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## Two Distinct Genotypes of *prtF2*, Encoding a Fibronectin Binding Protein, and Evolution of the Gene Family in *Streptococcus pyogenes*

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The group A *Streptococcus* (GAS) is an important pathogen that is responsible for a wide range of human diseases. Fibronectin binding proteins (FBPs) play an important role in promoting GAS adherence and invasion of host cells. The *prtF2* gene encodes an FBP and is present in approximately 60% of GAS strains. In the present study we examined 51 *prtF2*-positive GAS strains isolated from the Northern Territory of Australia, and here we describe two genotypes of *prtF2* which are mutually exclusive. The two genotypes have been identified previously as *pfbp* and *fbaB*. We show that these genotypes map to the same chromosomal location within the highly recombinatorial fibronectin-collagen-T antigen (FCT) locus, indicating that they arose from a common ancestor, and in this study these genotypes were designated the *pfbp* type and the *fbaB* type. Phylogenetic analysis of seven *pfbp* types, 14 *fbaB* types, and 11 *prtF2*-negative GAS strains by pulsed-field gel electrophoresis (PFGE) produced 32 distinct PFGE patterns. Interpretation of evolution based on the PFGE dendrogram by parsimony suggested that the *pfbp* type had a recent origin compared to the *fbaB* type. A comparison of multiple DNA sequences of the *pfbp* and *fbaB* types revealed a mosaic pattern for the amino-terminal region of the *pfbp* types. The *fbaB* type is generally conserved at the amino terminus but varies in the number of fibronectin binding repeats in the carboxy terminus. Our data also suggest that there is a possible association of the *pfbp* genotype with *sof* (84.2%), while the *fbaB* genotype was found in a majority of the GAS strains negative for *sof* (90.6%), indicating that these two *prtF2* subtypes may be under different selective pressures.

One mechanism for adherence of *Streptococcus pyogenes* (group A streptococcus [GAS]) to host cells and tissues is mediated by the interaction with the host ligand, fibronectin. Strains of GAS encode several proteins that have the capacity to bind fibronectin (9, 10, 16, 18, 19, 31, 32, 36, 37). This in itself strongly suggests that the fibronectin binding protein (FBP)-fibronectin interaction may play an important role in the progression of GAS infection and disease. Whereas many different FBPs in GAS have been described, not all strains are genetically totipotent for each of these FBPs (12, 14, 24, 40). For example, the *sfbl*, *sof*, and *prtF2* genes encoding FBPs are present in approximately 52, 44, and 60% of GAS strains isolated from the Northern Territory of Australia, respectively (12).

GAS is a human-specific pathogen and can cause a wide range of diseases, from benign mucosal and skin infections to life-threatening diseases and sequelae, such as acute poststreptococcal glomerulonephritis and rheumatic heart disease (11). Diversity in the repertoire of the genes encoding FBPs may have implications for GAS tissue tropism, persistence within the human host, and the spectrum of diseases that the strains can cause. For instance, Neeman et al. (29) have shown that there is an association between *sfbl*-positive GAS strains and persistence after antibiotic treatment. Likewise, an association between *prtF2* and GAS invasive diseases has been observed (12, 37). *SfbI*, *Sof*, and *PrtF2* are distinct proteins, and while

*sfbl* and *prtF2* are located in the same chromosomal location called the fibronectin-collagen-T antigen (FCT) locus (5), the *sof* gene is situated outside this locus.

*PrtF2* was originally described by Jaffe et al. (18). Subsequently, Rocha and Fischetti (32) described another FBP designated PFBP. *PrtF2* and PFBP have very high sequence identity and possess similar domains. More recently, Terao et al. (37) identified *FbaB*, an FBP from the M3 and M18 GAS serotypes. This protein and PFBP also have the same leader sequence and exhibit high sequence similarity in the C-proximal region of the protein, which contains the fibronectin binding domains. These observations raise the important question of the evolutionary relationship between the FBP genes.

In order to address this question and increase our understanding of the evolution of *PrtF2*, we selected 51 *prtF2*-positive and 11 *prtF2*-negative genotypically distinct GAS strains. Here we report characterization of the two distinct genotypes of *PrtF2* by PCR and DNA sequence analysis and examination of the strains by pulsed-field gel electrophoresis (PFGE) to determine the evolutionary relationship of the *prtF2* genotypes. The epidemiological and evolutionary implications of the data are discussed below.

### MATERIALS AND METHODS

**Bacterial strains.** Sixty-two GAS isolates belonging to distinct genotypes as judged by Vir typing (17) or *emm* sequence typing (1) were selected for this study. These strains were isolated from patients in the Northern Territory of Australia and have been described previously (12). In GAS, genomic diversity is predominantly due to recombination (13). Thus, GAS isolates from a defined geographical region, such as the Northern Territory, where the diversity of GAS

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TABLE 1. Primers used for gene detection

Primer	Sequence (5' to 3')	Gene detected	PCR conditions	Size of amplicons (bp)
VPrtF2-F	ATAGGATTGTCCGGAGTATCA	<i>pfbp</i> type	30 cycles of 95°C for 60 s, 50°C for 60 s, and 72°C for 60 s	3,332–3,322 <sup>a</sup>
VPrtF2-R	TTATGTTGCTTCTCACCAG	<i>fbaB</i> type		
PFBP-F	GTACGTTAAGCGCTTGAAAAG	<i>pfbp</i> type	30 cycles of 95°C for 60 s, 66°C for 60 s, and 72°C for 60 s	2,144–1,922 <sup>a</sup>
ManR4	CAACCCATTAGCAGCATCATTCCC	<i>fbaB</i> type		
SfbXF1 <sup>b</sup>	GCAGTGATTCTAGGCTTAGCAAGCATA	<i>sfbX</i>	30 cycles of 95°C for 60 s, 62°C for 60 s, and 72°C for 60 s	1,941
SfbXR1 <sup>b</sup>	GTTTTGTCGGTGTTCGACGTTTTT			
FbaA-F	AGTCTACTACTACGCCAGTTG	<i>fbaA</i>	30 cycles of 95°C for 60 s, 50°C for 60 s, and 72°C for 60 s	541
FbaA-R	CTGTCTTGACAATGAGCGAT			

<sup>a</sup> The amplicon sizes differ depending on the variation in the N-terminal and/or fibronectin binding domains.

