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Molecular Epidemiology and Genomics of Group A Streptococcus

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Abstract

Streptococcus pyogenes (group A streptococcus; GAS) is a strict human pathogen with a very high prevalence worldwide. This review highlights the genetic organization of the species and the important ecological considerations that impact its evolution. Recent advances are presented on the topics of molecular epidemiology, population biology, molecular basis for genetic change, genome structure and genetic flux, phylogenomics and closely related streptococcal species, and the long- and short-term evolution of GAS. The application of whole genome sequence data to addressing key biological questions is discussed.

Keywords

Group A streptococcus; *Streptococcus pyogenes*; Genomics; Population genetics; Population biology; Epidemiology; Evolution; Ecology; Streptococci

1. Taxonomy, habitats and disease

The importance of *Streptococcus pyogenes* as a human pathogen led to development of well-used clinical microbiology tools for its identification. Most notably, *S. pyogenes* forms large colonies and produces β-hemolysis following growth on blood agar, and is serologically distinguished from many other streptococcal species by its group carbohydrate that is

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covalently linked to the peptidoglycan cell wall. The term group A streptococci (or GAS) is commonly used as an alternative to *S. pyogenes.* Taxonomy based on 16S rRNA places *S. pyogenes* in what was historically referred to as the pyogenic (pus-forming) division of streptococci. The closest genetic relative of GAS is *S. canis* (group G streptococci; GGS) (Facklam, 2002). Other close genetic relatives, all of which lie within the pyogenic division and display β-hemolysis following growth on blood agar, include *S. dysgalactiae* subspecies *equisimilis* (SDE; largely group C streptococci [GCS] and GGS), *S. dysgalactiae* subspecies *dysgalactiae* (SDD), *S. equi* subspecies *zooepidemicus* (Sz) and *equi* (Se) (both are GCS), and *S. agalactiae* (group B streptococci [GBS]). Organisms that are (mostly) restricted to humans are GAS and SDE, whereas *S. canis*, SDD and *S. equi* subspecies Sz and Se primarily cause disease in other mammalian hosts. Environmental reservoirs appear to be non-existent or highly limited for the streptococcal species that cause human disease.

GAS is a human-specific pathogen that is highly prevalent worldwide, causing ~750 million infections per year (Carapetis, 2007; Carapetis et al., 2005), mostly at the throat (pharyngitis, tonsillitis) and skin (non-bullous impetigo). The epithelium of the throat and skin are the primary ecological niches of GAS and importantly, the tissue sites for most new GAS acquisitions and transmissions. Invasive disease is a relatively rare outcome of GAS infection, whereby the organism gains access to normally sterile tissue via the upper respiratory tract (URT) or breaks in the skin; from the bloodstream, GAS can disseminate to numerous deep tissues within the host. Although invasive GAS disease exacts a heavy toll in terms of morbidity and mortality, it is often an evolutionary dead end because an organism infecting deep tissue usually lacks an efficient means for transmission to a new host.

Symptomless throat infections are also known to occur, whereby the patient lacks overt clinical symptoms yet mounts a specific immune response to GAS antigens (i.e., clinically inapparent). Asymptomatic carriage of GAS at the URT - which is presumed to elicit little or no immune response due to the semi-quiescent state of the organism - can often achieve rates of >20% among school-aged populations (Kaplan, 1980). Although the organism is believed to be in a semi-quiescent, slow-growing state during carriage at the URT, transmission to new hosts can occur. Skin carriage appears to occur under endemic conditions (Anthony et al., 1976), but overall, it is less well documented. Thus, at least for GAS strains colonizing the oropharynx, a commensal-like state appears to be the predominant lifestyle.

The relative prevalence of pharyngitis and impetigo due to GAS varies in accordance with geographical location and season. Pharyngitis prevails in temperate regions and peaks in winter months, during which people spend extended time indoors and transmission occurs via a respiratory route. Impetigo is associated with warm, humid climates and is mostly observed in tropical and sub-tropical regions, or during summer months. Children are the primary targets of superficial GAS infections, and impetigo tends to afflict a slightly younger age cohort. Overall, there are spatial (geography) and temporal distances (winter versus summer seasons) that act to physically separate many organisms having a predilection for causing pharyngitis from those strains having a high tendency to cause impetigo. It is of great interest as to whether the spatial-temporal distances between GAS strains causing pharyngitis versus impetigo reduces the number of opportunities for

horizontal gene transfer (HGT) which in turn, can shape the population genetic structure of a bacterial species (addressed in Section 3).

2. Molecular epidemiology

Pioneering work by Dr. Rebecca Lancefield aimed to understand the basis for protective immunity to GAS infection, and led to the development of a serological typing scheme based on the antiphagocytic M protein surface fibrils (Lancefield, 1962). More than 80 distinct M types were identified, whereby protective immunity to GAS is M type-specific. The M type-specific determinants map to the fibril tips, encoded by the 5' end of *emm* genes. More recently, a sequence-based *emm* typing scheme was implemented, based on extensive nt sequence differences at the 5' end of the *emm* gene, whereby a unique *emm* type is defined as having <92% sequence identity over the nt sequence corresponding to the first 30 codons of the mature M protein (Beall et al., 1996). Among the 234 *emm* types recognized to date are >1200 distinct allelic forms of the *emm* type-specific regions of *emm* genes, known as *emm* subtypes (Beall, 2014). Virtually all contemporary epidemiological studies define GAS isolates according to their *emm* type and therefore, *emm* type provides the primary framework for understanding the population biology and genetic structure of this species.

All GAS isolates harbor an *emm* gene. In addition, many GAS strains have paralogous *emm*-like genes lying immediately upstream and downstream of *emm*, and a few strains have only the downstream *emm*-like locus. Thus, a given GAS strain can have one or two *emm*-like genes, in addition to *emm*; the upstream *emm*-like gene is often referred to as *mrp*, and the downstream *emm*-like gene is often referred to as *enn.* The paralogous *mrp* and *enn* genes lack *emm* type-specific determinants, and are also distinct from the *emm* locus within their 3' end regions which encode a peptidoglycan-spanning domain. Based on the structure of the cell wall-spanning domain, there are four major forms or subfamilies (SF) of *emm* and *emm*-like genes, whereby *emm* is either SF-1 or SF-2, *mrp* is always SF-4, and *enn* is SF-1 or SF-3 (Hollingshead et al., 1994). Furthermore, there are five distinct chromosomal arrangements (Haanes et al., 1992; Hollingshead et al., 1993; Podbielski, 1993) of *emm* and *emm*-like genes and their SF forms, designated *emm* patterns A through E; *emm* patterns B and C are rare and grouped together with pattern A strains (referred to as *emm* pattern A–C), due to their structural similarities (i.e., all have an SF-1 *emm* gene and lack *mrp*). Pattern D and E strains have *mrp* and the SF-3 form of *enn*, but are distinct in that their *emm* genes are of the SF-1 and SF-2 forms, respectively. The SF-1 (58 aa residues) and SF2 (39 aa residues) forms of the peptidoglycan-spanning domain are markedly different in length, the functional significance of which has yet to be determined.

The *emm* pattern genotype has been determined for 170 of the 234 currently recognized *emm* types (McGregor et al., 2004; McMillan et al., 2013), including the most common ones. Thirty-six (21%) *emm* types are pattern A–C, 64 (38%) are *emm* pattern D, and 66 (39%) are *emm* pattern E; two *emm* types have a rearranged *emm* region (no pattern group is assigned), and only two (fairly rare) *emm* types are found in association with >1 *emm* pattern group. Importantly, the *emm* pattern group displays a statistically significant association with tissue site of infection, whereby GAS strains of the *emm* pattern A–C genotype show a statistically significant association with pharyngitis (throat specialists) and *emm* pattern D

isolates show a statistically significant association with impetigo (skin specialists) (Bessen, 2009; Bessen et al., 2000b; Bessen and Lizano, 2010; Bessen et al., 1996).

For the vast majority of *emm* types examined (97%), multiple isolates sharing an *emm* type belong to the same *emm* pattern group (McGregor et al., 2004). Therefore, *emm* type is highly predictive of *emm* pattern grouping, and reasonable inferences can be made for *emm* pattern group based on knowledge of the *emm* type. The initial findings on the strong correlations between *emm* pattern grouping and preferred tissue site of infection is confirmed in a meta-analysis of 5,439 GAS isolates obtained from 23 pharyngitis and 6 impetigo population-based surveys conducted throughout the world (Bessen et al., 2011; Steer et al., 2009b), whereby *emm* pattern group is inferred from the *emm* type (Table 1). Table 1 data show that overall, pattern A–C strains represent 46.6% of pharyngitis isolates but only 8.2% of impetigo isolates. In sharp contrast, the *emm* pattern D strains represent 49.8% of impetigo isolates, but only 1.7% of pharyngitis isolates. Pattern A–C versus D fractional contents for each of the 29 surveys were compared by the paired t-test (2-tailed); data reveal highly significant differences for both pharyngitis (t = 7.135E-06) and impetigo (t = 8.73E-04) strain collections. In contrast, *emm* pattern E isolates account for almost equal fractions of throat and skin infections (51.7 and 42%, respectively); as a group, they are designated “generalists.” In conclusion, *emm* pattern is a genotypic marker for preferred tissue site of infection.

In four of the 23 collections of pharyngitis isolates, *emm* pattern D isolates outnumber *emm* pattern A–C isolates and comprise >20% of the GAS isolates (Table 1; bold); all four surveys were conducted in tropical or sub-tropical regions. Regardless of tissue site of isolation, data support the notion that *emm* pattern D and E strains tend to predominate in tropical regions whereas pattern A–C and E strains represent the vast majority of isolates in temperate regions. This regional dominance is reflected in invasive disease isolates, which are largely collected from temperate regions (Beall, 2014; Fiorentino et al., 1997; Haukness et al., 2002; Kiska et al., 1997; Rogers et al., 2007; Steer et al., 2009b). It is very important to emphasize that not all so-called "skin infections" have a similar clinical course or share the same set of host risk factors. Superficial non-bullous impetigo caused by GAS (as depicted in Table 1 data) is a self-limiting infection (i.e., can naturally resolve in ~two weeks), that is clearly distinct from wound and deep soft tissue infections or invasive disease in which GAS gain entry through breaks in the skin and requires antibiotics (and sometimes surgery) for treatment (Bisno and Stevens, 2009). The GAS strains causing impetigo (i.e., largely patterns D and E) are mostly sampled from tropical or subtropical regions (because that is where the disease is most prevalent), whereas GAS strains causing invasive skin infections (i.e., largely pattern A–C and E) are mostly sampled from temperate regions (because that is where the most resources are to support extensive epidemiologic sampling).

Another key feature revealed by Table 1 data is the relative degree of genetic diversity (*D*) amongst GAS derived from population-based collections of pharyngitis versus impetigo isolates. A *D* value equal to 1 signifies that all isolates are genetically distinct. Using *emm* type as the genetic marker, the mean average for *D* is 0.8722 and 0.9593 for the sets of pharyngitis and impetigo isolate studies, respectively. Importantly, the difference in *D* values for pharyngitis versus impetigo collections is highly significant (t = 2.34E-06;

unpaired, 2-tailed); this finding is consistent with the observation that GAS pharyngitis tends to be dominated by a relatively small number of clones. An analysis that included all GAS, irrespective of clinical association, also shows fewer numbers of *emm* types accounting for a large proportion of isolates in resource-rich countries, where URT infections tend to predominate (Steer et al., 2009b). The biological basis for the difference in diversity among GAS in host communities experiencing pharyngitis versus impetigo may be tied to the mechanisms for transmission - respiratory droplets versus direct contact - with the respiratory route being highly efficient for at least a subset of strains (i.e., high basic reproductive rate, R_0) and resulting in a lower *D* value.

Despite differences in strain diversity among pharyngitis and impetigo isolates, within a well-circumscribed community over a narrow time frame, the number of distinct *emm* types circulating is quite large [for e.g., (Shulman et al., 2009; Steer et al., 2009a)]. This finding is indicative of high rates of GAS migration. The high level of GAS diversity holds true even for remote populations, such as an island community of aboriginal Australians at the Top End of the Northern Territory (Bessen et al., 2000a), where the *D* value for impetigo isolates over a 2-year surveillance period exceeds 0.95 (Table 1).

Although GAS strains most often recovered from cases of pharyngitis tend to belong to a more limited set of *emm* types, there is very high diversity among the classical throat strains (i.e., *emm* pattern A–C) in terms of the number of *emm* subtypes characterized (i.e., alleles based on the 5' end of the *emm* gene). The average mean number of *emm* subtypes identified for *emm* pattern A–C strains is 16.8 subtypes per *emm* type, compared to only 3.4 and 5.3 for *emm* pattern D and E strains, respectively, based on data available at (Beall, 2014). This 3- to 5-fold difference in the number of *emm* subtypes between throat specialists and the other two *emm* pattern groups may be partly due to sampling bias (i.e., skewed in favor of GAS invasive and pharyngitis isolates in resource-rich nations), or biological factors, or a combination of both.

Nucleotide sequence alignment of multiple *emm* subtype alleles for each *emm* type-specific region, collectively derived from >500 GAS isolates (N = 105 *emm* types), reveals an average ratio of non-synonymous substitutions per non-synonymous site (Ka) and synonymous substitutions per synonymous site (Ks) of 4.9, 1.5 and 1.3 for *emm* types of the *emm* pattern A–C, D and E groups, respectively (Bessen et al., 2008). Thus, diversifying (positive) selection acting on the *emm* type-specific region appears to be strongest for the classical throat strains (*emm* pattern A–C). Because *emm* types associated with GAS are largely restricted to this bacterial species, and each *emm* type-specific region has <92% nt sequence identity with all other *emm* types, the observed genetic changes giving rise to *emm* subtypes most likely originate as mutations arising among GAS, rather than *emm* gene acquisitions following HGT from another species, such as SDE which also have *emm* genes but they are (largely) of distinct *emm* types (Ahmad et al., 2009; Beall, 2014; McMillan et al., 2010).

A new cluster typing system based on GAS *emm* types was recently developed, using the portion of *emm* genes encoding the entire surface-exposed region of M proteins, for >1,000 *emm* genes corresponding to 175 *emm* types (Sanderson-Smith et al., 2014). Phylogenetic

analysis revealed two main clades (X and Y), and 16 well-supported clusters accounting for 82% of *emm* types, with each cluster composed of multiple taxa. Of the pattern E *emm* types, 98% belong to clade X, whereas 92% of pattern A–C *emm* types fall into clade Y. Thus for these two *emm* pattern groups, the excluded cell-wall-spanning domain (SF-1 for pattern A–C, SF-2 for pattern E) is tightly linked to the phylogeny that is based on the surface-exposed portion of M proteins. In contrast, the pattern D *emm* types (SF-1) form three discrete groupings, and are present in both clades X and Y.

Collectively the M proteins bind numerous host factors such as IgA, IgG, fibrinogen and plasminogen. The M protein types assigned to an individual *emm*-cluster have high sequence similarity and as might be expected, share functional properties as well (Sanderson-Smith et al., 2014). Importantly, type-specific regions of M proteins belonging to the same cluster often elicit cross-protective antibodies; this may aid in the design of highly efficacious vaccines with broad coverage that are based on a limited selection of type-specific epitopes. Whereas 90% of pattern D and E *emm* types belong to one of the 16 *emm*-clusters, nearly half of pattern A–C *emm* types are standalone and do not cluster with any other *emm* type, highlighting once again, a distinct dynamic for the evolution of many pattern A–C *emm* types.

