive infection isolates. Thirteen profiles, representing the majority of the strains, were shared between these two classes of isolates. Significant differences were observed in the frequency of occurrence of certain prophage toxin gene profiles and infection type. M28 strains are highly diverse in prophage-encoded virulence gene content and integration site, supporting the key concept that prophages are critical contributors to GAS genetic diversity and population biology. Nucleotide sequence variation in the *emm* gene (encodes M protein) was also examined. Only three allelic variants were identified in the hypervariable portion of the *emm28* gene. All but one strain had the same inferred amino acid sequence in the first 100 amino acids of the mature M28 protein. In contrast, size differences in the *emm28* gene and inferred protein due to variable numbers of C-terminal repeats were common. The presence of macrolide resistance genes (*mefA*, *ermB*, and *ermTR*) was analyzed by PCR, and less than 2% of the strains were positive.

Research on group A *Streptococcus* (GAS) host-pathogen interactions has been aided greatly by the recent publication of genome sequences from serotype M1, M3, M6, and M18 strains (1, 5, 16, 38, 48, 51). The availability of multiple genome sequences has also increased our understanding of GAS molecular population genetics, species diversity, and strain variation within and between M-protein serotypes. The majority of differences in gene content between strains are located in 35- to 45-kb insertions corresponding to prophages, prophage-like elements, and other exogenous sequences (1–6, 16, 38, 48, 51) (for simplicity, the term prophage will be used to refer to prophages or prophage-like elements). The genome of each sequenced GAS strain is polylysogenic, and 23 distinct prophages have been described (1–6, 16, 38, 48, 51). Twenty of these 23 prophages encode one or two proven or putative extracellular virulence factors, including pyrogenic toxin superantigens (*speA*, *speC*, *speH*, *speI*, *speK*, *speL*, *speM*, and *ssa*), DNases (*spd1*, *spd3*, *spd4*, *sdn*, and *sda*), a phospholipase A₂ (*slaA*), macrolide resistance (*mefA*), and a novel cell surface protein of unknown function (1–5, 16, 37, 38, 48, 51).

The contribution of serotype M28 GAS strains to human morbidity and mortality has often been underappreciated in part because serotypes such as M1 and M3 attract widespread investigative attention. However, serotype M28 strains are also leading causes of invasive infections and pharyngitis episodes. For example, surveillance of GAS disease in the United States has documented that serotype M28 strains are among the top five most common causes of invasive and pharyngeal infections (30, 39, 46, 47). Serotype M28 strains have also been documented to be abundant causes of human infections in countries other than the United States (10, 11, 12, 15, 17, 25, 27, 32, 33, 43, 46, 53–56). Despite the importance of serotype M28 strains in human infections, relatively few studies have investigated genetic diversity and prophage content in these organisms. The goal of the present work was to analyze genetic diversity among serotype M28 strains cultured from patients in three distinct geographic sites, with special attention given to analysis of prophage-related proven and putative virulence genes and chromosomal integration site.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 568 serotype M28 GAS strains representing three distinct sets of strains were studied (Fig. 1).

(i) **Strains from Ontario, Canada.** Two hundred forty-six strains were obtained from a population-based study of invasive GAS infections in Ontario,

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FIG. 1. Temporal distribution of *emm28* isolates studied.

Canada. These strains represent all sterile-site serotype M28 isolates reported from 1991 to 2002. This ongoing study has been well described (11, 27, 34, 46). Invasive disease was defined as isolation of GAS from a normally sterile site or tissue or from a wound accompanied by necrotizing fasciitis. The major disease types represented by the 246 Ontario isolates included soft tissue (31%), postpartum/gynecological (17%), bacteremia (12%), arthritis (11%), necrotizing fasciitis (7%), and other/unknown (22%). The majority of infections occurred in females (65%), males accounted for 34% of the infections, and patient gender was unknown for 1% of the cases. The majority of gynecological infections (e.g., peripartum and postpartum infections) occurred in females between 30 and 39 years of age.

(ii) **Strains from Finland.** One hundred eleven strains were collected in Finland between 1995 and 2002 from a national population-based study of invasive infections (35, 45). All isolates were from blood or cerebrospinal fluid.