<sup>b</sup> The primer sequences used for amplification of *sfbX* were obtained from reference 19.

strains and the disease burden are high, offer an opportunity to discern the lineage of a single locus in relation to the population structure.

**Screening for genes encoding fibronectin binding proteins.** All GAS strains were screened for genes encoding FBPs, including *prtF2*, *pfbp*, *fbaB*, *sfbI*, *sof*, *sfbX*, *fbp54*, and *fbaA*. The *prtF2*, *sfbI*, *sof*, and *fbp54* status of these strains has been described previously (12). However, *prtF2* PCR performed with primers situated within the fibronectin binding repeat domains described by Delvecchio et al. (12) does not differentiate between the two genotypes of *prtF2* (*pfbp* and *fbaB*). Therefore, two sets of PCR primers were designed and utilized in this study. The first amplification with primers VPrtF2-F and VPrtF2-R designed for the signal sequence and cell wall anchor region, respectively, distinguished between the two genotypes of *prtF2* and confirmed the mutual exclusiveness of the two *prtF2* genotypes, and the second PCR amplification with primers PFBP-F and ManR4 designed for the flanking region of the *prtF2* open reading frame also distinguished between the two genotypes and confirmed the location of the genotypes in the chromosome. Primers SfbXF1 and SfbXR1 were used to screen for *sfbX* in all strains (19), and primers FbaA-F and FbaA-R were used to screen for *fbaA* in all strains (Table 1). PCRs were carried out in 50- $\mu$ l (total volume) mixtures containing 2  $\mu$ l of DNA template extracted with a QIAGEN DNeasy tissue kit, 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 2 mM MgCl<sub>2</sub>, 50 pmol of each primer, each deoxynucleoside triphosphate at a concentration of 200  $\mu$ M, and 1 U of *Taq* DNA polymerase.

**DNA sequence analysis.** Complete nucleotide sequences of seven *pfbp*-type and 11 *fbaB*-type genes were determined. PCR products obtained with primers PFBP-F and ManR4 (Table 1) were used as templates after the amplicons were purified with a QIAquick PCR purification kit (QIAGEN, Melbourne, Australia). The nucleotide sequences of both strands of DNA were determined by primer walking. DNA sequencing reactions were performed with an ABI Prism BigDye cycle sequencing kit (Applied-Biosystems, Melbourne, Australia), and the products were electrophoresed by using an ABI prism 377 DNA sequencer (Perkin-Elmer, Foster City, Calif.). Compilation and analysis of DNA sequence data were performed by using the Auto Assembler software (Perkin-Elmer). An amino acid analysis was performed by using programs accessed via the Australian National Genomic Information Services ([www.angis.org.au](http://www.angis.org.au)). ClustalW (38) was used to produce multiple-sequence alignments of the *pfbp* and *fbaB* sequences determined in this study and previously determined sequences for these genes, including those of the M3 GAS strain SSI-1 (accession number AB084272) (37), the M5 GAS strain Manfredo (<http://www.sanger.ac.uk>), the M12 GAS strain A735 (accession number AF071083) (32), the M18 GAS strain MGAS8382 (accession number AE009964) (33), the M49 GAS strain 100076 (accession number U31980) (18), and the M49 GAS strain B737 (accession number AY049089) (5).

**PFGE and phylogenetic analysis.** PFGE was carried out by using the following modification of the method described by Chatellier et al. (7). Briefly, single colonies of each GAS strain were used to inoculate 2 ml of Todd-Hewitt broth (Difco) supplemented with 1% yeast extract and grown overnight at 37°C. Cells were harvested by centrifugation, washed twice with TSE buffer (10 mM Tris-Cl, 1.0 M NaCl, 50 mM EDTA [pH 8.0]), and resuspended in 200  $\mu$ l of Tris-EDTA buffer (pH 7.5). An equal volume of prewarmed 1.5% low-melting-point preparative-grade agarose (Bio-Rad, Richmond, Calif.) was mixed with each cell suspension, transferred into gel block molds, and allowed to solidify. The blocks were treated for 4 h at 37°C with 400  $\mu$ l of lysis buffer (6 mM Tris-Cl [pH 7.6], 100 mM EDTA [pH 7.5], 1 M NaCl, 0.5% Brij 58, 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine) containing freshly added lysozyme (1 mg/ml), mutanolysin (100 U/ml), and RNase (20  $\mu$ g/ml). The lysis buffer was replaced with 300  $\mu$ l of deproteinization solution (1  $\mu$ g of proteinase K per ml, 1% sodium lauroyl sarcosine, 500 mM EDTA [pH 8.5]), and the blocks were incubated overnight at

50°C. The blocks were prepared for restriction enzyme digestion by washing them three times in Tris-EDTA buffer (pH 7.5) for 30 min each time; the first wash solution contained 0.5 mM phenylmethylsulfonyl fluoride. The blocks were stored in 1 M EDTA (pH 8.5) at 4°C until they were used.

Prior to digestion with restriction enzyme, 2- to 3-mm slices were aseptically cut from the blocks, rinsed for 10 min with sterile distilled water, and equilibrated for 30 min in SmaI restriction buffer. The slices were then incubated overnight at 25°C in fresh restriction buffer containing 20 U of SmaI restriction enzyme (Roche). The digested DNA was resolved by PFGE by using a CHEF-DRTM electrophoresis cell (Bio-Rad, Sydney, Australia) and the following parameters: 0.5 $\times$  Tris-borate-EDTA running buffer, 6 V/cm, and linearly ramped switch times of 2 to 40 s at 10°C for 23 h.  $\lambda$  DNA concatemers (New England Biolabs, Beverly, Mass.) were included as molecular size standards. The gel was then stained with ethidium bromide (1  $\mu$ g/ml) for 30 min and visualized with UV illumination by using a GelDoc 1000 image analysis station (Bio-Rad, Sydney, Australia).