While *emm* typing has emerged as the dominant typing scheme for GAS, two other serology-based typing schemes have an important place in understanding the molecular epidemiology and genetic organization of this species. Serum opacity factor (SOF) is an LPXTG-anchored, multifunctional surface protein that also appears in a secreted form; SOF binds fibronectin and enzymatically disrupts the structure of high-density lipoproteins present in blood (Courtney and Pownall, 2010). The original SOF typing scheme was serologically based, wherein SOF type-specific serum neutralized enzymatic activity. More recently, *sof* sequence types have been defined based on a 450-bp region at the 5' end of the *sof* gene (Beall et al., 2000); the genome map position of *sof* lies ~16.5 kb from *emm*. All (or nearly all) *emm* pattern E strains have a *sof* gene and an enzymatically active SOF protein (Beall et al., 2000; Johnson et al., 2006; Kratovac et al., 2007). Several *emm* pattern D strains also have a *sof* gene; several of these (*emm59*, *emm81*, *emm85*) are assigned to *emm*-cluster E6 within clade X (Sanderson-Smith et al., 2014); interestingly, *emm12* (pattern A–C) has a partial *sof* gene. Despite the close physical distance between *sof* and *emm* on the chromosome, several *emm* types are found in association with >1 *sof* type (and vice versa), indicative of HGT of *emm* or *sof* to new genetic backgrounds (Beall et al., 2000). Attempts to generate a phylogeny for the *sof*-specific determinants have been largely unsatisfactory (i.e., poorly supported trees) due to extensive intergenic recombination (Wertz et al., 2007). While there are currently >65 recognized *emm* types assigned to the *emm* pattern E group (McGregor et al., 2004; McMillan et al., 2013), the characterization of distinct *sof* types has not yet kept pace.

The third serological typing scheme for GAS is T-typing, based on the trypsin-resistant T-antigens that were more recently identified as surface pili which mediate adherence of GAS to host epithelium and promote biofilm formation (Manetti et al., 2007; Mora et al., 2005). The *tee6* gene (Schneewind et al., 1990) maps to a genomic region containing genes known to encode microbial surface components recognizing adhesive matrix molecules

(MSCRAMMs) (Hanski and Caparon, 1992; Podbielski et al., 1999), and has been designated the FCT region (FCT stands for Fibronectin- and Collagen-binding proteins and T-antigen) (Bessen and Kalia, 2002). Pilus structural components and enzymes (sortases) that mediate their assembly are encoded within the FCT region, whereby the pili correspond to T-antigens (Barnett and Scott, 2002; Lizano et al., 2007; Mora et al., 2005). Nine distinct FCT region forms are currently recognized, each containing between five and ten ORFs and differing in their content of genes encoding pilus structural components, enzymes involved in their assembly, other MSCRAMMs and transcriptional regulators (Bessen and Kalia, 2002; Falugi et al., 2008; Koller et al., 2010; Kratovac et al., 2007). The FCT region lies ~250 kb from the *emm* region on the genome. Despite their physical distance, there is strong linkage between FCT region form and *emm* pattern (Kratovac et al., 2007), suggesting that the FCT region gene products may play an adaptive role in establishing tissue tropisms.

3. Population genetics

MLST based on seven core housekeeping genes is routinely used to define clones of GAS (Enright et al., 2001). The MLST data posted at www.mlst.net currently lists 628 sequence types (ST) of GAS (i.e., *S. pyogenes*), based on allelic profiles at the seven loci (Aanensen, 2014). Numerous investigators from throughout the world have generously contributed to this rich data set. A population snapshot generated by the eBURST algorithm (Feil et al., 2004; Francisco et al., 2012) reveals 91 clonal complexes (CCs), in which the connected STs are single locus variants (SLVs) sharing 6 of the 7 housekeeping alleles. A high proportion of STs (40.1%) differ from all others by > 2 alleles (singletons), and the largest CC contains only 3.66% of the total STs. This places the population structure of GAS at the far end of the spectrum for bacteria, approaching that of the gut pathogen *Helicobacter pylori*; organisms falling within this part of the scale are characterized by high levels of genetic diversification due to both point mutation and homologous recombination (Turner et al., 2007).

Several methods have been used to assess the relative amount of recombination and mutation that contributes to genetic change in GAS based on the seven core housekeeping genes. Statistical tests were used to measure the congruence between pair wise combinations of phylogenetic trees corresponding to each of the seven housekeeping genes, based on representative strains from each deep branch (Feil et al., 2001). Of the 42 possible pair wise tree comparisons, no significant congruence between trees was observed. The lack of congruence is indicative of relatively high levels of recombination within GAS. Using the multilocus infinite alleles model and an expanded set of isolates, high rates of recombination were again predicted for GAS (Hanage et al., 2006). The value for the estimated rate of occurrence of mutation (θ) was 7.1, which is similar to that calculated for *S. pneumoniae* ($\theta = 7.4$); the estimated rate of recombination (ρ) was even higher for GAS (51.2) than for pneumococcus ($\rho = 29.6$). *S. pneumoniae* is similar to GAS in its lack of congruency among the topologies of phylogenetic trees based on housekeeping gene sequences (Feil et al., 2001). Using ClonalFrame, the ratio of nt changes as the result of recombination relative to point mutation (r/m) was calculated as 17.2 for GAS (Vos and Didelot, 2009). Together, the findings on housekeeping genes support the notion that GAS exhibit high levels of recombination that at least by some measures, is roughly comparable to that observed for the pneumococcus.

In a more extensive analysis of a highly diverse set of ~600 GAS isolates collected from >25 countries, representing 156 *emm* types and 259 STs, 56 SLVs were detected by eBURST (Bessen et al., 2008). The genetic mechanism of descent was estimated as recombination for ≥33 of the 56 SLVs, yielding a recombination to mutation ratio of ≥1.4, which is perhaps a bit lower than expected in light of the other types of measurements mentioned above. Interestingly, when stratified according to *emm* pattern group, the ratio of recombination to mutational events is 0.14, 4.0 and 3.0 for *emm* patterns A–C, D and E, respectively. In addition, *emm* pattern A–C strains show the highest level of congruence for housekeeping gene tree topologies, indicative of relatively lower levels of recombination, whereas *emm* pattern D strains are least congruent (Kalia et al., 2002). Therefore, different methodologies point to the same general trend, whereby the throat specialists have the highest tendency to diversify by mutation, and skin specialists and generalists have a greater tendency to diversify by recombination.

Molecular evolutionary analysis of *emm* type-specific determinants adds further support for a large role for mutation (relative to recombination) in genetic diversification among the classical throat strains. Antibodies to the M type-specific determinants are bactericidal and act by mediating opsonophagocytosis of the GAS organism (Cunningham, 2000; Lancefield, 1962); thus, strong diversifying selection acting on *emm* type may arise from host immune pressures. The average mean Ka to Ks ratio for pattern A–C *emm* type-specific regions exceeds the values observed for pattern D and E *emm* types by ~3- to 5-fold (described in Section 2) (Bessen et al., 2008). If indeed the respiratory route of transmission by classical throat strains leads to a high R_o, herd immunity may build quickly whereby selection pressure favors the emergence of immune escape mutants. Alternatively, the reduced level of diversifying selection among pattern D and E *emm* type-specific determinants, relative to pattern A–C *emm* types, may be the result of functional constraints. Human complement regulator C4b-binding protein (C4BP) binds to the N-terminal hypervariable region of many M proteins and confers resistance to phagocytosis; binding of C4BP was observed for 97 and 100% of the *emm* pattern D and E strains tested, respectively, compared to only 38% of the pattern A–C strains (Persson et al., 2006). Although there is no clear cut binding motif for C4BP, it seems plausible that purifying (negative) selection acting on pattern D and E *emm* type-specific regions may preserve functional activity, yielding both fewer *emm* subtypes (Section 2) and lower Ka/Ks values.

For the same set of >500 GAS isolates used to calculate Ka/Ks ratios, the average mean number of HGT events per *emm* type was measured, whereby an *emm* type found in association with >1 ST differing by ≥5 of the 7 housekeeping alleles is defined as a HGT event. Data indicate an average of 0.17, 0.60 and 0.75 HGT events per *emm* type for the pattern A–C, D and E groups, respectively (Bessen et al., 2008). Thus, as compared to the classical throat strains, immune escape by *emm* pattern D and E strains may be more often driven by serotype replacement mediated via HGT and homologous recombination of the *emm* gene onto a new genetic background. Also, the data supports the notion that for the classical throat strains, but not skin strains or generalists, *emm* type is a fairly good marker for clone or clonal complex as defined by MLST (Bessen et al., 2008; Enright et al., 2001). In summary, differences among the *emm* pattern-defined groups of GAS in terms of their

mechanisms for genetic change point to different dynamics in shaping their population structures.

As described in Section 1, GAS strains may encounter some degree of ecological separation by virtue of their tissue site preferences for infection (throat versus skin), geographic partitioning (temperate versus tropical regions) and temporal distances (winter versus summer seasons). Ecological barriers can give rise to allopatric speciation and therefore, signatures of early stages of speciation within the GAS population were sought. Numerous analyses of housekeeping alleles - including phylogenetic trees of concatenated alleles, splits graphs, fixed nt differences, distribution of shared alleles - all provide strong support for ample recombination of housekeeping genes among all three *emm* pattern groupings (Kalia et al., 2002). These findings extend to isolates known to be recovered from the URT versus impetigo lesions, regardless of *emm* pattern group (Kalia et al., 2002). A phylogenetic tree based on concatenated nt sequences of the 7 housekeeping genes used in MLST, for 114 strains representing 114 STs and 113 distinct *emm* types, shows only a few branches with strong bootstrap support; nt diversity (π) is very low (Bessen, 2009). Strains assigned to *emm* pattern groups A–C, D and E are scattered throughout the tree and there is a lack of strong clustering. A SplitsTree graph of concatenated housekeeping alleles for 97 GAS isolates representing 95 *emm* types also shows a lack of clustering in accordance with *emm* pattern group (Bessen et al., 2011). Thus despite spatialtemporal distances between many GAS strains, there is no clear evidence for diminished opportunities for HGT of core housekeeping genes between strains of different *emm* pattern groups. Against a background of random genetic change in core housekeeping genes, loci exhibiting a high degree of linkage with *emm* pattern genotypes (Section 8), and which also map to distal positions on the genome, are strong candidates for playing a direct role in the adaptations leading to throat or skin infection.

4. Molecular mechanisms underlying genetic change

The footprints of past homologous recombination events are clearly evident within core housekeeping genes of GAS. Yet, one of the major unresolved puzzles of this species concerns the molecular mechanisms governing HGT that lead to high rates of homologous recombination. GAS are rich in bacteriophage (Section 6) and theoretically, non-specific packaging of chromosomal fragments into bacteriophage capsids might provide a means for HGT of bacterial DNA (i.e., generalized transduction). GAS produce numerous DNases, and GAS DNA is presumably protected during phage-mediated transfer.

Natural transformation is another plausible mechanism for HGT among GAS, yet it has been difficult to demonstrate experimentally and when it is achieved, transformation efficiencies are relatively low. Using a mouse model, in vivo transfer of a DNA marker between two GAS strains was demonstrated and the *sil* locus appears to be involved; in vitro transfer of DNA could also be demonstrated, albeit at a very low frequency (Hidalgo-Grass et al., 2002). The *sil* locus is regulated by quorum-sensing, and the SilCR autoinducing peptide can be sensed across multiple streptococcal species (i.e., SDE) (Belotserkovsky et al., 2009). However, any involvement of *sil* in DNA transfer involving GAS is unlikely to be a

universal mechanism because the *sil* locus is present in only a minority of GAS strains (Plainvert et al., 2014).

Several *Streptococcus* species, such as *S. pneumoniae* and *S. mutans* in particular, readily undergo transformation and the genes that confer competence for the uptake of DNA uptake are well-characterized (Martin et al., 2006). For homologs present in GAS, activation of putative late competence genes by a small-peptide pheromone (XIP) and ComR, mediated via the alternative sigma factor and master competence regulator SigX/ComX, has been successfully demonstrated (Mashburn-Warren et al., 2012; Woodbury et al., 2006). However, uptake of DNA into the GAS cell was blocked; failure to import DNA could not be explained by production of DNases (Mashburn-Warren et al., 2012). Transformation at a low efficiency is detected for GAS grown as biofilms, whereby integration of a DNA marker is increased slightly following addition of XIP and is dependent on ComR, which is also required for biofilm formation (Marks et al., 2014). Although transformation rates in this study are ~100,000-fold less than those observed for the highly competent pneumococcus, natural transformation of GAS is enhanced by biofilm formation in mammalian cell culture and in nasal-associated lymphoid tissue (NALT) of mice.

Horizontal transfer of DNA between different GAS strains seems most likely to occur during co-colonization of the URT, or co-infection of impetigo lesions with multiple strains (Carapetis et al., 1995). GAS can grow as biofilms or form microcolonies during infection within both of these tissue niches (Akiyama et al., 2003; Roberts et al., 2012). Interspecies gene exchange is also known to occur (Section 7). Quorum sensing among GAS is mediated via transcriptional regulators of the Rgg-family and peptide pheromones (Chang et al., 2011). The short hydrophobic peptide (SHP) pheromones of GAS, and the Rgg-like regulators (Rgg2, Rgg3) that detect them, mediate cross-species signaling between GAS and other member species of the pyogenic division, specifically GBS and SDE (Cook et al., 2013). These bidirectional quorum sensing systems may influence numerous biological properties, and possibly promote interspecies exchange of DNA. Other ecological considerations that may affect HGT include bacteriocin production by GAS and related species (Wescombe et al., 2009; Wescombe and Tagg, 2003), leading to competition between GAS strains or between GAS and other species, and further modulation of the microbiome.

A mutator phenotype leading to high rates of mutation has been characterized for GAS. This defect in DNA mismatch repair (MMR) is controlled by excision and re-integration of a streptococcal phage-like chromosomal island (SpyCI) at a site between the *mutS* and *mutL* loci on the genome (Scott et al., 2008), which is discussed in detail in Section 6. The presence of SpyCI in GAS leads to a ~100-fold increase in the rate of spontaneous mutation, to as high as ~10^{-7} to 10^{-8} mutations per generation.

5. Whole genome sequences

The GAS genome is remarkable for its content of prophages, SpyCIs, and other mobile genetic elements (MGEs) such as integrative and conjugative elements (ICEs). An early analysis of 11 GAS genomes (of eight *emm* types) yielded a pan-genome of ~2,500 genes

and a core-genome of 1,297 genes (Lefebure and Stanhope, 2007); the non-core genome contains a mix of prophages, and other MGEs and accessory gene regions (AGRs). Core-genome phylogenies based on concatenated sequences of the 1,297 genes were constructed for the 11 GAS strains, and non-core gene gain and loss was enumerated at the branch point for each strain. High levels of gene gain and gene loss along each branch were observed, even for GAS isolates sharing the same *emm* type. This study also estimated that up to ~37% of the genes in the GAS core-genome show evidence for recombination (Lefebure and Stanhope, 2007). A few core genes under positive selection were observed for some strain-specific branches, but some branches had no core genes under positive selection.

Estimation of prophage gain and loss in GAS is problematic due to multiple copies of phage genes having high similarity, their rapid evolution, and the difficulty in distinguishing vertical inheritance from horizontal transfer. By using a synteny-based method to sidestep these problems, 12 GAS whole genome sequences represented by nine *emm* types were assessed for genetic flux, which was found to be dominated by gain and loss of prophage genes (Didelot et al., 2008). Estimation of the rates of genomic flux indicates, at least for some strains, that prophage integration may have accelerated in recent time. This finding is consistent with another study (five GAS genomes of four *emm* types) showing that for GAS species-specific genes, those that underwent recent HGT have higher Ka/Ks ratios (by ~3-fold) and a faster rate of evolution; this finding may be the result of habitat-driven adaptation (Marri et al., 2006). Yet, in another phylogenomic analysis that used the same five GAS strains but considers a slightly larger (and possibly somewhat distinct) set of GAS genes, the proportion of genes with signatures of positive (diversifying) selection is about equal (~8%) for accessory and core genes (Anisimova et al., 2007).