(iii) **Strains from Houston, Texas.** Two hundred eleven strains were cultured from pediatric patients with pharyngitis in Houston, Texas, between November 2001 and January 2003. These patients were seen at one private-practice pediatric outpatient clinic affiliated with the Texas Children's Hospital and Baylor College of Medicine. In 2002, the clinic had 49,836 patient visits. A rapid GAS antigen test (Directigen 1-2-3 Group A Strep Test; Becton-Dickinson) was performed on a pharyngeal swab obtained from all patients seen during the study period with signs and symptoms consistent with GAS pharyngitis. Individuals with a positive rapid antigen test were cultured, and beta-hemolytic organisms with colony morphology consistent with GAS were confirmed by group-specific antigen typing (BBL Streptocard; Becton-Dickinson). A total of 1,445 GAS isolates were obtained. The M type of the organism was inferred on the basis of the results of DNA sequence analysis of a 300-bp region of the 5' end of the *emm* gene encoding M protein (see below). These organisms represent a comprehen-

sive convenient sample of all GAS isolates causing pharyngitis in a city with a large multiethnic population.

Strains used as experimental controls. GAS strains MGAS8232 (serotype M18), MGAS315 (serotype M3), MGAS10394 (serotype M6), and MGAS6708 (serotype M1 [also known as strain SF370]) were used as positive controls for strains containing prophage-associated virulence factor genes (1, 5, 16, 48). Strains MGAS5005 (serotype M1) and MGAS9429 (serotype M12) were used as positive controls for the prophage-encoded *speA* and *sda* genes, respectively. The genomes of these six strains have been sequenced (1, 5, 16, 48, 51; unpublished data). Serotype M12 strains MGAS10380, MGAS10377, and MGAS10378 were used as positive controls for the *ermTR*, *mefA*, and *ermB* genes encoding macrolide resistance.

GAS strains were grown overnight at 37°C in an atmosphere of 5% CO₂ on Trypticase soy agar supplemented with 5% sheep blood (Becton-Dickinson, Sparks, MD). Bacteria were transferred to Todd-Hewitt broth supplemented with 1% yeast extract (Difco, Sparks, MD) and grown overnight at 37°C in an atmosphere of 5% CO₂.

Isolation of chromosomal DNA. GAS chromosomal DNA was isolated using the PureGene DNA purification kit (GentraSystems, Minneapolis, MN). The protocol supplied by the manufacturer was used with the following modifications: addition of mutanolysin (10 µl of 10 U/µl; Sigma-Aldrich, St. Louis, MO), lysozyme (2 µl of 20 mg/ml; Sigma-Aldrich, St. Louis, MO), and RNase A (5 µl of 10 mg/ml; Sigma-Aldrich, St. Louis, MO).

Analysis of the *emm* gene. The *emm* gene was amplified by PCR using primers *emm1b* and *emm2* (Table 1) (36). PCR was performed in a reaction mixture volume of 25 µl under the following conditions: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min 45 s, and a final extension step at 72°C for 5 min. Size variation in the *emm* PCR product was assessed by gel electrophoresis using 1.5% agarose gels. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Purified PCR product was used as template for cycle sequencing with either *emm1b* or *emm2* primer under the following conditions: 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reactions consisted of 8 µl template, 3 µl BigDye Terminator v.3.0 (Applied Biosystems, Foster City, CA), 1.2 µl of 2 µM primer, and double-distilled H₂O to a final volume of 15 µl. CentriSept 96-well plates (Princeton Separations, Adelphia, NJ) were used to remove excess nucleotides and primer, and the reaction products were analyzed with an ABI Prism 3700 DNA instrument (Applied Biosystems, Foster City, CA). Sequence data were analyzed by BLAST search performed against the Centers for Disease Control and Prevention (CDC) *Streptococcus pyogenes emm* sequence database (<http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm>). To be identified as a particular *emm* type, *emm* sequences had to have greater than 98% identity with the *emm* sequence of the CDC reference strain. The reference

TABLE 1. Primers used for screening prophage-associated virulence genes, macrolide resistance genes, and *emm* analysis