PFGE restriction fragment patterns were visually assessed by using the criteria of Tenover et al. (35) and were analyzed by using the GelCompar software (version 4.2; Applied Maths, Kortrijk, Belgium). Genetic similarities were compared by clustering methods (unweighted pair group method with arithmetic means) by using the Dice coefficient. A tolerance in the band positions of 0.2% was used for comparisons of fingerprint profiles. MacClade v. 3.08 (27) was used to assign genotypes to ancestral nodes of the PFGE dendrogram.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the 18 genes sequenced in this study have been deposited in the GenBank database under the following accession numbers: NS101, AY612216; NS1140, AY612217; NS125, AY612218; NS178, AY612219; NS179, AY612220; NS192, AY612221; NS195, AY612222; NS210, AY612223; NS235, AY612224; NS240, AY612225; NS265, AY612226; NS436, AY612227; NS506, AY612228; NS53, AY612229; NS564, AY612230; NS581, AY612231; NS730, AY612232; NS803, AY612233.

## RESULTS

***pfbp* and *fbaB* are two distinct mutually exclusive genotypes of *prtF2*.** Analysis of GAS chromosomal DNA from 51 *prtF2* GAS strains chosen for this study (Table 2) with PCR primers VPrtF2-F and VPrtF2-R revealed amplicons that were in two size classes, an approximately 1.9-kb class and an approximately 3.3-kb class (data not shown). None of these 51 strains yielded both amplicon sizes, suggesting that these classes are mutually exclusive. The *prtF2* gene is known to reside within the FCT locus of GAS (5). PCR performed with primers PFBP-F and ManR4 at the known chromosomal position of the *prtF2* open reading frame yielded amplicons that were approximately 2.6 and 4.0 kb long (data not shown), indicating that the chromosomal position of *prtF2* is within the FCT locus in the 51 *prtF2* GAS strains examined, adjacent to the flanking *Spy0136* open reading frame (Fig. 1). To confirm that these amplicons are indeed located within the FCT locus, we sequenced a region upstream of the *prtF2* open reading frame to confirm the presence of the *msmR* gene (data not shown) and the junction region between *prtF2* and the downstream *Spy0136* gene from a representative group of 18 *prtF2*-positive

TABLE 2. Characteristics of GAS strains used in this study

Isolate	Vir type	<i>emm</i> sequence type	<i>prtF2</i> type	Other FBP genes				
				<i>sfbI</i>	<i>sof</i>	<i>sfbX</i>	<i>fbp54</i>	<i>fbaA</i>
NS8	25	<i>emm85</i>	<i>pfbp</i>	+	+	+	+	+
NS83	37.1	<i>stns554</i>	<i>pfbp</i>	+	+	+	+	+
NS192	3.2	<i>emm106</i>	<i>pfbp</i>	+	+	+	+	+
NS210	34	<i>emm22</i>	<i>pfbp</i>	+	+	+	+	+
NS226	14.1	<i>emm4.2</i>	<i>pfbp</i>	+	+	+	+	+
NS240	116	<i>st2904</i>	<i>pfbp</i>	+	+	+	+	+
NS730	2.2	<i>emm90</i>	<i>pfbp</i>	+	+	+	+	+
NS878	124	<i>emm76.1</i>	<i>pfbp</i>	+	+	+	+	+
NS1133	17.1	<i>emm101</i>	<i>pfbp</i>	-	-	-	+	+
NS1	23	<i>emm100</i>	<i>fbaB</i>	-	-	-	+	+
NS6	12.2	<i>emm123</i>	<i>fbaB</i>	-	-	-	+	+
NS13	24	<i>emm53</i>	<i>fbaB</i>	-	-	-	+	+
NS50	5	<i>st854</i>	<i>fbaB</i>	-	-	-	+	+
NS80	69	<i>emm70</i>	<i>fbaB</i>	-	-	-	+	+
NS88.2	17.4	<i>emm98.1</i>	<i>fbaB</i>	-	+	+	+	+
NS90	52	<i>stns90</i>	<i>fbaB</i>	+	-	-	+	-
NS101	33.1	<i>emm110</i>	<i>fbaB</i>	+	+	+	+	+
NS125	20.1	<i>emm95</i>	<i>fbaB</i>	-	-	-	+	+
NS178	26	<i>emm54</i>	<i>fbaB</i>	-	-	-	+	+
NS195	120	<i>emm19</i>	<i>fbaB</i>	+	-	-	+	-
NS205	20.2	<i>emm56</i>	<i>fbaB</i>	-	-	-	+	+
NS223	4	<i>emm91</i>	<i>fbaB</i>	-	-	-	+	+
NS225	115	<i>emm99</i>	<i>fbaB</i>	-	-	-	+	+
NS235	118	<i>emm24</i>	<i>fbaB</i>	-	-	-	+	+
NS476	39	<i>emm80</i>	<i>fbaB</i>	-	-	-	+	+
NS501	61	<i>emm14</i>	<i>fbaB</i>	+	-	-	+	+
NS1140	101	<i>emm57</i>	<i>fbaB</i>	-	-	-	+	-
NS20	3.4	<i>emm75.1</i>	-	+	+	+	+	+
NS25	18	<i>emm55</i>	-	+	-	-	+	-
NS204	121	<i>emm2</i>	-	+	+	+	+	+
NS216	122	<i>stns216</i>	-	-	-	-	+	-
NS344	127	<i>emm1</i>	-	-	-	-	+	+
NS931	57	<i>emm65</i>	-	+	-	-	+	+
NS14	96	<i>emm102</i>	<i>pfbp</i>	+	+	+	+	+
NS32	29.2	<i>emm101</i>	<i>pfbp</i>	+	-	-	+	+
NS179	7.2	<i>emm9.1</i>	<i>pfbp</i>	+	+	+	+	+
NS199	55	<i>emm112</i>	<i>pfbp</i>	+	+	+	+	+
NS236	111	<i>emm77</i>	<i>pfbp</i>	-	+	+	+	+
NS436	3.3	<i>emm11</i>	<i>pfbp</i>	-	+	+	+	+
NS564	119	<i>emm44/61</i>	<i>pfbp</i>	+	+	+	+	+
NS672	112	<i>emm28</i>	<i>pfbp</i>	+	+	+	+	+
NS1099	126	<i>emm101</i>	<i>pfbp</i>	-	-	-	+	+
NS1185	135	<i>emm80</i>	<i>pfbp</i>	-	+	+	+	+
NS50.1	12.1	<i>emm108</i>	<i>fbaB</i>	-	-	-	+	+
NS53	29.1	<i>emm71</i>	<i>fbaB</i>	-	-	-	+	+
NS180	110	<i>emm74</i>	<i>fbaB</i>	-	-	-	+	+
NS182	60	<i>emm109</i>	<i>fbaB</i>	-	+	+	+	+
NS265	11	<i>emm56</i>	<i>fbaB</i>	-	-	-	+	+
NS282	17.2	<i>st6030.1</i>	<i>fbaB</i>	-	-	-	+	+
NS506	134	<i>emm14</i>	<i>fbaB</i>	+	-	-	+	+
NS581	42	<i>emm42</i>	<i>fbaB</i>	-	-	-	+	+
NS803	131	<i>emm97.1</i>	<i>fbaB</i>	-	-	-	+	+
NS804	130	<i>emm97.1</i>	<i>fbaB</i>	+	-	-	+	+
NS836	46	<i>stek249</i>	<i>fbaB</i>	-	-	-	+	+
NS1030	77	<i>stck401</i>	<i>fbaB</i>	+	-	-	+	+
NS1033	3.22	<i>stns1033</i>	<i>fbaB</i>	+	-	-	+	+
NS1353	44	<i>stns90</i>	<i>fbaB</i>	+	+	+	+	+
NS351	22	<i>emm58</i>	-	+	+	+	+	+
NS1045	117	<i>emm60</i>	-	+	+	+	+	+
NS1096	32	ND <sup>a</sup>	-	-	+	+	+	+
NS1122	65	<i>emm65</i>	-	+	+	+	+	+
NS1210	125	<i>emm75</i>	-	+	+	+	+	+