As of June 2014, 20 gapless and annotated whole genome sequences have been reported for GAS (NCBI, 2014) (Table 2). Also included in Table 2 are new GAS draft genomes of strains of four additional *emm* types; each draft genome remains in multiple contigs separated by gaps. Together, these 24 GAS strains comprise a diverse group, represented by 13 *emm* types, 17 STs, all three *emm* pattern groups, and six FCT-region forms. Genome size ranges from 1.75 to 1.93 Mb among 20 the completed genomes, and the G+C content ranges from 38.4 to 38.7%. There are >200 incomplete GAS genomes with partial sequences available (NCBI, 2014), including published draft genomes of several additional *emm* types (Table 3), and thousands of other GAS genomes which were primarily sequenced in a search for SNPs, indels and recent events involving MGEs [e.g., (Beres et al., 2010; Nasser et al., 2014; Shea et al., 2011); Section 9]. Computational algorithms taking a genome wide approach have identified small regulatory RNAs (sRNA) in GAS, of which ~50 have been characterized to date (Patenge et al., 2012; Perez et al., 2009; Tesorero et al., 2013).

The 24 strains listed in Table 2 comprise a genetically diverse set, and many were selected for study based on their disease association. A wide range of geographic sources is also represented. Nonetheless, it is important to bear in mind that these strains may not be representative of the species as a whole and therefore, meta-analyses may be somewhat biased in unknown ways. For example, strains of pattern D *emm* types in clade X (Sanderson-Smith et al., 2014), which include *emm59* and *emm85*, have several features that are more typical of pattern E strains, such as *sof* genes and FCT-4 regions encoding pili

(Bessen, 2012; Fittipaldi et al., 2012a). Several of the specific strains listed in Tables 2 and 3 are discussed in detail in Section 9.

The number of complete prophage genomes per strain ranges from none (MGAS15252) to six (Table 2). For those strains of the same *emm* type that have the same number of prophages (e.g., *emm1*, *emm3* and *emm12*), the nature of the prophages and their loci of integration can differ (Section 6). Also, the number of prophages differs for the two *emm59* isolates. About half of the GAS genomes have a SpyCI, and about half have one or more ICEs (Table 2). The genetic structures of prophages, SpyCIs, and ICEs are discussed in detail in Section 6.

The core-genome, which largely excludes prophages, other MGEs and non-phage AGRs (the latter is discussed in Section 8), can be defined by whole genome multiple alignment [e.g., Mugsy (Angiuoli and Salzberg, 2011)], combined with identification of regions of the alignment that are shared by all genomes. These core locally collinear blocks (LCBs) of the alignment were identified and ungapped segments concatenated using Phylomark (Sahl et al., 2012). Alignment of the 24 genomes of Table 2 yields a core-genome of 1,510,896 bp, whereby 2.4% of the sites are informative. The GAS core-genomes yield a star-like phylogeny (data not shown). A minimum evolution tree (Figure 1A) shows that only organisms sharing an *emm* type are very closely related and monophyletic, whereas the genetic distance between isolates of any two distinct *emm* types is relatively high. A SplitsTree graph of networked evolution adds further support for the highly recombinogenic nature of GAS, yet even here, long branches corresponding to distinct *emm* types predominate (Figure 1B). One of the interesting unresolved questions of GAS phylogenomics concerns the (seeming lack of) genetic intermediate or transitional forms among organisms of different *emm* types. The sequencing of genomes from strains of many more of the 234 recognized *emm* types may help to clarify this issue. Low levels of nt sequence diversity (with little/no phylogenetic signal) and high rates of recombination add to the challenge of tracing the ancestry of strains representing each *emm* type.

The size of the pan-genome of GAS, based on the 24 genomes listed in Table 2, can be estimated by tallying the cumulative lengths of all LCBs (i.e., core and non-core). Using default parameters for alignments, maximum gap size allowed for LCBs, and minimum length for LCBs (LCBs < 50 nt are excluded) (Angiuoli and Salzberg, 2011), the GAS pan-genome is estimated to be 3,113,976 bp in size. ORFs were queried for their distribution among the 24 genomes using Sybil (Riley et al., 2012); ORFs can be assigned to gene clusters and these are synteny-based assignments (Angiuoli et al., 2011). Based on whole genome sequences of the 24 GAS strains (Table 2), an estimated 1,200 core clusters of orthologous proteins, together with 1,503 non-core clusters (i.e., orthologs present in 2 to 23 genomes) and 1,240 singleton proteins (i.e., genome-specific, and not included in synteny-based clusters) comprise the GAS pan-genome, for a total of ~3,943 genes/proteins. Note that estimates for the size of the GAS pan-genome vary, in part, based on the methodologies and parameters employed, and the number and nature of the strains compared [e.g., (Beres et al., 2006)].

Figure 2 illustrates the distribution of whole genome alignment-derived LCBs based on the 24 GAS genomes listed in Table 2. Figure 2A includes all 5,210 individual LCBs. The alignment contains a relatively small number of large core LCBs shared by all strains (N = 159) and shared but non-core LCBs (N = 700). However, Figure 2A is dominated by the 4,351 strain-specific shorter LCBs that are approximately equally distributed across all 24 strains. The shared non-core LCBs are concentrated towards the top and bottom (tree on the left side) and are the primary drivers of strain clustering (tree on the top). Although the tree branches of the strain clusters (top tree) are fairly deep, clustering in accordance with *emm* type is still evident.

In order to better visualize the distribution of shared non-core LCBs, all LCBs <1,000 nt were removed (Figure 2B). This step eliminates most, but not all strain-specific LCBs, as well as very short LCBs. The core LCBs are concentrated in the upper third of the panel. Strain clustering (tree on top) is largely driven by LCBs that are shared among a subset of strains, and many branch lengths are fairly short. For example, three *emm1* strains (M1_A-C_ii to iv) uniquely share a relatively large number of LCBs depicted below the large block of core LCBs; strain M1_A-C_i (SF370; historic *emm1*; Section 9) also clusters with these three M1 strains but with a longer branch, reflected by the absence of some of the M1-specific LCBs in this strain. In addition to clustering in accordance with *emm* type, there is (partial) clustering in accordance with *emm* pattern group; possibly, this is related to linkage between AGRs and *emm* pattern group (discussed in Section 8). One striking feature is a fairly large number of strain-specific LCBs in strains M85_D and M95_D (Figure 2B); these are draft genomes and therefore, it is possible that at least some of the sequences correspond to redundant low quality contigs.

The presence or absence of genes among 97 GAS strains of 95 different *emm* types was assessed by comparative genome hybridization (CGH) using a GAS pan-genome microarray based on 14 whole genome sequences (Bessen et al., 2011). Of the >3,400 microarray targets, 393 were identified as having a differential distribution among 15 to 85% of the GAS strains. After excluding genes associated with prophage or SpyCIs, the differentially distributed target genes formed 22 AGRs, ranging from 1 to 43 genes per AGR. Fourteen of the 22 AGRs are represented by a single form, whereas the eight other AGRs have multiple (i.e., two or more) forms. Numerous AGRs are associated with transposons, suggesting that Tn-mediated insertions or deletions may have played a role in gene acquisition or loss. *emm* and *sof* lie within AGR-21/21X, whereas the FCT region lies within AGR-2/2X; both AGR-2 and AGR-21 have multiple forms. One of the AGRs (AGR-13) corresponds to an ICE-like region (Beres and Musser, 2007). The distribution of AGRs among GAS strains is discussed in detail in Section 8. Based on TA- and GC-skewing for 12 of the sequenced GAS genomes, it was concluded that *emm* lies within an ancient pathogenicity island of ~47 kb (Panchaud et al., 2009), which extensively overlaps with AGR-21/21X.

Of the 24 genomes listed in Table 2, four (16.7%) show evidence for chromosomal inversion relative to the majority of GAS genomes (Figure 3). For two of the strains - SSI-1 (*emm3*) and Manfredo (*emm5*) - a ~1.3 Mb inversion centered on the *ori* occurred following a recombinational event immediately downstream from the *comX/sigX* gene (corresponding to ORF SPy0300 in the SF370 M1 genome) and at a second *comX1*-like gene and glycerate

kinase gene (corresponding to SPy1901–SPy1902 of the SF370 genome) (Holden et al., 2007; Nakagawa et al., 2003). Strain HKU16 (*emm12*) has a slightly larger chromosomal inversion (Tse et al., 2012), wherein the two crossover points lie in the vicinity of SPy0205 to SPy0207 and SPy2006 to SPy2010; the latter region encodes the virulence factors laminin-binding protein Lmp, the fibronectin-binding protein (FnBP) FbaA, and C5a peptidase (*scpA*) of the SF370 genome. Strain M1_476 has a remnant of a chromosomal inversion that partially reverted, and spans from a transposase corresponding to the vicinity of SF370 genome SPy0216–SPy0219 through to *comX* (on the right side of the *ori*) and SPy2006–2007 (encoding Lmp and FbaA) through to SPy1901–SPy1902 (on the left side of the *ori*). Thus, the *comX* region appears to be a hotspot for chromosomal inversion; the function of this region is discussed in Section 4. In addition, it may be noteworthy that *fbaA* (SPy2007) has a differential distribution among GAS strains, wherein *fbaA* is present in 100% of *emm* pattern D skin specialists but in only 25% of pattern A–C throat specialists evaluated (Bessen et al., 2011). Therefore, based on in silico analysis, this crossover hotspot (as well as the *comX* region) may directly impact GAS virulence or other key biological properties.

6. Mobile genetic elements

Beginning with the first sequenced GAS genome (Ferretti et al., 2001), and confirmed by each subsequent one (Table 2), it is clear that lambdoid prophages, *S. pyogenes* phage-like chromosomal islands (SpyCIs), integrative and conjugative elements (ICEs), and other mobile genetic elements (MGEs) are prominent features of the GAS genome. Among the completed and publicly available genomes, the number of endogenous prophages ranges from as few as zero to as many as six and together, can account for almost 10% of the total genome (Banks et al., 2004b). One GAS genome, MGAS15252, remarkably contains no intact prophages although it carries a SpyCI integrated into DNA mismatch repair gene *mutL* (Scott et al., 2012). The contribution of prophages and other MGEs to their host cell phenotype is evident through toxigenic conversion, antibiotic resistance, and modulation of host cell gene expression.

The earliest report that linked GAS phages to virulence showed that the “scarlatina toxin” of scarlet fever can be transmitted to a new GAS strain following exposure to a sterile filtrate from a toxigenic isolate (Frobisher and Brown, 1927). Numerous studies followed over the ensuing decades, but it was not until genome sequencing of multiple strains of GAS was complete that the true extent and diversity of endogenous prophages became more fully apparent. With few exceptions, GAS prophages follow the general structure of lambdoid phages, with some genes common to many distinct phages, whereas other genes are seemingly unique to one particular phage (Banks et al., 2002; Canchaya et al., 2002; Desiere et al., 2001). Associated with GAS prophages is a wide range of virulence-associated genes that encode exotoxins (including many superantigens) and enzymes such as DNases (i.e., streptodornases) (Table 4).

Botstein proposed that the product of phage evolution is a family of interchangeable genetic modules, each associated with a particular biological function (Botstein, 1980). This modular view is embodied by GAS prophages. For example, a given integrase gene and its

targeted integration site may be associated with several different virulence genes (Table 4). In contrast, some modules are very strongly linked, such as the SF370.1-like integrase and the virulence genes *speC-spd1* (Table 4; locus code D). Some prophages may be associated with a single *emm* type, as may be the case for ΦNZ131.3 and *emm49* strains (McShan et al., 2008); host range restriction, which may or may not apply here, could potentially limit recombination opportunities between phages inhabiting GAS of other *emm* types. Highly conserved sequences within GAS prophage genomes, such as the paratox sequence (Aziz et al., 2005) or the hyaluronidase gene family, may help promote recombination between similar modules in other phages. In addition, the gene pool for GAS phages extends to other streptococcal species. Phages sharing highly similar genes or larger genetic blocks with GAS have been identified in *S. equi* subsp., *S. agalactiae*, *S. equisimilis* subsp., and *Streptococcus thermophilus* (Bai et al., 2013; Canchaya et al., 2002; Davies et al., 2007b; Desiere et al., 2001; Holden et al., 2009).

Although *Escherichia coli* phage lambda is the paradigm for site-specific integration (Hendrix et al., 1983), its use of an intergenic attachment site on the bacterial chromosome is somewhat atypical. An extensive analysis of bacterial genome prophages showed that tRNA genes, the tmRNA gene, and intragenic targets account for nearly 70% of prophage attachment sites (Fouts, 2006). Attachment sites within coding regions can be further subdivided according to whether the phage integrates into the 5’ or 3’ end of the gene (Canchaya et al., 2002). For tRNA genes, the sequence duplication between the host gene and phage attachment site ensures that gene function is preserved following integration at the 3’ end (Fouts, 2006). By contrast, integration into the 5’ end of a gene has the potential to disrupt gene function, as observed for the SpyCIs that integrate into the 5’ end of DNA mismatch repair gene *mutL* (Scott et al., 2012; Scott et al., 2008).

Numerous GAS genes are targeted by phages for site-specific integration, with integration occurring at either the 5’ and 3’ ends (Table 4). Some sites are frequently exploited by phages (e.g., the genes for tmRNA and DNA-binding protein *hup*; locus codes H and J, Table 4), whereas other integration sites are presently known by single representatives. The prophages that integrate into the promoters at the 5' ends of genes Spy49_0371 and *yesN* may interrupt the expression of these genes by separating the ORF from its transcriptional start site. GAS prophages integrate directly into the 5’ end of structural genes encoding a conserved dipeptidase, DNA translocation machinery channel protein ComEC, DNA-binding protein HU, RecX, and a gamma-glutamyl kinase (Table 4). Presently, the impact of these site-specific recombination events on the host cell phenotype is mostly unknown. The *emm3* prophages Φ315.1 and ΦSPsP5 target a highly unusual chromosomal attachment site via integration into a direct repeat sequence for the CRISPR type II system (i.e., CRISPR-1 locus; Table 2). None of the spacers or direct repeats typically associated with this CRISPR in other GAS strains are evident in the *emm3* isolates, and it is possible that the site-specific integration event led to inactivation of the CRISPR anti-phage system (Nozawa et al., 2011).

Phylogenetic analysis shows that the genome prophages are highly diverse, despite the frequent sharing of lysogeny modules, virulence genes, or both (Figure 4A). Some lysogeny modules are strongly linked to particular virulence genes, such as the integrases that target the dipeptidase gene paired with *speC-spd1*, or integrases targeting $tRNA_{ser}$ paired with

DNase genes (Table 4). Other modules are associated with multiple virulence genes, such as integrases targeting the tmRNA gene (locus code H, Table 4). However, these apparent linkages do not necessarily result from clonality: the prophages integrated into the $tRNA_{ser}$ (locus code N) form a closely related branch of the tree, whereas those targeting the dipeptidase gene (locus code D) are somewhat diverse (Figure 4A). As mentioned, prophage integration at the dipeptidase gene has the potential alter its expression, and it may be that dipeptidase inhibition enhances the functional impact of the prophage-encoded *speC-spd1* virulence cluster.

The impact of phages and other MGEs upon their bacterial host cells can be divided into two broad categories: phage-encoded genes that contribute to the phenotype of the host cell (e.g., toxigenic conversion) and alterations of host cell gene expression due to integration of the phage. The impact of phage-encoded virulence genes that are mobilized by specialized transduction is well known, whereas the impact of site-specific integration on host cell gene expression is only beginning to be understood. An important class of phage-encoded GAS virulence genes includes the erythrogenic (pyrogenic) exotoxins that function as superantigens. Not all streptococcal superantigens are phage-associated (e.g., streptococcal mitogenic exotoxin Z [SmeZ]). However, the exotoxin genes that are phage-encoded are uniform in their association with phages, and they are not observed to occur as non-phage-associated genes, although a phage toxin gene may be part of a decaying prophage remnant that becomes fixed in the bacterial genome. Interestingly, some virulence genes often occur in genetically linked pairs that may enhance virulence through synergistic interactions (e.g., *speC-spd1* and *speH-speI*). It has been proposed that the hyaluronidase that forms part of the phage tail fiber structure acts as a virulence factor, but such a role appears to be unresolved (Starr and Engleberg, 2006), and the function of hyaluronidase may be restricted to degrading the GAS capsular polysaccharide for the sole purpose of allowing phage attachment. Included among GAS prophages are genes with identifiable functions (Canchaya et al., 2002; Desiere et al., 2001), plus numerous ORFs encoding proteins of unknown function. Some of the uncharacterized genes may encode additional virulence factors as well as sRNAs and short peptides that participate in genetic regulatory networks within the bacterial host cell (Gottesman and Storz, 2011; Tesorero et al., 2013).