Amplicon type	Gene	Forward primer	Reverse primer	Amplicon size (bp)
Prophage-associated virulence factor	<i>spd1</i>	CCCTTCAGGATTGCTGTCAT	ACTGTTGACGCAGCTAGGG	400
	<i>spd3</i>	CAAATTGACTGACGCTAATGG	CCGCTTCTCAAACCTCTTCG	481
	<i>spd4</i>	TGGCATTGCTTCATAGTAAAGG	ATCTACCTGAAGCTTTGTCGTG	494
	<i>sda</i>	AGCATAGCCCCAAAAATGTG	TGCTACAGCATTGAAGACACG	379
	<i>sdn</i>	AACGTTCAACAGGCGCTTAC	ACCCCATCGGAAGATAAAGC	489
	<i>slaA</i>	CTCTAATAGCATCGGCTACGC	AATGGAAAATGGCACTGAAAG	440
	<i>speA</i>	ATGGAAAACAATAAAAAAG	TTACTTGGTGTTAGGTAGCTTC	755
	<i>speC</i>	TCTAGTCCCTTCATTGGTG	GTAAATTTTTCACGACACA	459
	<i>speH</i>	GTGAATGTCCAGGGAAAAGG	GCATGCTATTAAGTCTCCATTG	317
	<i>speI</i>	AATGAAGGTCGCGCATTTTC	TCTCTCTGTCAACATGTCCTG	516
	<i>speK</i>	GTGTGTCTAATGCCACCGTCT	GGAACATATATGCTCCTAGAT	564
	<i>speL</i>	CAGCACCTTCTCTTTCTCG	GGAAAAAGAGGGACGCAAG	459
	<i>speM</i>	GGATGAGTGAATAAATCGGTAAAC	AGTCTGGGACGATGATAA	425
	<i>ssa</i>	TGATCAAATATTGCTCCAGGTG	TCCACAGGTCAGCTTTTACAG	502
Macrolide resistance	<i>ermB</i>	GAAAAGGTACTCAACCAAATA	AGTAACGGTACTTAAATTGTTTAC	525
	<i>ermTR</i>	GAAGTTTAGCTTTCTTAA	GCTTCAGCACCTGTCTTAATTG	420
	<i>mefA/E</i>	AGTATCATTAATCACTAGTGC	TTCTTCTGGTACTAAAAGTGG	345
<i>emm</i>	<i>emm</i>	GGGAATTCTATTSGCTTAGAAAATTAA	GCAAGTTCTTCAGCTTGTTT	Variable

TABLE 2. Multiplex primers used for screening prophage-associated virulence genes

Arbitrary position (chromosomal location of prophage-associated virulence factor gene)	Multiplex primer
1	GCTCTTGCGCAATTGTTTT
2	CGTCTTGCAATCCAGTGATG
3	AGTTGGTTGTTTTGGTCA
4	GTAAAGGCGCATCCACATC
5	AGCCAAAAGCGAGGAAA
6	GCAGAGCACCTCCAG
7	GAAAGGGCATCCCTAAATC
8	TGTCTGCTGTGGATTAAGTTGG
9	ACCAGCAACAACAGCAGA
10	AGAAGGTTGCTCCCCAAAT
11	GGCGACCATCACTAATCA
12	TTCTCACCTTAGCAACTGCTTG
13	TCAGTTGCCCATTAATGCT
14	CGTTTCGAAAAATCACTAAATGC
15	ACGCGTATGGAAGTTCTGGT
16	TCAAAGGCTAGACTAGATTTTCC

strain sequences are available from the CDC at the following URL: <http://www.cdc.gov/ncidod/biotech/strep/emmtypes.htm>. To identify putative *emm* polymorphisms, SeqManII software (DNASTAR, Madison, WI) was used to align the *emm* sequences with the *emm28* alleles present in the CDC *emm* database.

PCR screening for genes encoding macrolide resistance. The presence of genes (*ermB*, *ermTR*, and *mefA*) encoding macrolide resistance (9, 44, 52) was assessed by PCR (Table 1). The amplification conditions used for the genes were as follows: for *ermB* and *mefA*, initial denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 1 min, primer annealing at 57°C for 1 min, and extension at 72°C for 1 min, and a final extension step at 72°C for 5 min; and for *ermTR*, initial denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 30 s, primer annealing at 42°C for 30 s, and extension at 72°C for 1 min, and a final extension step at 72°C for 5 min.

Identification of prophage-associated virulence genes and virulence gene profiles. A two-step PCR-based method was used to determine the presence and genome location of putative prophage elements and prophage-associated virulence genes, as described recently (1, 6). Strains used as positive controls included MGAS8232 (serotype M18), MGAS315 (serotype M3), MGAS10394 (serotype M6), and SF370 (serotype M1) (1, 5, 16, 48). Strains MGAS5005 (serotype M1) and MGAS9429 (serotype M12) were used as positive controls for the *speA* and *sda* genes, respectively (51; unpublished data). Prophage-associated virulence genes that could not be mapped to any of the known prophage locations in strains SF370, MGAS315, MGAS10394, and MGAS8232 were localized using unpublished genome sequence data available for strain MGAS9429 (serotype M12) and PCR with different combinations of primers directed against genes located in regions of the chromosome flanking known prophage integration sites (Table 2). The PCR amplification conditions used for all 14 virulence genes were as follows: initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 3 min, and a final extension step at 72°C for 5 min (Table 1). The combination of prophage-associated virulence genes was defined as the virulence gene profile.