<sup>a</sup> ND, not determined.

strains. The nucleotide sequence data generated were aligned with selected FCT junction sequences deposited in the GenBank and other databases. The *prtF2*-positive nucleotide sequences were highly conserved across this junction sequence

(>93%), whereas the *prtF2*-negative nucleotide sequences (M1 and M6) exhibited significant homology only from nucleotide 207 of the junction sequence (data not shown).

To further characterize these amplicons, we selected seven of the larger amplicons (NS179, NS192, NS210, NS240, NS436, NS564, and NS730) and 11 of the shorter amplicons (NS1140, NS101, NS125, NS178, NS195, NS235, NS265, NS506, NS53, NS581, and NS803) and subjected these amplicons to complete DNA sequence analysis. The *prtF2* open reading frame in amplicons that were these two sizes exhibited high levels of nucleotide sequence identity within the N-terminal signal sequence (>98%), part of the upstream fibronectin binding domain (>98%), and the C-terminal anchor region (>94%). A moderate level of identity (>91%) within individual fibronectin binding repeats was also found; there were differences in the number of fibronectin binding repeats within this domain (Fig. 2). The central domain exhibited no significant homology for the two types of amplicons. The larger amplicons displayed a high degree of similarity to the previously published *pfbp* gene sequence (32), and the smaller amplicons displayed a high degree of similarity to the previously published *fbaB* gene sequence (37).

The central domain of the 11 *fbaB*-like amplicons sequenced showed a high degree of similarity (>99%) (Fig. 2); however, this domain did not exhibit significant similarity with other sequences deposited in the GenBank database (data not shown). The fibronectin binding repeat domains (FBRD), as defined by Jaffe et al. (18), of the 11 *fbaB*-like amplicons varied and contained either two (NS265), three (NS53, NS101, NS125, NS178, NS195, NS235, NS506, NS581, and NS803), or four (NS1140) fibronectin binding repeats. By contrast, three intact fibronectin binding repeats were found in all seven *pfbp*-like amplicons sequenced (Fig. 2). The *pfbp*-like central domains displayed >87.1% similarity and contained distinct DNA cassettes flanked by highly conserved junction sequences, indicating that horizontal gene transfer may have generated the gene mosaic pattern found within this region (Fig. 3). The nucleotide alignment of the *pfbp*-like sequences is available at the GenBank website (alignment accession number ALIGN\_000736; ftp://ftp.ebi.ac.uk/pub/database/embl/align/ALIGN\_000736.dat) (data not shown).

Given (i) that the two mutually exclusive amplicons from the 51 *prtF2*-positive strains were amplified by using the same primers sets, (ii) the fact that each of these open reading frames mapped to the same position at the end of the FCT region, and (iii) that the downstream sequences across the junction of the 18 open reading frames analyzed and *SpyO136* are virtually identical, we suggest that *pfbp* and *fbaB* represent two distinct genotypes of *prtF2*. These two genotypes were designated the *pfbp* type and the *fbaB* type in this study.

**The *pfbp* lineage is more recent than the *fbaB* lineage.** PFGE analysis of 32 strains, including 7 *pfbp*-positive, 14 *fbaB*-positive, and 11 *prtF2*-negative GAS strains, revealed that they produced 32 PFGE patterns, which is consistent with the conclusion that the strains are genetically distinct. Genetic similarity was determined by band-based clustering in the PFGE profiles by using 0.2% tolerance (Fig. 4). The strains were very diverse (<45% similarity) when diversity was measured by using the criteria of Tenover et al. (35). The isolates subjected to PFGE belonged to diverse *emm* sequence types, with the