Several studies indicate that the expression of GAS prophage-encoded virulence genes is not autonomous, and may be linked to bacterial cell gene regulation. Expression of pyrogenic exotoxin C (*speC*) is up-regulated when GAS are co-cultured with human pharyngeal cells (Broudy et al., 2001). These growth conditions also stimulate prophage induction, which is apparently induced by a soluble factor released by the pharyngeal cells (Broudy and Fischetti, 2003; Gottesman and Storz, 2011; Holden et al., 2009). Similar findings were independently obtained when co-culture of GAS with pharyngeal cells lead to expression of prophage-encoded virulence genes for pyrogenic exotoxin K (*speK*), phospholipase A2 (*sla*), and streptodornase (*sdn*) (Banks et al., 2003a). GAS regulatory proteins can directly interact with phage genes. The GAS transcriptional regulator Rgg/RopB regulates expression of the prophage DNase Spd3 gene by binding its promoter (Anbalagan and Chaussee, 2013; Anbalagan et al., 2012; McShan et al., 2008), and the peroxide response regulator PerR enhances expression of the related prophage DNase Sda1 gene (Wang et al.,

2013). The ability to selectively enhance the expression of DNases may facilitate survival of GAS when entangled in neutrophil extracellular traps (NETs) (Buchanan et al., 2006; de Buhr et al., 2014; Wartha et al., 2007). The interaction between GAS phage and non-phage genes and their products is an evolving field that is bound to shed important new light on the pathogenesis of GAS infections.

SpyCIs are a unique group of MGEs found frequently in GAS genomes. These phage-like elements integrate into the MMR gene *mutL* (Scott et al., 2008). SpyCIs have small genomes (12–17 kb) containing lysogeny and DNA replication modules, but they lack identifiable genes for DNA packaging or capsids. By integrating into the MMR operon, SpyCIs silence not only *mutL* but also the downstream genes *lmrP* (multidrug efflux pump), *ruvA* (Holliday junction helicase subunit), and *tag* (base excision repair). The consequence of this gene silencing is that the GAS cell adopts a complex mutator phenotype (Scott et al., 2012; Scott et al., 2008). Remarkably, SpyCI are dynamic elements. When GAS cells reach early logarithmic growth, SpyCI excises from the genome and allows transcription of *mutL* and the downstream genes to proceed. As the GAS cells approach stationary phase, SpyCI re-integrates into *mutL* and once again silences the downstream genes. This cycle of excision and re-integration causes the GAS cell to alternate between a wild type and mutator phenotype. One exception to this cycle is found in the *emm5* strain (Manfredo), wherein a deletion in the SpyCIM5 integrase gene was compensated for by a novel promoter in the gene remnant that constitutively transcribes *mutL* and the other downstream genes. Outside of the highly conserved modules for lysogeny and replication, the remainder of SpyCI DNA is often quite divergent (Figure 4B), and includes many ORFs of unknown function, including potential sRNAs or peptides that may influence host cell function.

More than half of the GAS strains with completed genome sequences are host to a SpyCI (Scott et al., 2012; Scott et al., 2008) (Table 2). Some, but not all strains sharing an *emm* type, harbor a SpyCI (e.g., *emm1* strain SF370 versus the three other *emm1* strains; Table 2). Curiously, SpyCIM53 as defined by the primase C terminal 1 family protein target probe (based on strain Alab49), is present in only 20% of *emm* pattern A–C strains, but is detected in 70% of pattern D and 59% of pattern E strains evaluated by CGH (Bessen et al., 2011). Given the relatively low ratios for rates of recombination to mutation observed in housekeeping genes for *emm* pattern A–C organisms as compared to pattern D and E strains, and the large number of pattern A–C *emm* subtypes that presumably arise by mutation (Section 3), one might expect the throat specialists to have a higher concentration of mutator phenotypes. But that may not be the case if the distribution of SpyCIs is skewed in favor of skin specialist and generalist strains.

An efficient means for dissemination of SpyCIs must exist in spite of their lack of capsid genes. It may be that helper phages are tapped as a source of structural genes, as has been observed in the *Staphylococcus aureus* pathogenicity islands (SaPI) (Novick et al., 2010). The SpyCIs are not unique to GAS; genomes of *Streptococcus anginosus*, *Streptococcus intermedius*, SDE, *S. canis*, and *Streptococcus parauberis* display similar elements integrated into *mutL* (Nguyen and McShan, 2014). Although mutator phenotypes are common in wild populations of bacteria (Bucci et al., 1999; LeClerc et al., 1996; Oliver et al., 2000; Taddei et al., 1997), based on published reports to date, it appears that only in

streptococci has such a remarkable molecular switch evolved that allows the bacterial host cell to cycle between mutator and wild type in response to growth.

Clustered, regularly interspaced short palindromic repeat (CRISPR) elements and CRISPR-associated (Cas) proteins constitute a microbial immune system that can protect the bacterial host cell from invasion by MGEs, including phage (Barrangou et al., 2007). CRISPR expression appears to generate anti-phage mRNA that interferes with the genetic program of the lytic cycle (Barrangou et al., 2007; Bolotin et al., 2005; Mojica et al., 2005). Examination of 13 GAS genomes reveals two CRISPR-Cas loci, wherein a given strain has both, one, or neither locus (Nozawa et al., 2011). A CRISPR locus is characterized by a succession of ~21- to 47-bp direct repeat sequences, separated by unique spacer sequences (~30- to 36-bp). Expression of repeat/spacer-derived, short CRISPR RNA (crRNA) leads to the silencing of foreign nucleic acids in a sequence-specific manner (Deltcheva et al., 2011). The CRISPR spacers in GAS genomes are homologs of known prophage sequences, or they may match yet undiscovered sequences (McShan et al., 2008); no sequence homologies have yet been found with other MGEs such as ICEs (Nozawa et al., 2011). The CRISPR elements provide a glimpse of previous encounters with bacteriophages in the evolutionary past of each GAS genome, and as more phage sequence information is acquired, they may provide insight into subpopulations of phages that interact with GAS which differ by *emm* type or disease association.

For 13 GAS genomes examined, a statistically significant inverse relationship between the numbers of CRISPR spacers versus prophages was observed (Nozawa et al., 2011). These findings support the notion that in GAS, CRISPRs inhibit prophage incorporation into host cell genomes and this action is dependent on the number of spacers. In addition, GAS strains lacking CRISPRs possess statistically significantly more prophages than CRISPR-harboring strains (Nozawa et al., 2011). However, based on Table 2 data for 20 complete and assembled GAS genomes, which include three *emm* pattern D strains and a slightly different accounting for the number of prophages in each strain (i.e., Table 2 counts only complete lambdoid phage genomes), the difference in the number of prophages for CRISPR-1-positive GAS versus CRISPR-1-negative GAS is not statistically significant (Student t-test, 2-tailed). Similarly, no significant difference is observed in the number of prophages for CRISPR-2-positive versus CRISPR-2-negative GAS. Of the 20 GAS strains considered, six lack both CRISPR elements (Table 2). Considering the number of prophages, or the combined number of prophages plus SpyCIs, and the presence versus absence of both CRISPR elements, no significant differences are detected; a lack of significance is also evident for the combined number of prophages plus SpyCIs versus either CRISPR-1 or CRISPR-2 (Table 2). Findings from CGH for 97 GAS strains of 95 distinct *emm* types reveal that the CRISPR-1 element is present in 40, 48 and 52% of *emm* pattern A–C, D and E strains, respectively (Bessen et al., 2011). However, there are highly significant differences in the distribution of the CRISPR-2 locus in accordance with *emm* pattern group, whereby none of the pattern D strains harbor CRISPR-2, in contrast to 35% and 66% of pattern A–C and E strains, respectively. The biological significance of the lack of the CRISPR-2 element among skin specialist strains remains to be established.

Among the other MGEs inhabiting the GAS genome are ICEs or ICE-like regions of difference (RD) [reviewed in (Banks et al., 2002; Beres and Musser, 2007; Green et al., 2005)]. Several ICE or RD forms have been well-described in GAS. An ~10–15 kb ICE/RD, which varies in size among GAS strains, harbors genes encoding the bacteriocin known as streptin; this RD is equivalent to AGR-13 and is present in >50% of GAS isolates of 95 different *emm* types (Bessen et al., 2011). Within a far more restricted set of GAS strains lies a ~37 kb element that encodes the R28 surface protein (Green et al., 2005) (discussed in Section 7); this ICE/RD is present in *emm2* and *emm28* strains and is nearly identical in nt sequence in both strains, indicative of a recent common ancestor (Beres and Musser, 2007).

Genes conferring resistance to antibiotics have been localized to ICEs within GAS. The *mef(A)* gene, which encodes a macrolide efflux pump and gives rise to the M phenotype for erythromycin resistance, is present within a ~59 kb chimeric element composed of a transposon inserted into a structure with prophage features, and has been associated with an *emm6* outbreak clone (Φ10394.4) (Banks et al., 2003b). Also contained within this element is a type II restriction endonuclease-modification cassette, which may protect the host cell from subsequent infections by bacteriophage or other elements (Euler et al., 2007). In addition, *mef(A)* can be associated with other MGEs in GAS such as ΦM46.1, which also harbors the *tet(O)* gene (Brenciani et al., 2010). Numerous other ICEs (or plasmids) present in GAS harbor additional antibiotic resistance genes including *tet(M), erm(A)* and *erm(B)* (Banks et al., 2004a; Beres and Musser, 2007; Brenciani et al., 2012; Giovanetti et al., 2012; Tse et al., 2012; Varaldo et al., 2009; Willems et al., 2011; Woodbury et al., 2008). It was estimated that resistance to macrolides was acquired by GAS in at least 49 independent genetic events (Robinson et al., 2006), and tetracycline resistance has arisen ≥80 times (Ayer et al., 2007); both values may be gross underestimates.

7. Phylogenomics of GAS and closely related species

A very recent phylogeny was constructed using concatenated nt sequences of 136 core genes derived from whole genome sequences of 46 *Streptococcus* species (Richards et al., 2014). GAS is placed within the clade corresponding to the pyogenic division of streptococcal species. Its closest genetic relatives are *S. canis* and SDE, with SDD closely related to SDE; the *S. equi* subspecies Se and Sz lie on a closely related branch; *S. agalactiae* (GBS) also lies within the pyogenic division, but within a distinct sub-cluster. This finding (Richards et al., 2014) is generally consistent with the taxonomies derived from 16S rRNA sequences (Facklam, 2002), concatenates of inferred sequences of DNA replication and repair proteins (Beres et al., 2008), and a larger set of core genes from fewer pyogenic species (Lefebure et al., 2012), and phylogenetic reconstruction based on multilocus sequence analysis (MLSA) using seven housekeeping genes that are shared among numerous streptococcal species (Jensen and Kilian, 2012).

Gene families can arise and expand via intra-chromosomal gene duplication followed by neo- or sub-functionalization (i.e., paralogs) or alternatively, by xenologous gene displacement wherein a horizontally transferred ortholog from another lineage or species replaces the target gene, often via homologous recombination (Koonin et al., 2001). The latter is a form of replacing HGT. Analysis of 17 *Streptococcus* genomes, of which 12 are

GAS, reveals that HGT of orthologs far outweighs the contribution of gene duplication to the expansion and diversification of gene families (Treangen and Rocha, 2011). Thus, replacing HGT involving orthologous genes provides an efficient means to introduce novel functions that may provide a selective advantage.

Interspecific HGT events among species of the pyogenic division are well-documented. Foreign DNA can be introduced into a recipient genome by additive or replacing HGT, and each mechanism results in a distinct phylogenetic signature (Choi et al., 2012). Whole genome sequence data allows for a comprehensive overview of gene gain and loss that occurred during species formation and evolution. Whole genome sequence data are presently available for many species within the pyogenic division including *S. canis* (Richards et al., 2012), SDE (Shimomura et al., 2011; Suzuki et al., 2011), SDD (Suzuki et al., 2011), Sz (Beres et al., 2008; Holden et al., 2009), Se (Holden et al., 2009), and GBS [e.g., (Glaser et al., 2002; Tettelin et al., 2005; Tettelin et al., 2002)].

HGT events that replace recipient DNA sequences with donor DNA via homologous recombination were inferred using the ClonalOrigin model-based approach, for GAS, SDE and SDD (Choi et al., 2012). Using one or two genomes per species, alignments yielded a shared core-genome of ~1.5 Mb (which is about the same size of the estimated GAS pan-genome based on 24 strains [Section 5], although the methodologies differ and the model-based method may be less conservative); although only one or two genomes of each species could be included in this analysis, other GAS genomes yielded similar results. Data indicate elevated rates of replacing HGT between GAS and SDE in both directions, but with marked asymmetry favoring the GAS → SDE direction by ~2-fold. Elevated rates of replacing HGT were also observed for SDE → SDD, but not for SDD → SDE or between GAS and SDD in either direction (Choi et al., 2012).

A potential drawback to the model-based approach used (Choi et al., 2012) is that only two SDE whole genome sequences are presently available for evaluation. An oligonucleotide-based microarray containing probes for >200 GAS genes (derived from GAS strains of three *emm* types) was used to examine the genomic content of 57 SDE strains by DNA-DNA hybridization (Davies et al., 2007a). Two major clusters of SDE strains could be distinguished by the presence or absence of up to 35 GAS gene homologs, many of whose products likely serve housekeeping functions. These data, much of which likely represent replacing HGT, provide further support for the GAS → SDE direction of HGT. Additional replacing HGTs between GAS and SDE, occurring in both directions, involve alleles of at least three of the housekeeping genes used for MLST (Ahmad et al., 2009; McMillan et al., 2010). There is also evidence for HGT between GAS and SDE of a FnBP gene within the FCT region (Towers et al., 2004) and *emm* types 12 and 57 (McMillan et al., 2010; Simpson et al., 1992).

Population surveys that evaluate individual gene sequences but include many more strains of both GAS and SDE, show that for at least two genes essential for virulence, there is strong support for interspecies gene flow that is consistent with skewing in the SDE → GAS direction. One possible SDE → GAS replacing HGT involves the pilus transcriptional regulatory *rofA* locus within the FCT region: *rofA* alleles present in only 73 (64%) of the

114 GAS isolates, but the highly homologous *rofCG* alleles are present in all 33 (100%) SDE strains examined (Bessen et al., 2005). The remaining GAS isolates have *nra* replacing *rofA* at the same locus, whereby alleles of the *rofA* and *nra* lineages exhibit ~33% nt sequence divergence. In contrast, the maximal nucleotide sequence divergence between *rofA* alleles of GAS and the *rofCG* alleles of SDE is only 2.0%, and the extent of sequence differences within both the *rofA* and *rofCG* sets of alleles are roughly of the same order as the differences between the two populations, indicative of a recent common ancestor. In another example suggestive of replacing HGT, *ska* alleles of the well-supported *ska-2a* subcluster are present in all 33 (100%) SDE strains examined, but in only 15 (17%) of 90 GAS strains (Kalia and Bessen, 2004); *ska* encodes streptokinase, a plasminogen activator having a well-established role in virulence. The presumptive replacing HGTs of *rofA* and *ska-2a*, from SDE → GAS, may represent xenologous gene displacements. Of biological relevance is the finding that the distributions of *rofA/nra* and *ska* subcluster alleles among GAS also show statistically significant correlations with *emm* pattern grouping, and the orthologous forms show signs of positive selection (Bessen et al., 2005; Kalia and Bessen, 2004). It is not known whether these likely HGT events involving virulence genes were detected by the ClonalOrigin analysis (Choi et al., 2012), and DNA-DNA hybridization may not provide sufficient sensitivity (Davies et al., 2007a). More extensive whole genome sequence analysis of numerous strains belonging to both streptococcal species, as well as other related species, should permit a more precise analysis of the direction of net gene flow, as well as aid in identifying species-specific genes.