Statistical analysis. Chi-square analysis was used to assess associations between virulence factor profile and GAS disease type. A *P* value of ≤0.05 was considered to be significant.

RESULTS

***emm* gene typing and size variation.** Extensive allelic variation has been reported for strains of many M-protein serotypes in the region of the *emm* gene encoding the first 100 amino acids of the mature protein. For example, more than 20 serotype M1 and serotype M3 variants each have been identified (6, 22, 30, 36) (<http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm>). To determine whether allelic variation existed in the analogous region of the *emm28* gene, a 300-bp region of the 5'

FIG. 2. *emm28* gene PCR size variation among the strains studied. (A) The distribution of *emm* PCR size variants by geographic locality. Two main *emm28* size variants (850 bp and 950 bp) and a mixed-size variant (850 bp and 950 bp) were identified by PCR. The majority of the strains had the 850-bp size *emm* product. (B) Sequencing of the entire *emm28* gene in strains with representative size variants revealed the size difference to be caused by variation in the number of 105-bp repeat segments. The boxed insert contains the nucleotide sequence of the 105-bp repeat. R, repeat region.

end of this gene was sequenced in all 568 strains. Surprisingly, nucleotide sequence variation was relatively limited. Among the 357 strains causing invasive infections, only one strain had nucleotide sequence variation in this region that differed from the sequence of *emm28.0*. The sequence of this strain was identical to that of the *emm28.1* strain, except that it had a short deletion resulting in a change in amino acid sequence from KLEEKEKNL at positions 65 to 73 to KEL. Only two alleles, *emm28.0* and *emm28.4*, were identified among the 211 pharyngeal isolates. The *emm28.4* gene was represented in 42 (20%) pharyngeal strains.

Size variation in the *emm* gene in GAS has also been well documented (23, 26, 49). Size variation in *emm* is generally caused by differences in the number of repeat segments present in the 3' region of the gene. PCR analysis identified size variants of ~750 bp, ~850 bp, and ~950 bp (Fig. 2A). The majority of strains examined (371 isolates; 65.3%) had only a 850-bp amplicon. Some strains (55 isolates; 9.7%) had both the 850-bp and 950-bp amplicons. Of note, a significantly greater number of pharyngitis strains had the mixed *emm* gene amplicon (*P* ≤ 0.01). Sequencing of the entire *emm28* gene from representative isolates with the 750-bp, 850-bp, or 950-bp amplicon revealed that the size difference was caused by variation

FIG. 3. Frequency distribution of prophage-encoded virulence factor genes and common virulence factor profiles. Strains were screened for the presence of 14 prophage-encoded virulence factor genes by PCR. (A) Overall distribution of prophage-encoded virulence factor genes among the 568 *emm28* strains analyzed. (B) Frequency distribution of prophage-encoded virulence factor genes by geographic locality. (C) Comparison of prophage-encoded virulence factor gene frequency between invasive and pharyngitis isolates. Invasive strains had a significantly higher rate of occurrence of the *sdn* gene ($P < 0.01$). Pharyngitis isolates were characterized by more strains containing the *speI*, *speH*, and *speA* toxins ($P < 0.001$). (D) Virulence factor profile of the invasive and pharyngeal *emm28* isolates. Profiles were generated by combining all prophage-encoded virulence factor genes present, and an arbitrary letter code designation was assigned to each profile. The key to the code designation is provided in the boxed insert. Significant differences in overall prophage-encoded virulence factor gene content between the two GAS populations (invasive and pharyngeal isolates) were observed ($P < 0.001$ *, $P < 0.01$ [± in panel C], and $P < 0.025$ [± in panel D]).

in the number of 105-bp repeat segments present in the 3' region of the gene (Fig. 2B).

Frequency of occurrence of genes conferring macrolide resistance. Resistance to macrolide antibiotics has been documented to be an increasing problem for GAS in many geographic areas. Resistance to this class of antibiotics can be due to the *mefA* gene encoding a macrolide efflux pump or ribosomal modification by methylation caused by the *ermB* or *ermTR* gene (9, 29, 44, 52). Macrolide resistance genes have been reported to be transferred horizontally by mobile genetic elements, such as phages and transposons (4, 18, 19, 24, 41). As assessed by PCR, 1.2% of the 568 strains had the *mefA* gene, and virtually all of these PCR-positive strains were from pharyngitis cases. Less than 1% of the isolates contained the *ermB* or *ermTR* gene. Strains with these genes were found in each of the three patient populations.