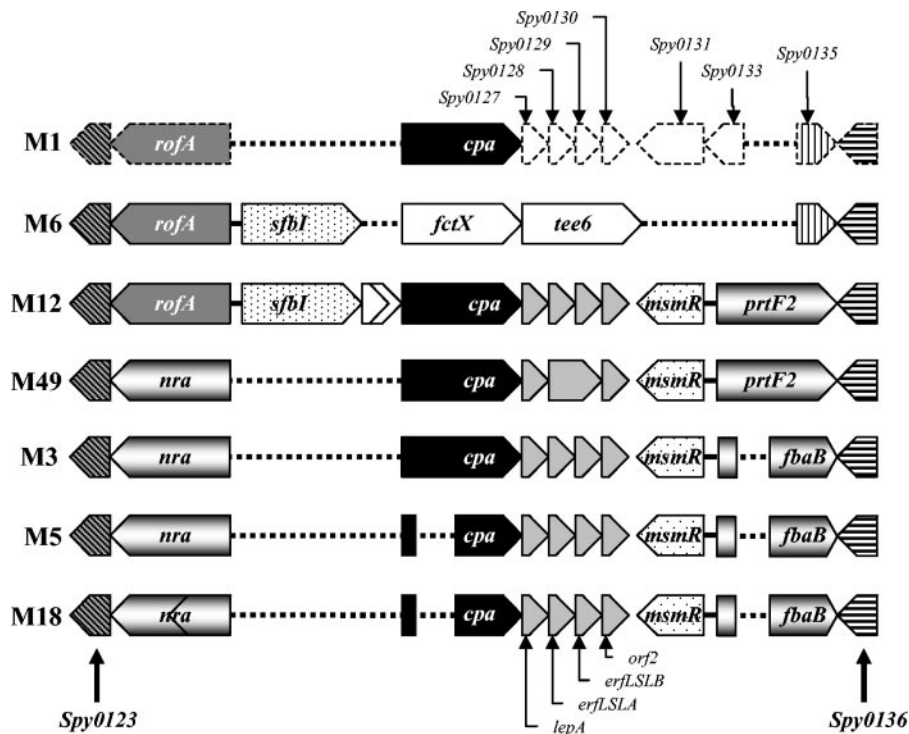


FIG. 1. Arrangement of the FCT region. The following strains were examined (accession numbers are indicated in parentheses): M1, strain SF370 (AE006482/3); M6, strain D471 (U01312, L10919, and AY049087); M12, strains A735 and A374 (AF447492, AY049088, and AF071083); M49, strains CS101/B737 (U49397 and AY049089) and 100076 (U31980); M3, strain MGAS315 (AE014138); M5, strain Manfredo ([http://www.sanger.ac.uk/Projects/S\\_pyogenes](http://www.sanger.ac.uk/Projects/S_pyogenes)); and M18, strain MGAS8382 (AE009963/4). The open reading frame designations are indicated above the M1 genome. The region is demarcated by two highly conserved open reading frames, *Spy0123* and *Spy0136* (striped arrows). With the exception of *sfbI*, which is used instead of *prtF1*, all other designations are those reported by Podbielski et al. (30) and Bessen and Kalia (5). The dotted lines represent gaps introduced to aid alignment. Open reading frames with no significant homology are not shaded. The chevron within *nra* of M18 indicates a stop codon caused by a single point mutation. Similarly, the single open reading frame in M49 spanning *eftLSLA* and *eftLSLB* is caused by a single point mutation that results in replacement of a stop codon (TAA) with a Glu (Q) codon (CAA).

exception of strains NS1122 and NS931, which were *emm* sequence type 65. However, despite having the same *emm* sequence type and a *prtF2*-negative genotype, NS1122 and NS931 were found to contain distinct Vir types and to produce genet-

ically unrelated PFGE fingerprint profiles, indicating that these strains are genetically distinct.

We examined the evolution of different *prtF2* genotypes (*pfbp* type, *fbaB* type, and *prtF2* negative) using MacClade (27)

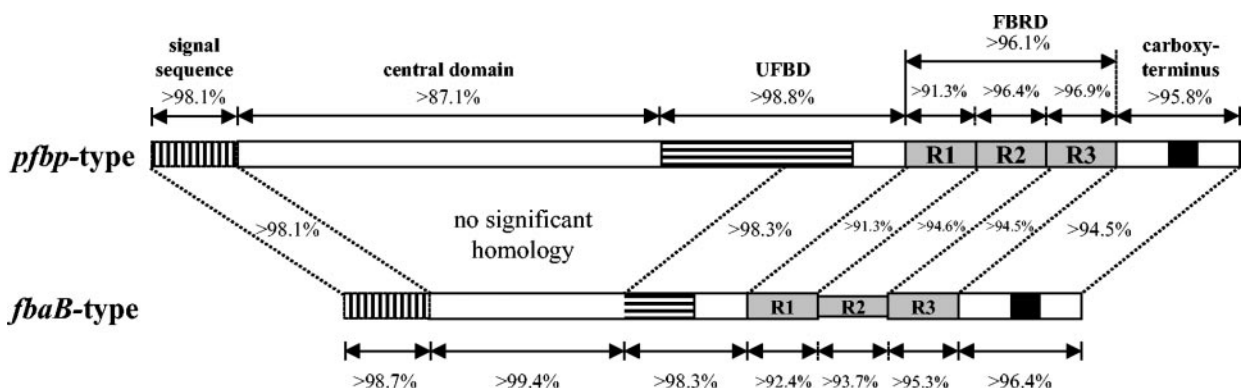


FIG. 2. Comparison of *pfbp*-type and *fbaB*-type variants. The percentages indicate the levels of identity observed in pairwise comparisons of DNA sequences both within and between variants. The two mature proteins differ significantly in the central domain but are highly similar in all other domains. The central domain is highly conserved in the *fbaB* type, but there is considerable variation in the *pfbp*-type central domain. The *pfbp*-type variants always possess three nonidentical fibronectin binding repeats in the FBRD, while the *fbaB*-type variants have variable numbers of repeats due to duplication or deletion of repeat 2 (R2). The overall homology of the FBRD of the *fbaB* type could not be determined due to the resultant gaps in the alignment; however, the individual repeats are highly homologous both within and between variants.

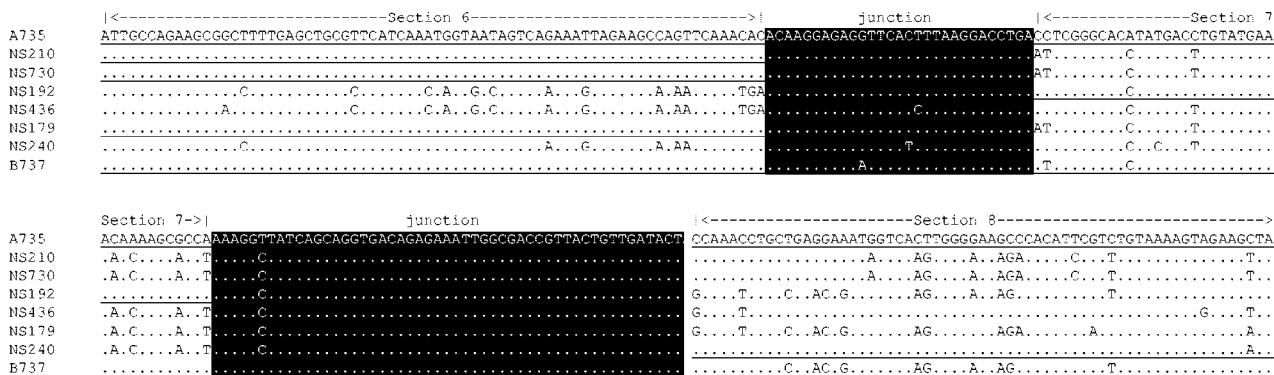


FIG. 3. Parts of the *pfbp*-type coding regions of the amino terminus have been horizontally transferred between different *pfbp*-type genes, producing mosaic alleles. Throughout the sequence highly conserved junction regions were identified (shaded) (>93.1% identity in pairwise comparisons) flanking distinct sections or cassettes, which appear to have been exchanged between alleles, resulting in a mosaic structure. Regions with identity to the *pfbp*-type gene of M12 strain A735 (*prtF2.12*) are underlined to illustrate the mobility of individual DNA cassettes.