Divergent (but homologous) regulatory genes and their targets can give rise to cross-regulation and add layers of complexity to transcriptional networks. RofA-like proteins (RALPs) are present in various combinations among the different GAS strains, and many of their target genes encode MSCRAMMs which also comprise a gene family (Kreikemeyer et al., 2007). The presence of RALPs and their target genes in GAS may have occurred by a mixture of replacing and additive HGT events (Section 8). Members of the Rgg family of transcriptional regulators (Rgg2 and Rgg3 [distinct from Rgg/RopB]) and their cognate pheromone peptides [Section 4; (Cook et al., 2013)], which are also present in multiple species of the pyogenic division, may have also evolved by similar mechanisms, leading to a complex interspecific communication network.

A parsimony-based analysis that considers the pan-genome was used to evaluate additive HGT events among GAS, SDE and SDD, however, findings failed to reveal a strong bias in directionality for additive transfers between GAS and SDE (Choi et al., 2012). Genome-wide phylogenetic analyses involving 15 *Streptococcus* species were used to ascertain the number and nature of genes that were gained on the lineage leading to GAS (13 strains); 113 genes were gained from a combination of sources that include other GAS strains, other *Streptococcus* species and other bacterial species, or they arose de novo among GAS; together the gained genes represent ~6% of the average GAS genome size and almost half of them are phage-associated (Lefebure et al., 2012). Gained genes with an established role in virulence in GAS include *speB*, plus its upstream transcriptional regulatory gene *rgg/ropB*. SpeB encodes a secreted cysteine protease that degrades numerous host proteins involved in

defense, as well as extracellular proteins of GAS (Nelson et al., 2011); the ancestral source of the *speB* gene remains unknown.

Horizontal movement of bacteriophage between member species of the pyogenic division is well-established. Transfer of phage is likely to have occurred between GAS and SDE (Davies et al., 2007b; Shimomura et al., 2011) and between GAS and Se (Holden et al., 2009). Many GAS-derived prophage mobilize genes encoding pyrogenic exotoxins via specialized transduction, and exotoxin genes that are highly homologous to those found in GAS are also present in other streptococcal species (Holden et al., 2009; Shimomura et al., 2011); these exotoxins can function as superantigens and contribute to pathogenesis in toxic shock syndrome. Interestingly, the two sequenced genomes of Sz lack both prophage and ICEs (Beres et al., 2008; Holden et al., 2009).

Several of the genes gained on the *S. pyogenes* species-specific branch share sequence homology with GBS genes, suggesting that GBS is a potential donor, although most of these appear to be housekeeping genes (Lefebure et al., 2012). HGT likely occurred between GAS and GBS involving the gene encoding the virulence factor C5a peptidase (Chmouryguina et al., 1996), which is present in (nearly) all strains of both species. In GBS, the C5a peptidase gene and an adjacent gene encoding a laminin-binding protein (*lmb*) share 98% nt sequence identity to GAS orthologs and localize to a composite transposon (Franken et al., 2001). Of potential relevance is the finding that in GAS, the *lmb* gene lies adjacent to a hotspot for chromosomal inversion (SPy2006–2007; Figure 3).

A virulence factor that appears to originate from GBS and was acquired by a limited number of GAS strains is the R28 surface protein (SpyM28_1336). The R28 protein is a chimera of three GBS surface proteins (proteins Rib, α, and β); this mosaic structure is indicative of past recombinational events (Stalhammar-Carlemalm et al., 1999). R28 is encoded within an exogenous ICE/RD of ~37 kb in *emm28* and *emm2* strains (Beres and Musser, 2007; Green et al., 2005; Sitkiewicz et al., 2011). R28-expressing GAS have a strong epidemiologic association with puerperal sepsis, also known as childbed fever, which is a pelvic infection that occurs during or shortly after childbirth. The R28 protein mediates adherence of GAS to cultured epithelial cells derived from human cervical tissue (Stalhammar-Carlemalm et al., 1999); GBS naturally colonize the vaginal epithelium. Thus, puerperal fever caused by GAS and neonatal sepsis caused by GBS are probably initiated by bacteria colonizing the vaginal epithelium, and the possible acquisition of the R28 gene by GAS from GBS may have helped create disease-specialist clones of GAS (Green et al., 2005). Interestingly, the FCT region of an *emm2* strain (FCT-6), which is rare among GAS of other *emm* types (Falugi et al., 2008; Kratovac et al., 2007), has high sequence similarity to the FCT-like region of a SDE strain (Shimomura et al., 2011) and to the pilus gene region of GBS (Beres and Musser, 2007), suggestive of acquisition of the FCT-6 region by GAS following HGT from another streptococcal species.

8. Long-term evolution: Population genomics and tissue tropisms

One of the striking features of GAS biology is the distinction in *emm* types among GAS causing disease in temperate versus tropical communities where pharyngitis and impetigo,

respectively, prevail (Section 2). The spatial-temporal distances that act to separate certain strains of GAS from one another may limit HGT due to reduced opportunities in sharing the same niche. Yet, the *emm* pattern groupings do not correspond to discrete evolutionary lineages based on core housekeeping gene sequences (Section 3). Thus, there may be a common housekeeping gene pool that occupies both ecological niches, perhaps via HGT events involving *emm* pattern E generalist strains. The lack of clear pathways of evolutionary descent for throat versus skin specialist strains, based on core genes, prompted evaluation of the distribution of accessory genes that may play a critical role in establishing tissue site preferences for infection.

A population genomics approach was used to identify non-phage accessory gene regions (AGRs) of GAS, whereby 22 AGRs were identified by CGH of 97 GAS strains (95 *emm* types), defined by their presence/absence in 15 to 85% of strains (Section 5). A SplitsTree graph based on the presence or absence of 21 AGRs (excluding AGR-21/21X which contains the *emm* pattern genotype) reveals strong clustering for the vast majority of pattern D strains, and for the vast majority of pattern E strains, with pattern A–C strains sprinkled throughout both the pattern D- and E-dominant clusters (Bessen et al., 2011). The AGR-based SplitsTree finding was confirmed by population genetic structure analysis of GAS, inferred using a Bayesian nonhierarchical clustering method. A finding of pronounced clustering of AGRs in accordance with *emm* pattern genotype is indicative of co-inheritance and strong linkage among select combinations of AGRs. Maintaining linkage among AGRs, against a background of extensive recombination among core genes (which disrupts nonrandom associations), is highly consistent with the hypothesis that certain combinations of AGRs are subject to strong co-selection pressures as a result of having critical roles in adaptation, such as a tissue tropism for infection.

The distribution among 97 GAS strains of the 393 differential gene targets was further evaluated using the Fisher exact test for independence, for all possible pairwise combinations (>77,000 tests) (Bessen et al., 2011). Eight genes displaying the strongest linkage (BH-corrected p-values < 0.00001), and belonging to 8 different AGRs, were selected for construction of a simplified evolutionary network. Based on presence/absence of 8 genes, 34 unique haplotypes (H) were defined for the 97 GAS strains. A simplified evolutionary network, whereby every strain is connected to >1 other strain by gain/loss of a single AGR, was constructed and includes 26 of the 34 haplotypes (Figure 5). Two major clusters connected by a single weak link (H24) are evident, with 88% of *emm* pattern D (skin) strains in cluster I (upper) and 89% of *emm* pattern E (generalist) strains in cluster II (lower). Of the 18 *emm*-types representing *emm* pattern A–C strains (throat), 61% fall within cluster I, whereas 17% are in cluster II (including *emm12*); the remainder fall outside the main network (including two representatives of the highly prevalent *emm1*, strains SF370 and MGAS5005 [Table 2]).

The *emm* pattern A–C (throat specialist) strains of cluster I belong to the connected haplotypes H17-H19-H23/H29 (Figure 5; upper). Assigned to these haplotypes are M-types associated with significant causes of pharyngitis and acute rheumatic fever (e.g., M-types 3, 5, 6, 14, 18 and 29) (Bisno, 1980; Johnson et al., 1992; Shulman et al., 2009; Steer et al., 2009b; Stollerman, 1991). Analysis of this major sub-cluster of *emm* pattern A–C strains

within cluster I may lead to deeper insight on the key molecular determinants that distinguish many throat and impetiginous skin infections. The transition between H17 and H19 is marked by the loss/gain of a FnBP gene (*prtF1*) in AGR-2/2X. This finding sparked an even more detailed analysis of gain/loss of other differentially distributed FnBP genes among H17–H19 strains, and new extended haplotypes were defined (Bessen et al., 2011). The extended haplotypes reveal a key transition in the tendency for GAS strains to infect different host tissue, that lies between H17x and H17y (i.e., extended haplotypes that are subsets of H17), based on a meta-analysis of 29 surveillance studies (Table 1) normalized for equivalent numbers of pharyngitis and impetigo isolates. The percentage of pharyngitis and impetigo isolates sharing an *emm* type assigned to a given haplotype indicates that H19x and H17y represent classical throat strains, and H18 and H17x represent classical skin strains. For *emm* types associated with H18 and H17x, impetigo isolates outnumber pharyngitis isolates by ~20-fold (i.e., 95% are impetigo -derived). In sharp contrast, for isolates having *emm* types associated with H17y or H19x (N = 654 isolates), all (100%) were recovered from pharyngitis. Thus, the genetic transition between H18/H17x and H17y/H19x coincides with a profound switch in phenotype for the tendency to infect different host tissue.

Differences in gene content for H18, H17y and H19x GAS strains were assessed. The difference between H17x and H18 is loss/gain of AGR-4; AGR-4 lacks known virulence genes. Importantly, the transition from H18/H17x (impetigo) to H17y (pharyngitis) involves loss of *fbaA*. The transition from H17y to H19x involves loss of *prtF2* and gain of *prtF1*. The *fbaA* locus belongs to AGR21/21X, whereas *prtF1* and *prtF2* belong to AGR-2/2X. Thus, the gain/loss of *fbaA*, *prtF1* and/or *prtF2* may impact virulence at the throat and skin. However, it remains possible that additional genetic changes contribute to the tissue-specific phenotype shift, such as SNPs in core or other accessory genes. Furthermore, other classical throat strains (e.g., M1, which lies outside the major haplotype network) might utilize alternative molecular pathways, as might also be the case for the *emm* pattern E generalists.

Gain/loss of other genes may also contribute to tissue site preferences for infection. Strong genetic linkage to *emm* pattern group is observed for several other genes having an established role in virulence or another important biological function (Table 5). Accessory genes that display linkage disequilibrium (i.e., $p < 0.05$) with the *emm* pattern marker for throat specialist versus skin specialist strains are strong candidates for conferring tissue-specific tropisms, particularly when they lie physically distant from AGR-21/21X on the chromosome. *emm* pattern-linked genes include numerous genes within AGR-2/2X and AGR-21/21X that encode transcriptional regulators (i.e., *mga-1/mga-2*, *rofA/nra*, *msmR*), and surface proteins having an established role in virulence (i.e., FnBP genes *prtF1*, *prtF2* and *fbaA*), as well as the plasminogen activator streptokinase (*ska*) (Table 5). Accessory genes whose presence/absence are strongly linked to *emm* pattern E (e.g., *sof*, *sfbX*, *spn/nga*, *sse*, *grab*, *ralp3*; Table 5) might play a dual role in infection of both the skin and throat epithelial tissue sites.

9. Short-term evolution: Comparative genomics in epidemics and outbreaks

The monophyletic grouping of many GAS strains in accordance with *emm* type (Figure 1) readily allows for inferring ancestral-descendent pairs, particularly when coupled with epidemiological collections of strains with known dates of recovery. Whole genome sequencing of multiple isolates of GAS sharing the same *emm* type has provided important new insights on the genetic differences between organisms recovered from different hosts within the same community, genetic changes that are associated with different disease manifestations, the identity of genes subject to strong diversifying selection over time, and the evolutionary history underlying the emergence of new epidemic clones. Whole genome sequencing of GAS is also having increased utility in the clinical microbiology lab and in investigating nosocomial outbreaks.

GAS isolates of the *emm3* type are among the most thoroughly studied by genomics. Organisms of the *emm3* type are highly prevalent among cases of pharyngitis, invasive disease, and acute rheumatic fever. Since the first reports of whole genome sequences for two *emm3* strains (Beres et al., 2002; Nakagawa et al., 2003) (Table 2), hundreds of genomes of *emm3* isolates have been fully or partially sequenced via several methods (Beres et al., 2010; Beres et al., 2006; Beres et al., 2004; Shea et al., 2011). Ninety-five invasive disease isolates recovered from Ontario, Canada within an 11-year period revealed a total of 4,269 polymorphisms within the *emm3* core-genome, with an average of 49 SNPs and 11 indels per isolate; ~65% of the polymorphisms are specific to a single isolate, and within a single isolate ~10% of the polymorphisms are specific to that isolate (Beres et al., 2010). Thus, the core-genomes of *emm3* isolates from the Ontario study are closely related, yet each and every isolate is unique. Two epidemic waves of invasive *emm3* infections (peaking in 1995 and 2000) show differences in *emm3* subtype alleles, wherein isolates recovered in the second wave have a duplication of the first four amino acid residues of the mature M3 protein; this change led to increased resistance to opsonophagocytosis by the organism (Beres et al., 2004). However, a major source of genomic diversity among the invasive *emm3* isolates involves the gain or loss of prophage.

A more precise model of invasive *emm3* disease in Ontario emerged following inclusion of a third epidemic wave (peak in 2005) and analysis of 344 GAS isolates. This study yielded 280 informative core-genome polymorphisms that were used to construct a phylogenetic tree based on biallelic characters defining 97 distinct haplotypes (Beres et al., 2010). Ten major subclones were identified. Based on progenitor-descendent pairs, the rate of accumulation of core-genome polymorphisms was estimated at 1.7 SNPs per organism per year. Data show that each successive epidemic wave consists of organisms that are genetically distinct from those which preceded it. Although some subclones appear to have evolved from strains that were circulating during an earlier wave, new migrants also contributed to the overall level of *emm3* invasive GAS disease.

The genome sequences of *emm3* invasive isolates from Ontario were compared to *emm3* organisms recovered from individuals with asymptomatic carriage in Texas (Beres et al., 2006). Despite differences in virulence in a mouse model for systemic infection, there were

no SNPs in the core-genome that differentiated the two clinical groups, suggesting that the two phenotypes arose multiple times via independent evolutionary pathways. A similar conclusion was reached in a more recent study that compared genome sequences of 86 pharyngitis isolates to those of 100 invasive disease isolates, all from Ontario (Shea et al., 2011). In terms of genetic diversity in the *emm3* core-genome, the pharyngitis and invasive isolates were highly similar, averaging 79 and 77 polymorphisms per isolate, respectively, relative to the MGAS315 reference strain. Data indicate that invasive isolates originate from all major lineages of pharyngitis isolates and they are more similar to the circulating pharyngitis strains from which they evolved as opposed to other invasive strains (Shea et al., 2011).

Despite the lack of "smoking gun" polymorphisms that clearly distinguish *emm3* GAS isolates associated with different clinical syndromes, there are distinctions between disease groups in terms of the genes undergoing strong diversifying selection, which is indicative of ongoing adaptations. For example, in pharyngitis isolates there is an overrepresentation of indels in the *hasA* and *hasB* genes that are essential for biosynthesis of the hyaluronic acid capsule, a key virulence factor (Shea et al., 2011). The numbers of polymorphic sites in genes encoding the CovRS/CsrR and Rgg/RopB transcriptional regulators are also skewed in invasive disease versus pharyngitis isolates (Beres et al., 2010; Ikebe et al., 2010; Olsen et al., 2012; Shea et al., 2011). Many of the SNPs result in non-synonymous substitutions which in turn, lead to extensive diversity in phenotype among GAS isolates that are otherwise highly similar in their core-genomes (Carroll et al., 2011).