Frequency of prophage-associated virulence genes. PCR was used to screen all 568 strains for 14 prophage-associated genes that encode extracellular proven or putative virulence factors (Fig. 3A). The majority of strains (84%) had the *speC* and *spd1* genes encoding streptococcal pyrogenic exotoxin C and a DNase, respectively. These genes are usually colocalized on the same prophage (16, 48). Other prophage-associated genes encoding extracellular proteins that were relatively common

included *sdn* (34%), *slaA* (16%), and *speK* (17%), encoding a DNase, phospholipase A₂, and streptococcal pyrogenic exotoxin K, respectively. None of the genes encoding the other nine extracellular proteins were present in greater than 5% of the 568 strains. The presence of the *ssa* (streptococcal superantigen A) and *spd4* (DNase) genes was very rare.

Association between prophage-associated virulence genes and disease types. Inasmuch as prophage-encoded extracellular proteins can influence host-pathogen interactions, next we compared the frequency distribution of the 14 prophage-associated genes among invasive infection and pharyngeal isolates (Fig. 3B and C). Significantly more strains cultured from invasive infections had the *sdn* gene ($P \leq 0.001$; 138 versus 55 strains). Although these genes were relatively rare in the study population, significantly more strains from pharyngitis patients had the *speI* and *speH* genes ($P \leq 0.01$; 16 versus 4 strains) and the *speA* gene ($P \leq 0.001$; 21 versus 5 strains). The *speI* and *speH* genes are encoded by the same prophage ($\phi 370.2$) in serotype M1 strain SF370 (16).

The patient and disease information available for the invasive strains obtained from patients in Ontario, Canada, were used to test the hypothesis that a difference existed in individual prophage-associated virulence genes and disease outcome.

TABLE 3. Prophage-associated virulence gene profiles of M28 isolates used in this study

Profile no.	No. of isolates			Prophage-associated virulence gene													
	Ontario, Canada (n = 246)	Finland (n = 111)	Houston, Texas (n = 211)	<i>speC</i>	<i>mf2</i>	<i>mf4</i>	<i>mf3</i>	<i>sda</i>	<i>ssa</i>	<i>speI</i>	<i>speH</i>	<i>sdn</i>	<i>sla</i>	<i>speK</i>	<i>speL</i>	<i>speM</i>	<i>speA</i>
1	114	43	115	+	+	—	—	—	—	—	—	—	—	—	—	—	—
2	39	21	11	+	+	—	—	—	—	—	—	+	—	—	—	—	—
3	22	4	5	—	—	—	—	—	—	—	—	+	—	—	—	—	—
4	16	16	16	+	+	—	—	—	—	—	—	—	+	+	—	—	—
5	9	0	0	—	—	—	—	—	—	—	—	+	—	—	+	+	—
6	6	2	3	+	+	—	—	+	—	—	—	—	—	—	—	—	—
7	6	1	1	—	—	—	—	—	—	—	—	+	+	+	—	—	—
8	5	8	2	+	+	—	—	—	—	—	—	+	+	+	—	—	—
9	5	0	0	+	+	—	—	—	—	—	—	+	—	—	+	+	—
10	3	0	14	+	+	—	—	—	—	+	+	—	—	—	—	—	—
11	3	0	0	+	+	—	—	+	—	—	—	+	—	—	—	—	—
12	3	0	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
13	2	4	0	+	+	—	+	—	—	—	—	+	—	—	—	—	—
14	2	1	0	+	+	—	—	+	—	—	—	—	+	+	—	—	—
15	1	3	2	+	+	—	+	—	—	—	—	—	—	—	—	—	—
16	1	1	0	—	—	—	—	+	—	—	—	—	—	—	—	—	—
17	1	0	2	+	+	—	—	—	—	—	—	+	+	+	—	—	+
18	1	0	3	+	+	—	—	—	—	—	—	+	—	—	—	—	+
19	1	0	1	—	—	—	—	—	—	—	—	+	+	+	+	+	—
20	1	0	10	—	—	—	—	—	—	—	—	+	—	—	—	—	+
21	1	0	0	+	+	—	—	—	—	+	+	—	+	+	—	—	—
22	1	0	0	+	+	—	—	—	—	—	—	—	—	—	—	—	+
23	1	0	0	—	—	—	+	—	—	—	—	+	+	+	—	—	—
24	1	0	0	—	—	—	+	—	—	—	—	+	—	—	+	+	—
25	1	0	0	—	—	—	—	+	—	—	—	—	+	+	—	—	+
26	0	2	0	+	+	—	+	+	—	—	—	+	—	—	—	—	—
27	0	2	0	+	+	—	+	—	—	—	—	—	+	+	—	—	—
28	0	2	0	—	—	—	+	—	—	—	—	—	—	—	—	—	—
29	0	1	0	—	—	—	—	+	—	—	—	+	—	—	—	—	—
30	0	0	1	+	+	—	+	+	—	—	—	+	+	+	—	—	+
31	0	0	1	+	+	—	—	—	—	+	+	—	—	—	—	—	—
32	0	0	1	+	+	—	—	—	—	—	—	+	+	+	+	—	—
33	0	0	1	+	+	—	—	—	—	—	—	+	—	+	+	—	—
34	0	0	1	+	+	—	—	—	—	—	—	+	—	—	+	—	—
35	0	0	1	+	+	—	—	—	—	—	—	—	+	+	+	—	—
36	0	0	1	+	+	—	—	—	—	—	—	—	—	—	+	—	—
37	0	0	1	—	+	—	+	—	—	—	—	+	—	—	—	—	—
38	0	0	1	—	+	—	—	—	—	—	—	+	—	—	—	—	—
39	0	0	1	—	+	—	—	—	—	—	—	+	—	—	+	—	—
40	0	0	4	—	+	—	—	—	—	—	—	+	—	—	—	—	+
41	0	0	1	—	—	—	+	+	—	—	—	+	+	+	—	—	—
42	0	0	1	—	—	—	+	—	—	+	+	+	—	—	—	—	—
43	0	0	1	—	—	—	+	—	—	—	—	+	—	—	—	—	—
44	0	0	2	—	—	—	—	—	+	—	—	—	+	+	—	—	—
45	0	0	6	—	—	—	—	—	—	—	—	+	—	+	+	—	—
46	0	0	1	—	—	—	—	—	—	—	—	—	+	+	—	—	+