and the PFGE dendrogram (Fig. 4). The MacClade program placed the contemporary genotypes on the tips of the tree, and the evolution of different genotypes was inferred by minimizing the total number of changes over the tree (Fig. 5). When the

gain or loss of a genotype was equally weighted, the pattern of evolution shown in Fig. 5A was recovered. This indicated that the *fbab* genotype was the older genotype and that the *pfbp* genotype or the *prtF2*-negative genotype evolved from an *fbab*-

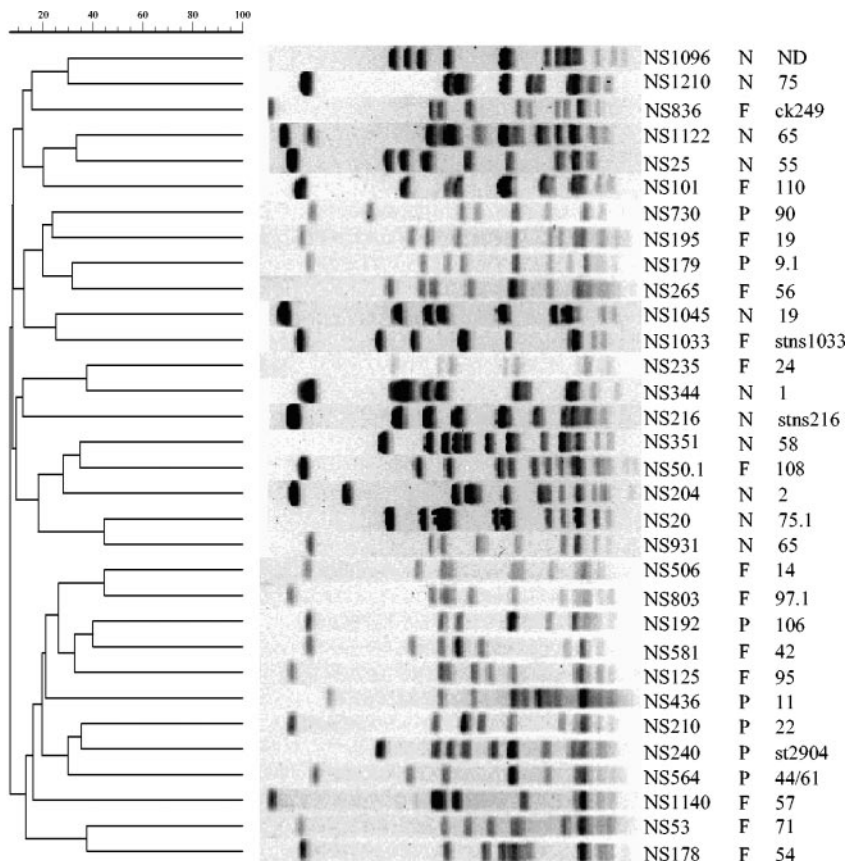


FIG. 4. Dendrogram generated by the GelCompar software, showing the genetic relationships among 21 *S. pyogenes* isolates possessing *prtF2* genotypes and 11 isolates not possessing the gene. The dendrogram was constructed by cluster analysis (unweighted pair group method with arithmetic means) of the PFGE patterns obtained after macrorestriction with the *Sma*I enzyme. A tolerance in the band positions of 0.2% was used for comparison of fingerprint profiles. PFGE fingerprint patterns are shown next to the corresponding branches of the dendrogram. The *prtF2* genotypes and *emm* sequence types are indicated after the strain number. P, *pfbp* type; F, *fbab* type; N, *prtF2* not present. The scale indicates percent similarity.