Prior to 2004, the worldwide recovery of *emm59* GAS strains was extremely rare. However, by 2006 a hypervirulent *emm59* clone emerged in western Canada and rapidly became a major cause of invasive GAS disease (Tyrrell et al., 2010). Genome sequencing of >100 isolates provided a magnified view of the unfolding emergence and epidemic spread of *emm59* GAS (Fittipaldi et al., 2012a; Fittipaldi et al., 2012b). The epidemic isolates are virtually identical with an average of only 7.3 SNPs and 4.3 indels relative to the epidemic reference strain MGAS15249 (Table 2). Thus, the contemporary *emm59* isolates descended from a recent common ancestor that underwent rapid geographic spread. When compared to a historic isolate recovered in the 1970s (MGAS1882; Table 2), the new epidemic *emm59* clone differed by only 118 biallelic SNPs and 12 indels in the *emm59* core-genome. Both isolates have a dearth of phage genes, whereby the older clone has only one prophage, which is absent from the epidemic clone, and the new clone has only a prophage remnant whose genes are absent from the historic clone. While the precise molecular steps and natural selection processes that led to the emergence of the new epidemic clone remain unresolved (Bessen, 2012), the contemporary and historic strains display distinct phenotypes in experimental models (Fittipaldi et al., 2012a).

The most common *emm* type recovered in association with GAS invasive disease in established market economy countries throughout the world is *emm1*; this *emm* type also ranks very high in prevalence for cases of pharyngitis (Beall, 2014; Shulman et al., 2009; Steer et al., 2009b). Studies on *emm1* isolates that spread globally in the late 1980s and early 1990s had two prophage which were absent from *emm1* isolates recovered prior to that period (Cleary et al., 1998). The very first sequenced genome of GAS is the *emm1* strain

SF370 (Table 2) (Ferretti et al., 2001), however, SF370 is more representative of the historic *emm1* clones (Sumby et al., 2005). The evolution of *emm1* isolates has been intensively studied by genome sequencing and other methods (Aziz et al., 2005; Aziz and Kotb, 2008; Maamary et al., 2012; Nasser et al., 2014; Sumby et al., 2005).

In a recent report based on >3,500 genome sequences, a detailed model for the most likely evolutionary history of contemporary *emm1* isolates is put forth (Nasser et al., 2014). In this model, a phage encoding an extracellular DNase (*sdaD2/sda1* gene) was acquired first, followed by acquisition of a second phage encoding the SpeA1 superantigen, whose gene subsequently underwent a non-synonymous point mutation to give rise to SpeA2 in the early 1970s; that mutational change confers an increased affinity of SpeA2 for HLA-DQ class II antigens (Kline and Collins, 1996). The final major change was acquisition of a 36-kb region from a probable *emm12* donor strain in ~1983. The newly acquired 36-kb region encodes the secreted toxins NAD+-glycohydrolase (Spn/Nga) and streptolysin O; about half the polymorphisms in the 36-kb region are concentrated in a highly divergent 2.6-kb region between the *slo* and *metB* genes (Nasser et al., 2014). This narrow region roughly corresponds to AGR-3 (Bessen et al., 2011). In addition, the contemporary MGAS5005 strain lacks the G330D polymorphism in Spn/Nga and truncated *ifs* (endogenous inhibitor of Spn/Nga) that are accurate predictors of NADase inactivity (Riddle et al., 2010), whereas the historic SF370 isolate has both the G330D polymorphism and a truncated *ifs* pseudogene (data not shown); thus, a recent acquisition of NADase activity via the 36-kb region by contemporary *emm1* strains may have contributed to its evolutionary success and/or hypervirulence. It is proposed that the 36-kb region was probably mobilized by generalized transduction and integrated into the *emm1* genome following homologous recombination (Nasser et al., 2014; Sumby et al., 2005).

Comparison of the polymorphic sequences of genomes from invasive- versus pharyngitis-derived *emm1* isolates failed to yield a clear disease association, supporting the idea that invasive *emm1* organisms evolve repeatedly from the pool of pharyngitis strains (Nasser et al., 2014), similar to the conclusion reached for *emm3* organisms. However, as previously observed for *emm3* strains (Shea et al., 2011), the *emm1* pharyngitis isolates have many more indels and SNPs in the capsular genes *hasA* and *hasB* as compared to *emm1* invasive strains, indicative of strong diversifying selection in pharyngitis strains (Nasser et al., 2014). In addition, numerous mutations in the transcriptional regulatory genes *covRS* and *rgg/ropB* in *emm1* strains and those of other *emm* types facilitate the transition between localized and invasive GAS infection (Cole et al., 2011; Garcia et al., 2010; Ikebe et al., 2010; Masuno et al., 2014). Mutations in *covRS* and *rgg/ropB* lead to the down-regulation of transcription of the gene encoding the broad-spectrum cysteine protease SpeB that, in turn, prevents the degradation of extracellular proteins such as DNase. In the absence of SpeB, the Sda1/SdaD2 DNase encoded by the phage recently acquired by contemporary *emm1* strains is spared degradation, and is able to digest the DNA-rich neutrophil extracellular traps (NETs), facilitating the escape of trapped GAS and acting as a selective force that promotes the emergence and invasive spread of *covRS* mutants (Walker et al., 2007). A recent study that sequenced the genomes of GAS (*emm1*) recovered from several different body sites of a single patient throws into question the exact anatomical location where isolates with

inactivating mutations in *covRS* or *rgg/ropB* begin to emerge (Flores et al., 2014). However, while the association of SpeB absence with invasive disease is very highly significant, it is an imperfect correspondence.

In 2011 there was an alarming increase in the number of cases (>1,000) of scarlet fever in Hong Kong, and many patients were hospitalized. The outbreak was largely attributable to *emm12* GAS strains that were resistant to macrolides and tetracycline (Hsieh and Huang, 2011; Lau et al., 2012; Luk et al., 2012). A complete genome (HKU16; Table 2) and draft genome (HKU30; Table 3) of organisms recovered from scarlet fever patients (Tse et al., 2012) were compared to two previously sequenced *emm12* genomes of non-scarlet-fever isolates (Beres and Musser, 2007). The pathognomonic scarlatina rash is triggered by superantigen activity (i.e., "erythrogenic toxin") and indeed, HKU16 and HKU30 have a novel prophage (ΦHKU.vir) harboring the superantigen genes *speC* and *sse* (Tse et al., 2012). Both strains also carry two prophage common to MGAS9429 that have *speH* and *speI* superantigen genes on one prophage, and the DNase *sdaD2/sda1* gene on the other. However, the Hong Kong scarlet fever outbreak was multiclonal in origin. Strain HKU16 has a chromosomal inversion that is lacking in HKU30 (Table 2). HKU16 also harbors a 65-kb ICE (ICE-*emm12*) that contains *erm(B)* and *tet(M)* genes. HKU30 lacks ICE-*emm12*, but has related genes with an overall 67% similarity and positioned at a different chromosomal location; thus, the two genetic elements share a common but distant evolutionary ancestor. Despite the multi-clonal nature of the outbreak, most GAS recovered from scarlet fever patients had ΦHKU.vir and ICE-*emm12* (Tse et al., 2012).

Whole genome sequencing was recently used to investigate an outbreak of puerperal fever (Section 7) in four hospitals in New South Wales, Australia during 2010 (Table 3) (Ben Zakour et al., 2012). Among the 11 GAS isolates from 9 patients were six different *emm* subtypes, indicative of independent sources of infection for at least five patients. The five *emm28.8* isolates were recovered from four patients in three hospitals; among them were 72 SNPs, but all had the ICE/RD harboring the gene for the R28 protein that promotes vaginal colonization (Section 7). Data indicate that the two patients from hospital A were infected with identical clones, wherein the second infection was the likely result of patient-to-patient or staff-to-patient transfer. The genomic contents of *emm28.8* isolates from patients in hospitals B and C were distinct from each other and from the hospital A isolates, indicative of an outbreak of a polyclonal nature (Ben Zakour et al., 2012). Whole genome sequencing of GAS is gradually becoming part of the clinical microbiology lab repertoire.

10. The shaping forces of natural selection

Although they are strictly human pathogens with no known environmental reservoir, the biological behavior of GAS is quite varied. The most common habitat for GAS is the non-diseased URT, wherein the organism assumes the commensal-like state of asymptomatic carriage. Next most often encountered by GAS is the purulent exudate (i.e., pus) triggered by infection at a superficial epithelial surface of the oropharynx or skin. For effective transmission to new hosts, it may be necessary for GAS to not only survive in this environment, but also produce new progeny. Once the protective barriers of the host are fully breached, and GAS gains access to normally sterile tissue such as the bloodstream, the

local microenvironment shifts drastically once again. The more highly specialized disease conditions caused by GAS, such as scarlet fever and puerperal fever, may be due to disease-specialist clones that carry a unique array of virulence factors which are tailored to that specific disease. Even among invasive diseases, specialist clones appear to exist, such as those that cause toxic shock syndrome.

The greatest challenges to GAS survival are likely encountered within its human host. Among the most prominent threats to GAS survival and reproductive growth are antibiotics, lantibiotics produced by resident organisms of the local microbiome, and innate and acquired host defenses. The emerging picture for the strategy used by this species is one that includes an uncanny ability to acquire DNA from a multitude of diverse sources, while maintaining a core-genome that is very highly conserved. This operates in parallel with extensive changes in gene clusters that encode surface proteins (e.g., *emm*) and/or mutations in various regulatory genes, whereby genetic change is often subject to positive selection and results in immune escape and/or gain or loss of function. It is the highly diverse non-core genome that confers much of the complexity in biological behavior that characterizes GAS and defines it as a major problem pathogen.

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Abbreviations

AGR	accessory gene region
C4BP	C4b-binding protein
Cas	CRISPR-associated proteins
CC	clonal complex
CGH	comparative genomic hybridization
CRISPR	clustered, regularly interspaced short palindromic repeat
D	genetic diversity
DNase	deoxyribonuclease
emm	encodes M protein
FCT region	encodes pili and T-antigen
FnBP	fibronectin-binding protein
GAS	group A streptococci
GBS	group B streptococci
GCS	group C streptococci

GGS	group G streptococci
H	haplotype
HGT	horizontal gene transfer
ICE	integrative and conjugative element
LCB	locally collinear block
MGE	mobile genetic element
MLST	multilocus sequence typing
MMR	mismatch repair
MSCRAMMs	microbial surface components recognizing adhesive matrix molecules
R_o	basic reproductive rate
RD	region of difference
Sda	streptococcal DNase (also, Sdn and Spd)
SDD	*Streptococcus dysgalactiae* subspecies *dysgalactiae*
SDE	*Streptococcus dysgalactiae* subspecies *equisimilis*
Se	*Streptococcus equi* subspecies *equi*
SF	subfamily
SLV	single locus variant
SOF	serum opacity factor
Spe	streptococcal pyrogenic exotoxin
Spn/Nga	NAD+-glycohydrolase
SpyCI	streptococcal phage-like chromosomal island
sRNA	small regulatory RNA
ST	sequence type
Sz	*Streptococcus equi* subspecies *zooepidemicus*
URT	upper respiratory tract.

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Highlights

- The epidemiology of group A streptococci (GAS) is reviewed, highlighting *emm* genes
- The genetic organization and population biology of GAS is discussed
- The core and non-core genomes of 24 GAS isolates are evaluated, including prophage
- Genes acquired by GAS genomes from related species are summarized
- Genome changes underlying long- and short-term evolution of GAS are delineated

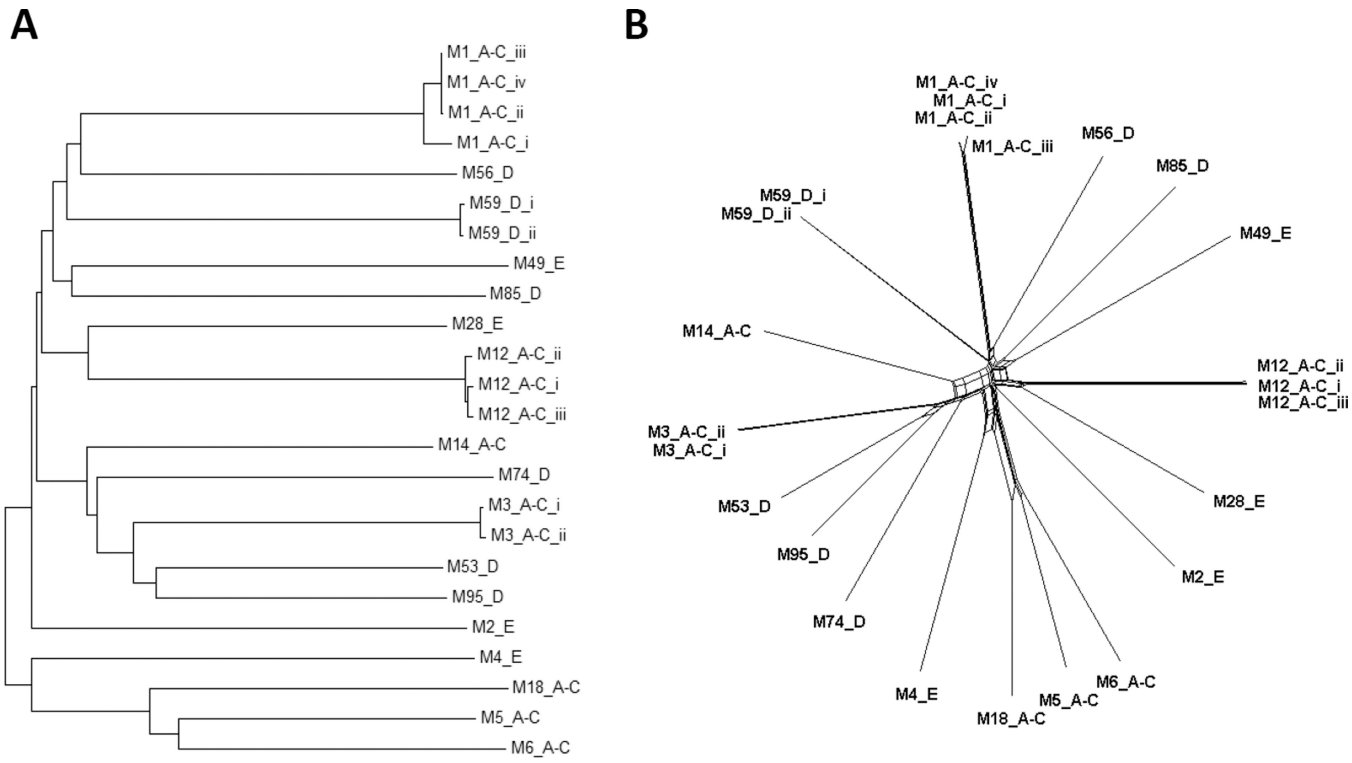


Figure 1. Phylogeny of the core-genome based on 24 GAS isolates

A core-genome based on the whole genome sequences of the 24 GAS isolates listed in Table 2 was defined; there are 1,510,818 positions (36,362 informative characters) in the dataset and no gaps. (A), Evolutionary history was inferred using the minimum evolution method with default parameters, conducted in MEGA6 (Tamura et al., 2013). (B), SplitsTree graph (SplitsTree v. 4.10) employs uncorrected P distance, the equal angle method for splits transformation (no weights), and the neighbor net network (Huson and Bryant, 2006); 72 splits are evident; the phi test finds statistically significant evidence for recombination.