Strains with the *speC* or *spd1* gene were significantly associated ($P \leq 0.05$) with puerperal sepsis and gynecological infections.

The virulence gene PCR results were used to generate a 14-gene profile for each strain (Fig. 3D). Overall, there were 46 distinct profile types identified for M28 isolates used in this study (Table 3). A total of 25, 15, and 30 prophage gene profiles were identified among the strains from Ontario, Canada; Finland; and Houston, Texas, respectively. There were 29 prophage gene profiles among the strains from invasive infections and 30 profiles among the strains causing pharyngitis. Thirteen profiles were shared between invasive and pharyngitis strains. There was no simple association between prophage gene profile and infection type. However, significant differences were observed in the frequency of occurrence of certain rare profiles and infection type (Fig. 3D).

Chromosomal locations of prophage-associated virulence genes. As assessed by genome sequencing and related genome-wide studies, most differences in gene content between GAS strains are due to prophage insertion and deletion events (1–6, 48). Fifteen prophage insertion sites have been identified in the serotype M1, M3, M6, and M18 strains whose genomes have been sequenced (1–3, 5, 16, 38, 48). Closely related prophages can have the same chromosomal integration sites, although different proven or putative virulence gene(s) can be located at the end of related prophages due to recombination events. We used PCR to map the prophage-associated virulence genes on the basis of the 15 chromosomal locations known to contain prophages or prophage-like elements (Fig. 4). New virulence gene-locus combinations were identified for five genes, including *spd3*, *speC*, *spd1*, *sda*, and *sdn*. Of note,