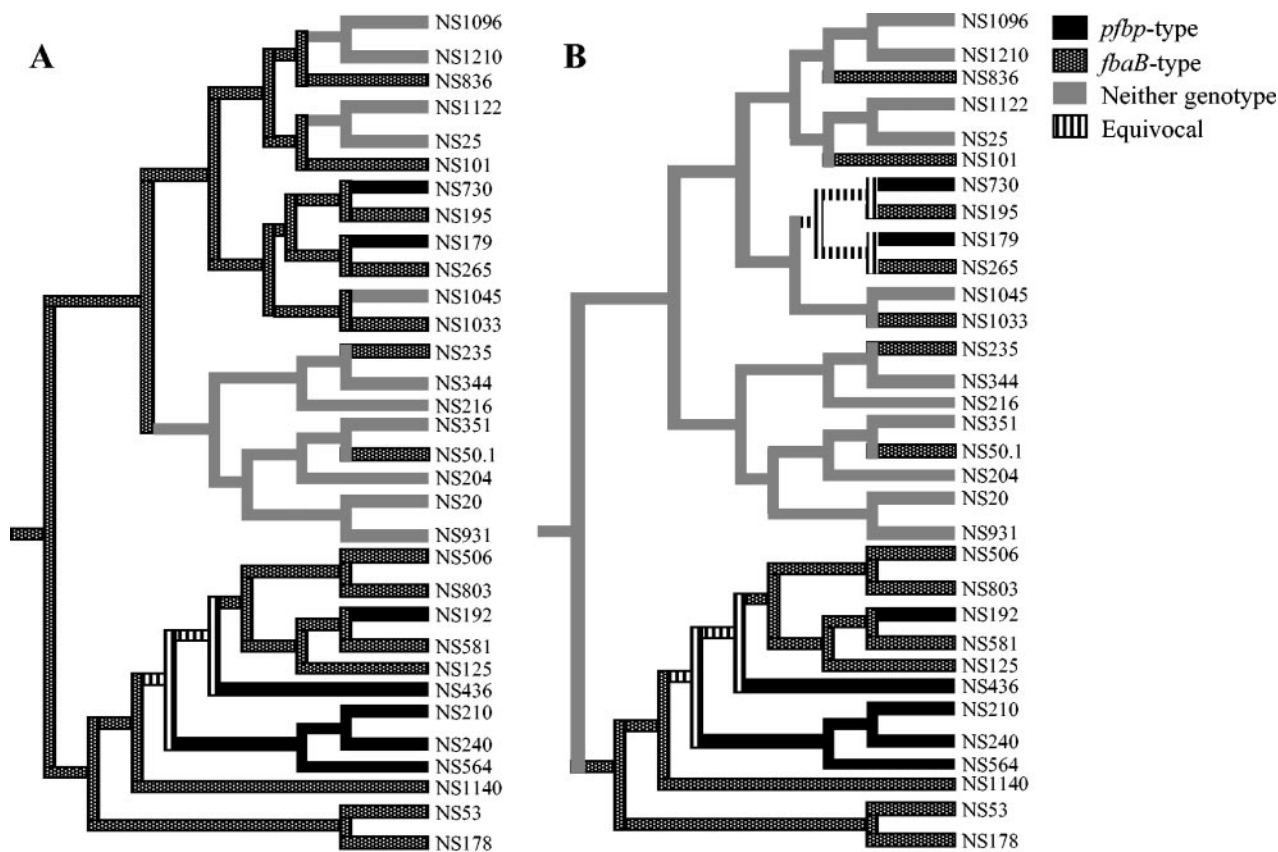


FIG. 5. Evolution of *prtF2* genotypes, inferred by parsimony by using MacClade v.3.08 (27), based on the PFGE dendrogram (Fig. 4). The trees indicate the evolution patterns obtained when the gains and losses of a genotype were equally weighted (A) and when gains were weighted higher than losses of a genotype (B).

positive progenitor. Strains NS50.1 and NS235, which are on a branch occupied predominantly by *prtF2*-negative strains, may have horizontally acquired the *fbaB* gene; inter- and intraspecies horizontal acquisition is common in GAS (5, 20, 21, 34). The analysis also indicated that the *fbaB* type can be lost from the genome, as exemplified by NS1045 and by the ancestral NS1122/NS25 strain and the ancestral NS1096/NS1210 strain. However, in our limited data set, loss of the *pfbp* type was not observed. The *pfbp* genotype may have been acquired in an *fbaB* background, as exemplified by NS179, NS730, NS436, and NS192 and in the ancestor of strains NS564, NS240, and NS210. In this analysis gene gains and losses were equally weighted, and a similar pattern emerged when losses were weighted lower than gains (i.e., when gains were penalized relative to losses).

As an alternative evolutionary scenario to examine the effect of positive selection pressure on the *pfbp* or *fbaB* genotype, a new MacClade analysis of the PFGE dendrogram was performed with gene gains weighted higher than losses (2:1 or higher). The resulting evolution pattern is shown in Fig. 5B. Here, the *prtF2*-negative genotype was present originally, the *fbaB* genotype generally evolved first, and then the *pfbp* genotype evolved in an *fbaB*-type genetic background. Potential horizontal gene acquisition of the *fbaB* type was observed in strains NS50.1, NS235, NS836, NS101, and NS1033, which are parts of branches dominated by *prtF2*-negative strains. An-

other difference between the two evolutionary patterns is the equivocal branch giving rise to strains NS265 and NS195 having the *fbaB* genotype and strains NS179 and NS730 having the *pfbp* genotype. This could indicate that NS179 and NS730 evolved from an *fbaB* ancestral genotype or vice versa.

We believe that the analysis shown in Fig. 5A (which is most commonly used) (27) represents the most reasonable working hypothesis for the evolution of different *prtF2* genotypes. Nevertheless, as Fig. 5B reveals, a small change in the weight given to gene gains and losses has an impact on the analysis, so that Fig. 5A should be considered only a starting point. Furthermore, these analyses assume that the topology shown in the 0.2% tolerance PFGE dendrogram (Fig. 4) is correct. Although some branches may be misplaced by this analysis, the evolutionary scenario described above represents a reasonable working hypothesis to direct subsequent investigations. Overall, the analysis above suggests that the *pfbp* type had a more recent origin and arose only in an *fbaB*-type genetic background.

**Distribution of *fbaB*-type, *pfbp*-type, and other GAS genes encoding fibronectin binding proteins.** GAS may possess genes encoding several FBPs, including *fbaB*, *pfbp*, *sfbI*, *sof*, *sfbX*, *fbaA*, and *fbp54*. We investigated the distribution of *sfbI*, *sof*, *sfbX*, *fbaA*, and *fbp54* in the 32 *fbaB*-type and 19 *pfbp*-type GAS strains examined in this study. The *fbaA* and *fbp54* genes were found in the majority of GAS strains possessing both the *pfbp*

and *fbaB* genotypes examined. Thirteen of 19 (68.4%) *pfbp*-type GAS strains possessed *sfbl*, *sof*, and *sfbX*. In contrast, only 2 of 32 (6.3%) the *fbaB*-type GAS strains possessed *sfbl*, *sof*, and *sfbX*, while in 21 of 32 (65.6%) *fbaB*-type GAS strains these genes were not detected (Table 2). GAS strains have been defined as belonging to either class I or class II M types based on epitopes present within the conserved C repeat region of M proteins (2, 3). Class II GAS strains are usually *sof* positive, while class I GAS strains are generally *sof* negative (2, 4, 15). Examination of the *sof* status of the 51 *prtF2*-positive GAS strains examined in this study revealed that 16 of 19 (84.2%) of the *pfbp*-positive strains possessed *sof*. By contrast, only 4 of 32 (13.3%) the *fbaB*-positive strains contained *sof*, suggesting that the *pfbp* type is linked to class II GAS and the *fbaB* type is linked to class I GAS.

## DISCUSSION

Strains causing GAS infections among the aboriginal communities of northern Australia have exhibited high diversity and turnover rates (6, 28). There is also little evidence of the emergence of a dominant clone, which has been a common cause of GAS invasive infections elsewhere in the world (8). Similarly, all strains used in this study were found to be genetically distinct, as indicated by *emm* typing, Vir typing, and PFGE profiles.