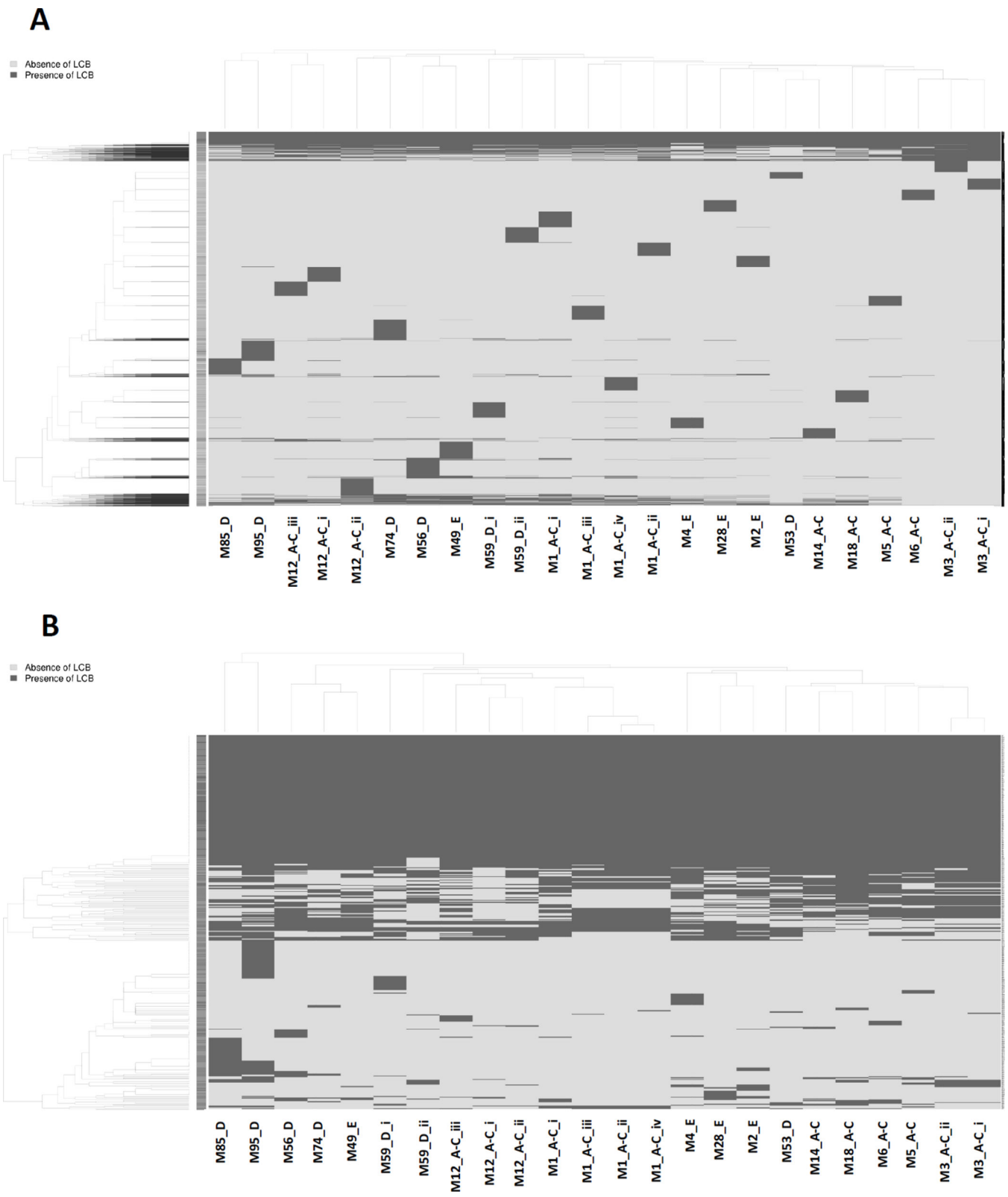


Figure 2. Distribution of whole genome alignment-derived locally collinear blocks (LCBs) based on 24 GAS isolates

The 24 GAS isolates listed in Table 2 were aligned with Mugsy using default parameters. Each row of the plot depicts an LCB from the alignment; all rows are the same height and are not drawn to scale based on LCB length. Columns represent the 24 GAS strains. A dark grey line indicates that an LCB is present in a strain; light grey lines represent LCB absence. Strains (top phylogeny, with taxon labels at the bottom) and LCBs (phylogeny on left side) are clustered using the hierarchical clustering method and the associated trees are shown. The unlabeled vertical bar towards the left end of the figure represents a log heat map of LCB lengths ranging from longest (dark grey) to shortest (light grey). (A), All LCBs. (B), LCBs <1,000 nt are excluded.

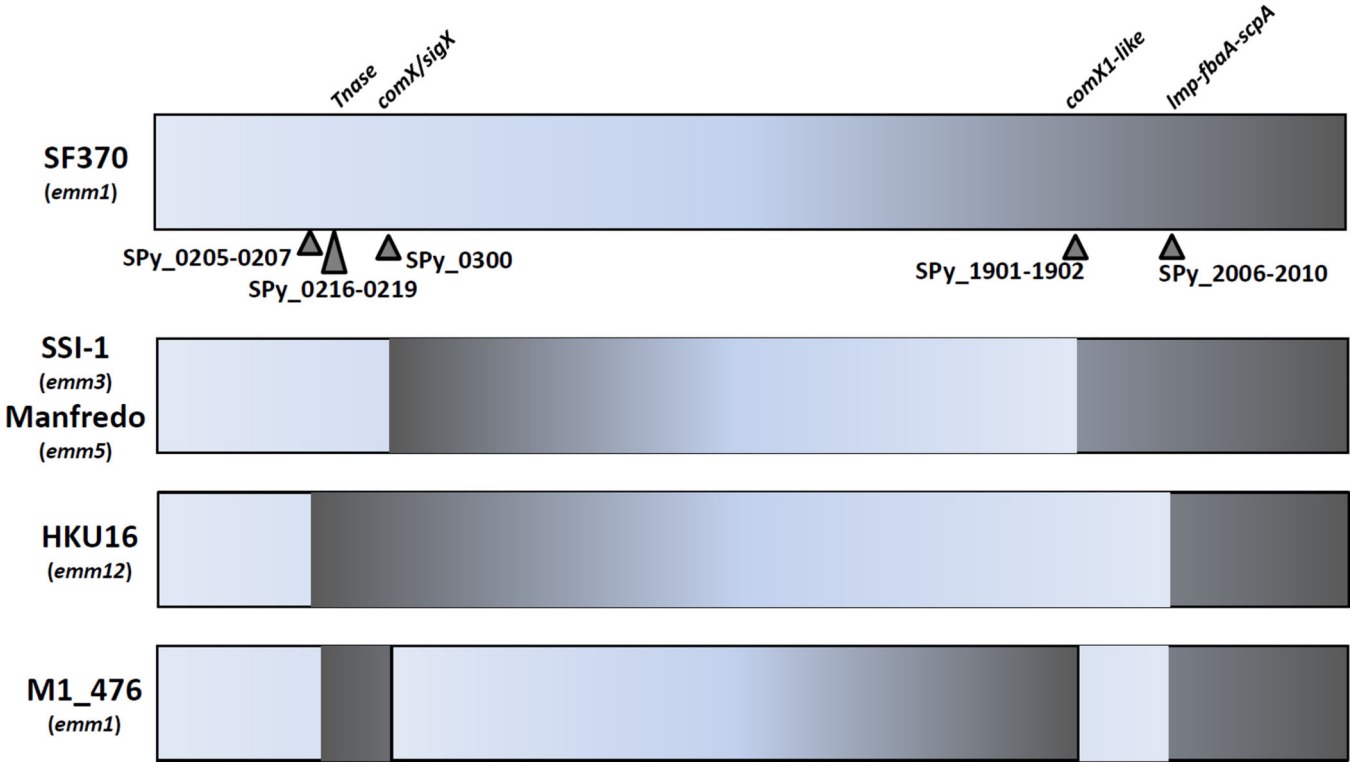
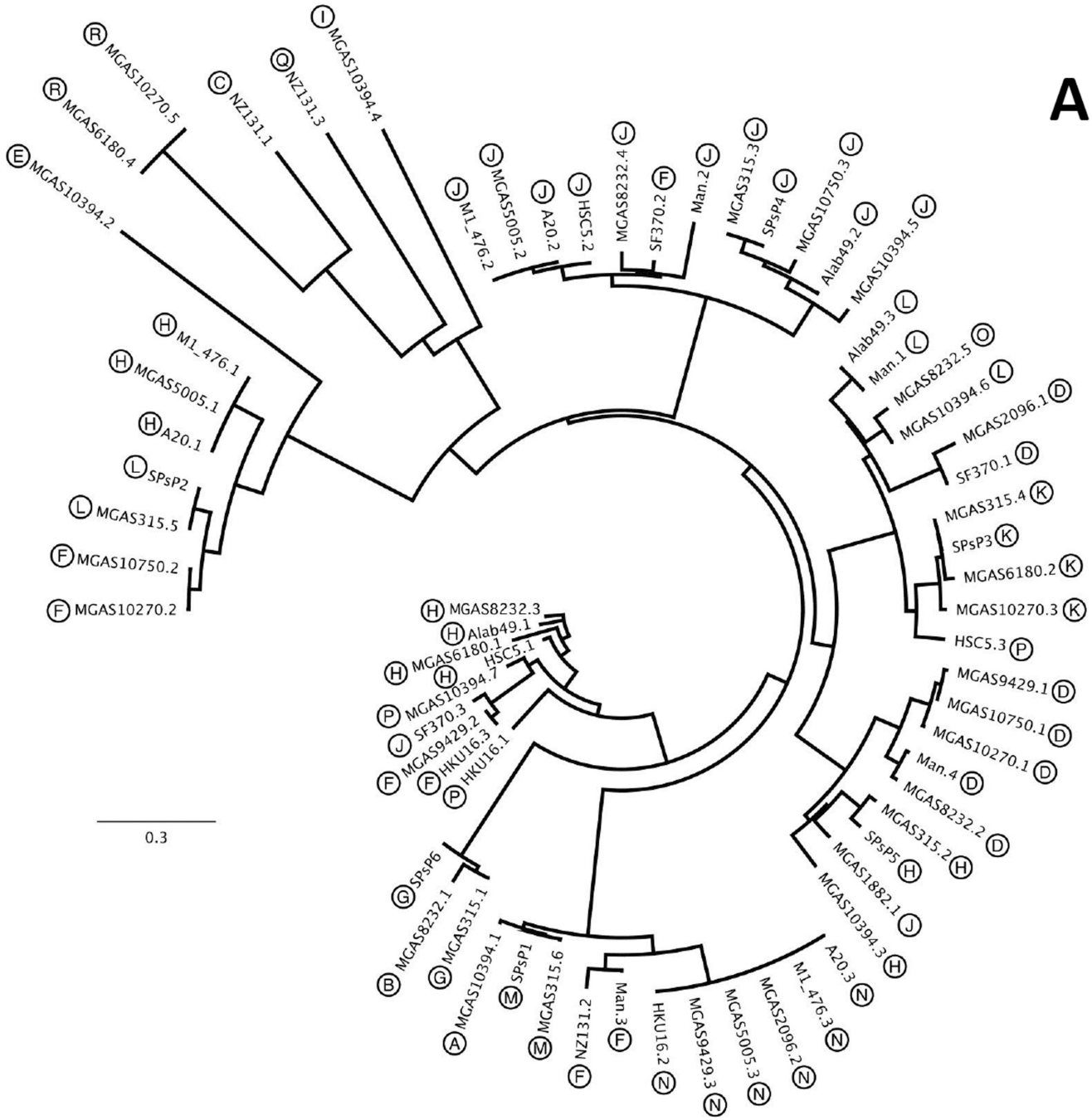


Figure 3. Chromosomal inversions

Chromosomal crossover points are depicted for GAS genomes that have undergone an inversion, relative to the SF370 reference strain; all ORF assignments are based on the SF370 genome (Ferretti et al., 2001).



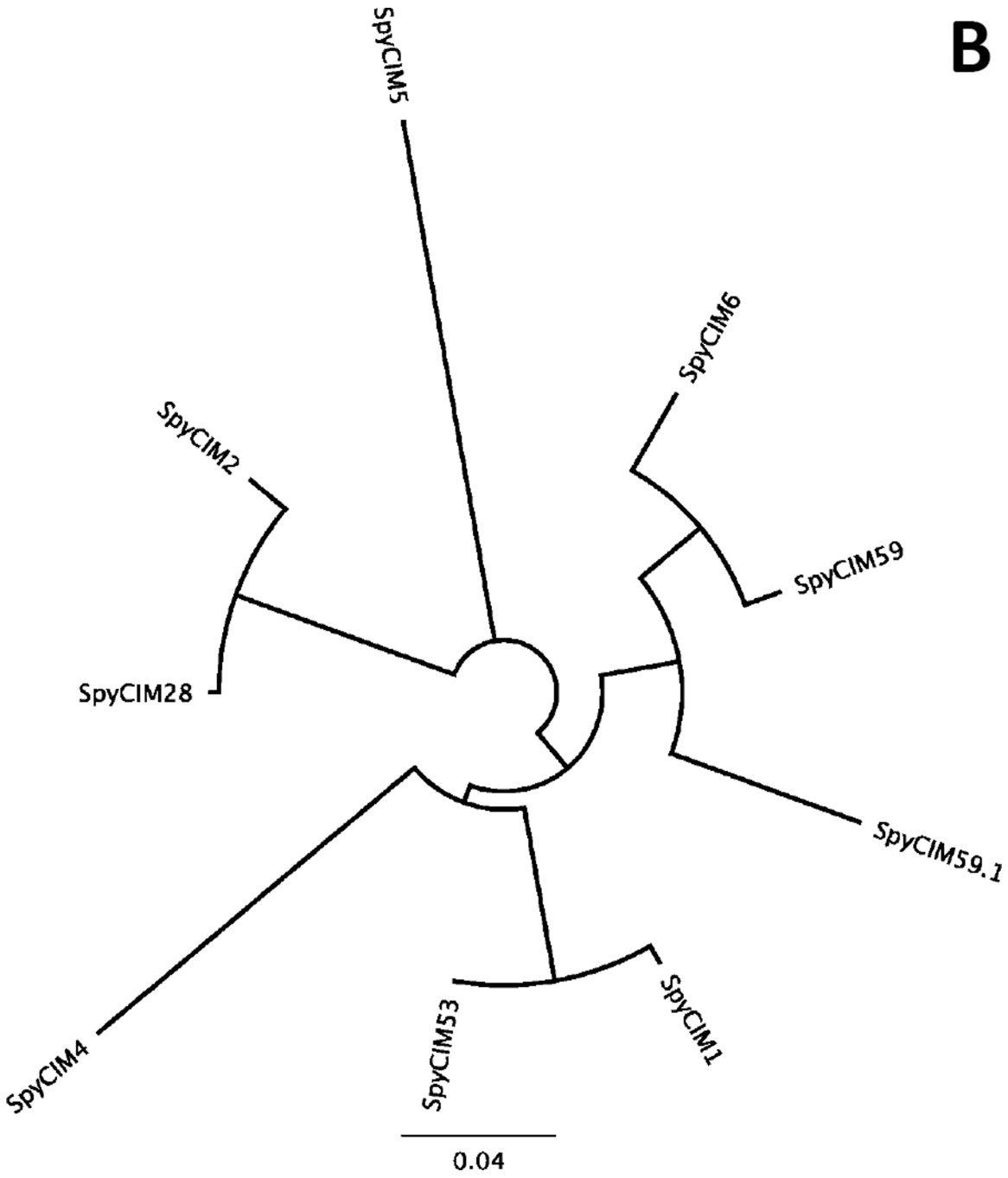


Figure 4. Prophage and SpyCI phylogenies

Phylogenetic trees of the endogenous lambdoid prophages (A), and SpyCIs (B), from the 20 complete and publicly available GAS whole genome sequences (Table 2) is presented. When possible, the ends of each prophage genome were identified by the flanking duplications generated by site-specific recombination. For this analysis, all ICEs (except 10394.4, which has prophage features) and other MGEs are omitted. The encircled letters next to each phage taxon label (A) match the target gene attachment site identifier listed in

Table 4. Trees were generated using the software package Geneious 6.1.7 (Biomatters Ltd.), employing the Tamura-Nei genetic distance model with neighbor joining and no outgroup.