FIG. 4. Schematic summarizing chromosomal sites of integration of prophage-encoded virulence genes. The large black circle denotes the core genome sequence shared by all sequenced GAS strains (serotype M1 strain SF370, serotype M3 strain MGAS315, serotype M6 strain MGAS10394, and serotype M18 strain MGAS8232). Short lines oriented perpendicular to the circle circumference represent the locations of conserved rRNA operons. Triangles represent prophage integration sites and the virulence gene(s) associated with each prophage. The triangles are color coded to correspond with the GAS serotype characterized and are numbered clockwise around the core chromosome. Stacked triangles indicate that prophage elements are inserted at the same site. The red triangles that lack internal numbers represent prophage-related virulence genes in M28 strains mapped to known GAS prophage integration sites. The chromosomal integration sites are numbered 1 to 16 around the inner aspect of the core chromosome based partially on the order of discovery. The genes and the proteins they encode follow: *sdn*, streptodornase (DNase); *speA*, streptococcal pyrogenic exotoxin A; *speC*, streptococcal pyrogenic exotoxin C; *speH*, streptococcal pyrogenic exotoxin H; *speI*, streptococcal pyrogenic exotoxin I; *speK*, streptococcal pyrogenic exotoxin K; *speL*, streptococcal pyrogenic exotoxin L; *speM*, streptococcal pyrogenic exotoxin M; *ssa*, streptococcal superantigen A; *slaA*, streptococcal phospholipase A₂; *spd1*, streptococcal phage DNase 1; *spd3*, streptococcal phage DNase 3; *spd4*, streptococcal phage DNase 4; *sda*, streptodornase-alpha; *mefA*, macrolide efflux A; R6, novel extracellular protein.

the tmRNA insertion site had an unusually diverse array of virulence genes among the serotype M28 strains, with nine different virulence genes (*speA*, *speC*, *spd1*, *speK*, *slaA*, *speL*, *speM*, *spd3*, and *sdn*) identified at this site (Fig. 4, position 5).

Using serotype M1 and M12 genome data, we identified a new phage insertion site (Fig. 4, position 12) located between the chromosomal positions of ϕ 315.6 and ϕ 8232.5 (51; J. M. Musser et al., unpublished data). Only two virulence genes (*sda* and *sdn*) were identified at this location in some *emm28* strains. In rare cases, a strain had the same virulence gene (*speC*, *spd1*, or *sdn*) inserted at two different locations in the genome.

Interestingly, none of the serotype M28 strains had a virulence gene located at the chromosomal location where ϕ 370.3, ϕ 8232.4, ϕ 10394.5, and ϕ 315.3 are inserted in the genomes of strains SF370, MGAS8232, MGAS10394, and MGAS315, respectively (Fig. 4, position 6). All published GAS genomes (serotype M1 strain SF370, serotype M3 strain MGAS315, serotype M3 strain SSI-1, serotype M18 strain MGAS8232, and serotype M6 strain MGAS10394) (1, 5, 16, 38, 48, 50) have a prophage-associated DNase gene, such as *spd1*, *spd3* or *spd4*, at this site. However, among the strains we studied, *spd3* was always located elsewhere in the chromosome (Fig. 4).

Although we were able to successfully identify the chromosomal locations of most virulence genes in the 568 strains studied, there were cases in which the target gene was present but located at an unknown chromosomal location compared to the serotype M1, M3, M6, M12, or M18 genomes. This occurred for some *emm28* strains which were PCR positive for *speK* (9/98), *slaA* (4/91), *speA* (3/26), *speI* (11/20), *speH* (11/20), *spd3* (11/26), *speC* (32/476), *spd1* (39/483), or *sdn* (45/193).

Prophage-associated virulence gene profiles and chromosomal locations. Prophage-associated virulence gene profiles were also generated for each strain on the basis of the combination of virulence gene content and chromosomal location. Four major profiles were identified on the basis of these criteria (Fig. 5A). These four major profiles accounted for 46.8%, 3.7%, 7.6%, and 6.1% of the total isolates included in this study. In each geographic area, there was considerable variation over time in the frequency of occurrence of each of the four prophage profiles. Differences were observed in the geographic distribution of these profile types (Fig. 5B to D). For example, profile 4 was relatively common in strains from Houston, Texas, and Ontario, Canada, but it was found for only a single isolate among the strains from Finland.

DISCUSSION

We analyzed genetic variation in *emm28* isolates from diverse geographic locations and disease types, with attention given to prophage-encoded virulence gene content and chromosomal location, *emm* gene allelic variation, and presence of genes encoding macrolide resistance. Several recent studies have examined GAS strains of various serotypes to determine whether there was a correlation between prophage-encoded virulence gene content and the ability to cause a specific disease (6, 21, 43, 55). However, there has not yet been an analysis of prophage-encoded virulence gene content that employed large population-based samples focused solely on type *emm28* GAS strains. Strains of this type are common causes of mild skin infections and pharyngitis and are also responsible for many episodes of severe invasive disease. For example, recent surveillance studies have found that *emm28* strains are among the predominant circulating *emm* types in many localities and are generally among the top five *emm* types identified (7, 11, 15, 25, 30, 32–34, 39, 43, 46, 47, 53–56). In addition, these strains have been recovered frequently from relatively unusual types of GAS infection, such as vulvovaginitis, perineal disease, puerperal sepsis, and other pregnancy-related infections (8, 10, 13, 15, 28, 31, 39, 40, 42, 55, 56).