The gene encoding the FBP PrtF2 is situated in the highly recombinatorial FCT region in GAS (5). PrtF2 facilitates the binding of host fibronectin, enabling GAS to adhere to and be internalized by host epithelial cells (18, 25). Epidemiological evidence suggests that the presence of this gene may confer upon the pathogen a greater propensity to cause invasive diseases (12). In the present study we examined 51 *prtF2*-positive GAS strains isolated from patients in northern Australia, and here we describe two genotypes of *prtF2*, which are mutually exclusive. Both genotypes map to the same chromosomal location within the FCT locus, between genes encoding the potential gene regulator (*msmR*) and a hypothetical protein (*Spy0136*), and the two types are designated the *pfbp* type and *fbaB* type.

Other researchers have shown that expression of PrtF2 is influenced by the global negative regulator *nra*, which is located within the FCT region (30). Recent evidence obtained by Kreikemeyer et al. (25) and the fact *nra* is not always found in *prtF2*-positive strains (5) suggest that *prtF2* expression is controlled by additional regulatory elements. Immediately upstream of *prtF2* is a putative transcriptional regulator gene that has been termed *msmR*. MsmR belongs to the AraC family of regulators (5). Furthermore, the GAS genes controlled by this putative regulator have not been identified yet. In all strains studied to date, *msmR* is present upstream of *prtF2*. This may suggest that *msmR* affects *prtF2* expression directly or regulates other genes that are required for PrtF2 function.

The major difference between the *pfbp* gene sequence (32) and the original *prtF2* gene sequence (18) is the presence of two single-base-pair sequence changes in *prtF2*. These changes result in a frameshift mutation that results in the loss of the additional 105 amino acids at the N terminus of PFBP, which was not identified in PrtF2. However, the upstream *prtF2* gene sequence (GenBank accession number U31980) contains ge-

netic information coding for part of the 105-amino-acid sequence. None of the 18 *prtF2* genes sequenced in this study contains a frameshift mutation similar to that reported by Jaffe et al. (18), suggesting that this mutation is infrequent. Clearly, however, the reported sequences of *prtF2* (18), *pfbp* (32), and *fbaB* (37) are closely related. We therefore suggest that *pfbp* and *fbaB* represent two distinct genotypes of *prtF2*.

Multiple-alignment analysis of the *pfbp*-type DNA sequences revealed the presence of a mosaic structure within the *pfbp*-type central domain. This mosaic structure is not present in *fbaB*-type sequences, suggesting that only the *pfbp*-type gene sequences are capable of the intergenomic exchange required to produce such arrangements. Several surface-exposed GAS proteins have been shown to display a mosaic gene structure. These include the proteins encoded by *emm* (41), *ska* (22), and *sfbl* (39). It is believed that this type of genetic recombination plays a fundamental role in providing a mechanism for evading host immune responses. This is highlighted by the serotype-specific protection displayed by anti-M-protein antibodies (26). Such horizontal gene transfer may also produce mosaic gene structures that generate functional diversity of surface-exposed proteins. For instance, various M or M-like proteins have been found to bind fibrinogen, Fc domains of various human immunoglobulins, plasminogen, and/or proteins involved in the complement cascade (23).

In this study, we used software that utilized both the PFGE dendrogram and the *prtF2* genotype status to infer the evolution of the different genotypes by minimizing the total number of changes over the tree. The results obtained with this methodology suggested that the smaller, more conserved *fbaB* genotype had a more ancient origin than the *pfbp* type. This pattern of inheritance was similar when gains or losses were equally weighted or when gains were weighted higher than losses. As the *pfbp* type appears only in an *fbaB*-type background, we hypothesize that the *pfbp* type arose from an insertional event within the *fbaB* type and that the genetic rearrangement seen in the central domain of this gene is the result of a subsequent process. This also suggests that acquisition of the *pfbp*-type gene may have some selectable advantage over acquisition of the *fbaB*-type gene. One possible advantage may be the result of extra functions gained by the protein through the additional *pfbp*-type central domain sequence. Additionally or alternatively, the mosaic structure of the *pfbp*-type central domain may allow immune evasion to occur.

Interestingly all *pfbp*-type genes sequenced contained three repeat regions in the FBRD, while *fbaB*-type gene sequences had two to four repeats. Jaffe et al. (18) localized the upstream fibronectin binding domain (UFBD) for PrtF2 between amino acids 679 and 783. Residues in the PrtF2 UFBD critical for fibronectin binding were mapped to positions 679 to 717. Homology of the FbaB UFBD region only begins at amino acid 740 of PrtF2. It is not known if the truncated UFBD of FbaB alters fibronectin binding. Perhaps three fibronectin binding repeats are a functional constraint for an intact UFBD in PFBP. Variations in the number of repeats in the FBRD of FbaB may be due loss of this constraint when the UFBD is truncated.

Thirteen of 19 (68.4%) *pfbp*-type GAS strains investigated in this study possessed *sfbl*, *sof*, and *sfbX*. In contrast, only 2 of 32 (6.3%) *fbaB*-type GAS strains possessed *sfbl*, *sof*, and *sfbX*.



Interestingly, 21 of 32 (65.6%) *fbaB*-type GAS strains lacked these three genes. The biological implications of the finding that the *pfbp* type is generally associated with other FBP genes (*sfbI*, *sof*, and *sfbX*) and the biological implications of the finding that the *fbaB* type is generally associated with fewer FBP genes are not known. A previous study showed that there is linkage association between the *sfbI* and *sof* genes (14). In this study we found that there is a linkage association between *pfbp* and *sof*. Sixteen of 19 (84.2%) *pfbp*-type strains possessed *sof*. By contrast, only 4 of 32 (13.3%) *fbaB*-type strains contained *sof*. Class I GAS strains are generally *sof* negative and have been associated with acute rheumatic fever, unlike class II GAS strains, which are generally *sof* positive and are usually associated with skin-tropic infections (2, 4, 15). Therefore, our data suggest that the *pfbp* type may be linked to class II GAS strains and the *fbaB* type may be linked to class I strains.

GAS is a highly specific yet extremely versatile pathogen of humans. An increasing number of studies are revealing the extent of the genetic diversity displayed by this species and are implicating horizontal gene transfer and recombination as major mechanisms influencing the generation of this diversity. Additional work that leads to a better understanding of the evolution of GAS and, in particular, the emergence of highly virulent strains is warranted and may provide new strategies for predicting GAS disease trends and pathogenic mechanisms.

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