Analysis of serotype M3, M6, M18, and other GAS has revealed that there is considerable variation in prophage con-

FIG. 5. Prophage-associated virulence gene profile type and temporal distribution. Profile types were numbered arbitrarily. (A) Schematics showing the four common prophage-associated virulence gene profile types identified in the study. Small numbers located inside the circles in panel A refer to the chromosomal integration site of each prophage (see Fig. 4). Temporal distribution of profile types in (B) Ontario, Canada; (C) Finland, and (D) Houston, Texas. The values in parentheses indicate the number and percentage of strains of a given type found over the entire study period.

tent within and between serotypes (1–6, 48). Beres et al. (6) recently reported a significant correlation between prophage content and chromosomal pulsed-field gel electrophoresis pattern in serotype M3 organisms. Closely related but distinct pulsed-field gel electrophoresis patterns have been identified in M28 strains by several investigators (12, 15, 21, 50, 55). Hence, we anticipated that M28 strains would be highly diverse in prophage-associated virulence gene content. Our results confirmed that this was the case and in addition showed substantial variation in the chromosomal location of these virulence genes. Analysis of 255 invasive serotype M3 isolates collected over 11 years from Ontario, Canada, identified nine distinct prophage genotypes (6). In striking contrast, 25 unique combinations of prophage-related virulence genes were present among the 246 invasive M28 isolates collected in the same geographic area (Ontario, Canada) over the same time period. What factors may contribute to this difference between M3 and M28 strains? In principle, it is possible that M28 strains simply had more prophage-associated virulence genes than M3 strains. However, this was not the case. On average, type M28 strains had fewer prophage-related virulence factor

genes than M3 strains. Four main profiles accounted for 78% of the M28 Ontario isolates, whereas three main profiles represented most M3 isolates. Two major differences between these populations include the high number of rare prophage virulence gene profiles present in M28 strains and different types of dominant prophage virulence gene profiles present in M28 and M3 strains. Similarly, European investigators (43, 55) screened M28 isolates for chromosomal and prophage-associated virulence genes and also found that M28 strains had a high level of diversity in prophage-related virulence factor gene profile types. Some possible explanations of this diversity are that *emm28* organisms are less clonal, unusually able to act as recipients of a broad range of phages, or are subject to higher recombination frequency among infecting phage. Clearly, additional study of this issue is needed.

The *emm28* strains we analyzed had prophage-associated virulence genes present at many locations recently reported for other GAS strains. Our analysis also identified several new chromosomal positions for these genes. DNA sequence analysis is needed to determine whether these virulence factors are encoded by prophages and whether the prophages that encode

these virulence factors are distinct from previously described prophages found in M1, M3, M6, and M18 strains (1–6, 16, 38, 48). Inasmuch as most GAS prophages encode one or more virulence factors, the possibility exists that periodic generation of new virulence gene combinations by recombination and lateral gene transfer will produce strains with altered virulence capacity. We note that we were unable to identify the chromosomal locations of prophage-associated virulence genes in some strains, indicating that additional insertion sites remain to be determined.

Knowledge of genetic diversity among bacterial strains provides insight into pathogen evolution and facilitates study of the relationships between strain genotypes and infection type. Importantly, the information we gained in this study about genetic diversity among *emm28* strains from widespread localities, coupled with the knowledge of a close linkage between *emm28* type and clone (14), permitted us to choose a genetically representative strain for whole-genome sequencing. Our findings, to be reported elsewhere (20), identified several new potential virulence factors and revealed significant new information that bears on the molecular basis of puerperal sepsis.

ACKNOWLEDGMENTS

We thank E. Conlon for assistance in preparing chromosomal DNA; A. Henion for help with statistical analysis; N. P. Hoe for extensive advice; K. D. Barbican, M. Liu, and G. Sylva for technical assistance and advice. We also appreciate the work of the multitude of physicians and support staff involved. We are indebted to patients at the Pediatric Medical Group (an affiliate of Texas Children's Pediatric Associates) in Houston, Texas and elsewhere for making this project possible.

This work was supported in part by NIH grant U01-AI-060595 to J.M.M.

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