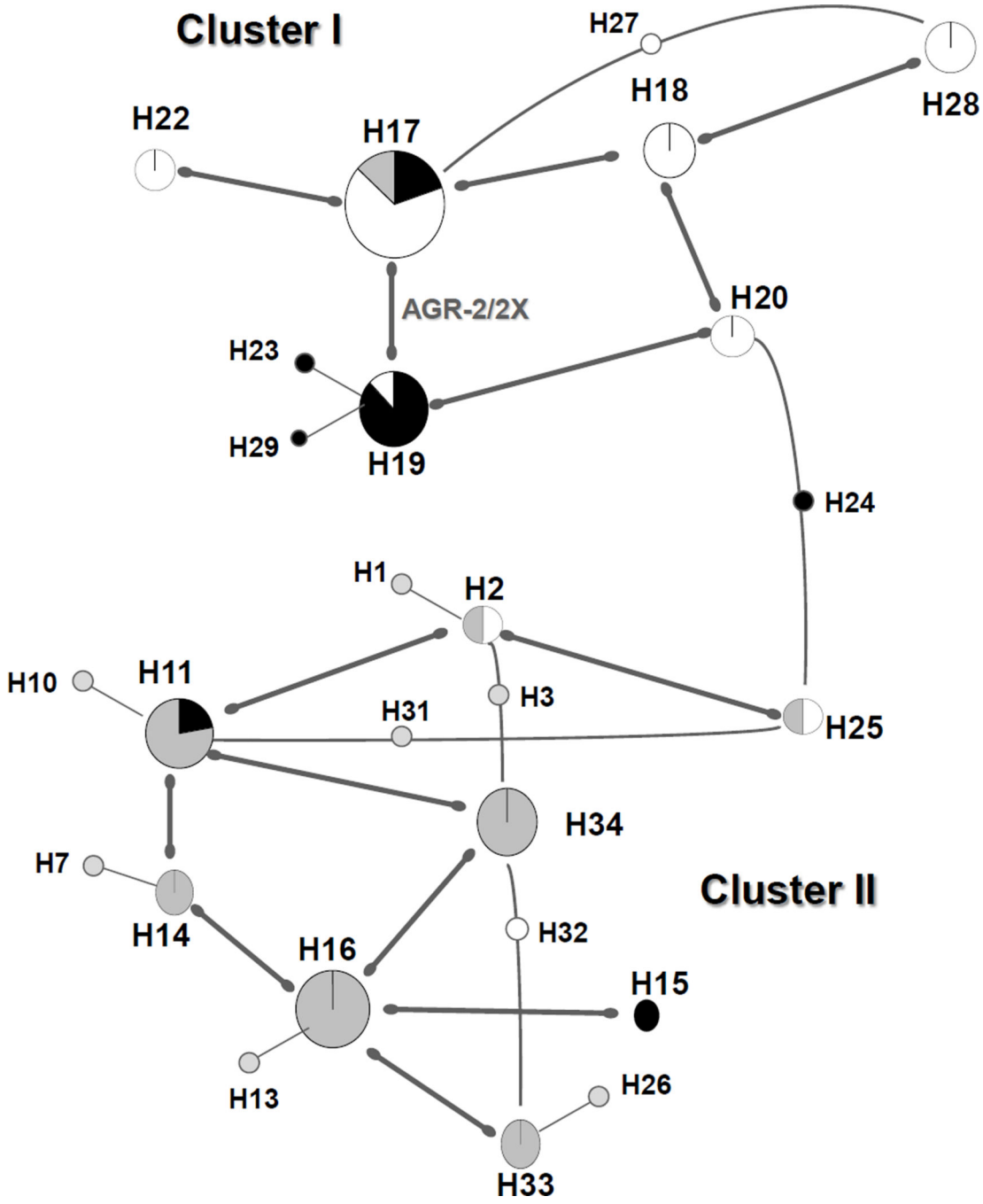


Figure 5. Model of evolution for GAS based on highly linked accessory gene region (AGR) genes

All possible pairwise comparisons between 393 differentially distributed GAS genes were made for 97 GAS strains of 95 *emm* types; eight AGRs having genes with the smallest *p*-values (Fisher's exact test), when paired with a gene from another AGR, were identified (AGRs 2X, 3, 4, 13, 14, 16B, 17A and 21X) (Bessen et al., 2011). The gene within each of the eight AGRs having the smallest *p*-value (i.e., strongest linkage disequilibrium) is used to define character states (0, absence; 1, presence) for their respective AGRs among the 97 GAS strains; 34 haplotypes (H) are defined according to the character states of these eight

AGR genes (Bessen et al., 2011). Single locus differences among haplotypes were established using the eBURST clustering algorithm (www.mlst.net) and redrawn; 26 of the 34 haplotypes differed from at least one other haplotype by only a single AGR gene, and they are depicted in the haplotype network shown; not shown are eight haplotypes that differ from the main network by ≥two loci. Circles denote each haplotype (H), whereby the size/area of the circle reflects the number of assigned GAS strains; *emm* pattern genotypes are represented by black (pattern A–C), white (pattern D) and gray (pattern E), and their fractional content is displayed. Lines connect all haplotype pairs that differ by a single locus; the set of thicker lines connect two core haplotypes, which are defined as being represented by >1% of the 97 GAS strains. H24 provides a link between the two major sub-clusters (I, upper; II, lower). Adapted from (Bessen et al., 2011).

Table 1

Inferred *emm* pattern group assignments based on *emm* type, for GAS recovered in 29 population- based surveys for pharyngitis or impetigo.

Study	Country	Years	Disease	No. of isolates *	No. pattern A–C	No. pattern D	No. pattern E	% pattern A–C	% pattern D	% pattern E	Simpson's *D* index #
(Mzoughi et al., 2004)	Tunisia	2003	pharyngitis	29	4	0	25	13.8	0.0	86.2	0.6995
(Tewodros and Kronvall, 2005)	Ethiopia	1990	pharyngitis	53	15	18	20	28.3	**34.0**	37.7	0.9625
(Majeed et al., 1992)	Kuwait	1980–9	pharyngitis	120	76	3	41	63.3	2.5	34.2	0.8924
(Dicuonzo et al., 2001)	Italy	2000	pharyngitis	112	57	1	54	50.9	0.9	48.2	0.8977
(Creti et al., 2004)	Italy	1996 – 2001	pharyngitis	80	42	0	38	52.5	0.0	47.5	0.8623
(Lorino et al., 2006)	Italy	2001–2	pharyngitis	123	56	0	67	45.5	0.0	54.5	0.8847
(Alberti et al., 2003)	Spain	1996–9	pharyngitis	520	165	5	350	31.7	1.0	67.3	0.8791
(Smeesters et al., 2006)	Belgium	2004	pharyngitis	163	79	0	84	48.5	0.0	51.5	0.8918
(Brandt et al., 2001)	Germany	1997	pharyngitis	216	110	0	106	50.9	0.0	49.1	0.8843
(Eisner et al., 2006)	Austria	1996 – 2003	pharyngitis	69	27	0	42	39.1	0.0	60.9	0.8819
(Dey et al., 2005)	India	2000–1	pharyngitis	51	2	20	29	3.9	**39.2**	56.9	0.9639
(Sagar et al., 2004)	India	2003	pharyngitis	29	3	8	18	10.3	**27.6**	62.1	0.9507
(Ma et al., 2002)	Japan	2000–1	pharyngitis	66	19	0	47	28.8	0.0	71.2	0.8308
(Wajima et al., 2008)	Japan	2003–6	pharyngitis	302	140	0	162	46.4	0.0	53.6	0.8330
(Rogers et al., 2007)	Australia	2001–2	pharyngitis	33	12	0	21	36.4	0.0	63.6	0.8693
(Dierksen et al., 2000)	New Zealand	1997	pharyngitis	145	29	2	114	20.0	1.4	78.6	0.7982
(Shulman et al., 2004) ^	USA	2000–2002	pharyngitis	1972	1045	6	921	53.0	0.3	46.7	0.8915
(Richter et al., 2005)	USA	2002–2003	pharyngitis	103	28	2	73	27.2	1.9	70.9	0.8736
(Haukness et al., 2002)	USA	1998	pharyngitis	61	36	0	25	59.0	0.0	41.0	0.9033
(Kiska et al., 1997)	USA	1993–4	pharyngitis	43	31	0	12	72.1	0.0	27.9	0.7342
(Mejia et al., 1997)	Mexico	1990	pharyngitis	51	40	1	10	78.4	2.0	19.6	0.8196
(Espinosa et al., 2003)	Mexico	1991–2000	pharyngitis	281	153	4	124	54.4	1.4	44.1	0.9076
(Smeesters et al., 2006)	Brazil	2004	pharyngitis	52	9	11	32	17.3	**21.2**	61.5	0.9495
Total	**All**		**pharyngitis**	**4674**	**2178**	**81**	**2415**	**46.6**	**1.7**	**51.7**	*0.8722*
(Tewodros and Kronvall, 2005)	Ethiopia	1990	impetigo	43	1	18	24	2.3	41.9	55.8	0.9779

Study	Country	Years	Disease	No. of isolates *	No. pattern A–C	No. pattern D	No. pattern E	% pattern A–C	% pattern D	% pattern E	Simpson's *D* index #
(Sakota et al., 2006)	Nepal	2000	impetigo	51	10	18	23	19.6	35.3	45.1	0.9443
(Bessen et al., 2000a) ^	Australia (tropical)	1994–6	impetigo	123	16	55	52	13	44.7	42.3	0.9584
(McDonald et al., 2007)	Australia (tropical)	2003–5	impetigo	126	15	67	44	11.9	53.2	34.9	0.9497
(Steer et al., 2009c) ^	Fiji	2006	impetigo	364	19	190	155	5.2	52.2	42.6	0.9570
(Smeesters et al., 2006)	Brazil	2004	impetigo	58	2	33	23	3.4	56.9	39.7	0.9685
Total	**All**		**impetigo**	**765**	**63**	**381**	**321**	**8.2**	**49.8**	**42.0**	*0.9593*

*Number of GAS isolates of known or inferred *emm* pattern. Among the 23 pharyngitis studies, 108 GAS isolates could not be assigned an *emm* pattern; among the 6 impetigo studies, 3 isolates could not be assigned an *emm* pattern (overall, 98.0% of isolates were assigned to an *emm* pattern group). Of the 111 isolates with unassigned *emm* pattern groups, 92 (*emm170, emm240*) were restricted to a single study (Alberti et al., 2003).

^All studies are derived from the original report by (Steer et al., 2009b), plus 3 additional studies (^). All studies meet the following criteria: (i), isolates are clearly defined as recovery from cases of pharyngitis (or tonsillitis) or impetigo; (ii), at least 25 isolates could be assigned to an *emm* pattern group based on *emm* type; and (iii), findings were published, as described (Bessen et al., 2011). Table 1 is an updated version of data reported in (Bessen et al., 2011), and incorporates recent changes to *emm* type assignments (Beall, 2014) and additional *emm* pattern determinations (McMillan et al., 2013).

#Simpson's diversity index (*D*) and standard deviation (Grundmann et al., 2001) is based on *emm* types that could be assigned to an *emm* pattern group. For (Alberti et al., 2003), inclusion of the unassigned *emm170* and *emm240* isolates increases the *D* value slightly to 0.8972.

Table 2

Assembled and annotated genomes of GAS

Strain	Taxon label[%]	*emm* subtype	*emm* pattern	*emm* clade: cluster *	*sof* gene	FCT region	ST	No. of prophages #	No. of SpyCIs @	CRISPR-1 locus	CRISPR-2 locus	No. of ICEs ^	Disease +	Origin	Genome size (bp)	Chromosomal inversion	Accession no.	Reference
SF370	M1_A-C_i	1.6	A-C	Y: A-C3	no	2	28	3	1	+	+	1	wound	USA	1852441	no	NC_002737	(Ferretti et al., 2001)
MGAS5005	M1_A-C_ii	1.0	A-C	Y: A-C3	no	2	28	3	0	+	+	1	invasive (CSF)	Canada	1838554	no	NC_007297	(Sumby et al., 2005)
M1_476	M1_A-C_iii	1.0	A-C	Y: A-C3	no	2	28	3	0	+	+	1	STSS	Japan	1831128	remnant	NC_020540.2	(Miyoshi-Akiyama et al., 2012)
A20	M1_A-C_iv	1.0	A-C	Y: A-C3	no	2	28	3	0	+	+	1	necrotizing fasciitis	Taiwan	1837281	no	NC_018936.1	(Zheng et al., 2013)
MGAS10270	M2_E	2.0	E	X: E4	yes	6	55	4	1	+	+	2	pharyngitis	USA	1928252	no	NC_008022	(Beres and Musser, 2007)
MGAS315	M3_A-C_i	3.1	A-C	Y: A-C5	no	3	15	6	0	+	−	0	STSS	USA	1900521	no	NC_004070	(Beres et al., 2002)
SSI-1	M3_A-C_ii	3.1	A-C	Y: A-C5	no	3	15	6	0	+	−	0	STSS	Japan	1894275	yes	NC_004606	(Nakagawa et al., 2003)
MGAS10750	M4_E	4.0	E	X: E1	yes	5	39	3	1	+	+	2	pharyngitis	USA	1937111	no	NC_008024	(Beres and Musser, 2007)
Manfredo	M5_A-C	5.0	A-C	Y: single	no	3	99	4	1	−	−	0	ARF	USA	1841271	yes	NC_009332	(Holden et al., 2007)
MGAS10394	M6_A-C	6.4	A-C	Y: single	no	1	382	6	1	−	−	1	pharyngitis	USA	1899877	no	NC_006086	(Banks et al., 2003b)
MGAS2096	M12_A-C_i	12.0	A-C	Y: A-C4	partial	4	36	3	0	+	+	2	AGN	Trinidad	1860355	no	NC_008023	(Beres and Musser, 2007)
MGAS9429	M12_A-C_ii	12.0	A-C	Y: A-C4	partial	4	36	3	0	+	+	1	pharyngitis	USA	1836467	no	NC_008021	(Beres and Musser, 2007)
HKU16	M12_A-C_iii	12.0	A-C	Y: A-C4	partial	4	36	3	0	+	−	0	scarlet fever	Hong Kong	1908100	yes	AFRY0000001	(Tse et al., 2012)
HSC5	M14_A-C	14.3	A-C	Y: single	no	4	680	3	0	−	−	0	not known	ND	1818351	no	NC_021807.1	(Port et al., 2013)
MGAS8232	M18_A-C	18.19	A-C	Y: single	no	3	42	5	0	−	−	0	ARF	USA	1895017	no	NC_003485	(Smoot et al., 2002)
MGAS6180	M28_E	28.4	E	X: E4	yes	4	52	3	1	+	+	3	puerperal sepsis	USA	1897573	no	NC_007296	(Green et al., 2005)
NZ131	M49_E	49.1	E	X: E3	yes	3	30	3	0	+	+	0	AGN	New Zealand	1815785	no	NC_011375	(McShan et al., 2008)
Alab49	M53_D	53.0	D	Y: D4	no	3	11	3	1	−	−	1	impetigo	USA	1827308	no	NC_017596	(Bessen et al., 2011)
AA472	M56_D	56.0	D	Y: D4	no	3	115	ND	ND	−	−	ND	impetigo	Australia	1733605[$]	no	JRLK00000000	This report (gaps remain)

Strain	Taxon label[%]	*emm* subtype	*emm* pattern	*emm* clade: cluster *	*sof* gene	FCT region	ST	No. of prophages #	No. of SpyCIs @	CRISPR-1 locus	CRISPR-2 locus	No. of ICEs ^	Disease +	Origin	Genome size (bp)	Chromosomal inversion	Accession no.	Reference
MGAS15252	M59_D_i	59.0	D	X: E6	yes	4	172	0	1	+	−	0	SSTI	Canada	1750832	no	NC_017040	(Fittipaldi et al., 2012a)
MGAS1882	M59_D_ii	59.0	D	X: E6	yes	4	172	1	1	+	−	0	AGN, pyoderma	USA	1781029	no	NC_017053	(Fittipaldi et al., 2012a)
AA216	M74_D	74.0	D	Y: single	no	3	120	ND	ND	−	−	ND	impetigo	Australia	1839747[$]	no	JRLJ00000000	This report (gaps remain)
SS1447	M85_D	85.0	D	X: E6	partial	4	109	ND	ND	+	−	ND	ARF	UK	1915158[$]	no	JRLL00000000	This report (gaps remain)
MGAS2111	M95_D	95.0	D	Y: single	partial	1	14	ND	ND	+	−	ND	not known	USA	2019649[$]	no	JRLI00000000	This report (gaps remain)

[%] Taxa labels are used in Figures 1 and 2

[*] *emm* clade and *emm* cluster assignments are predictions based on *emm* type (Sanderson-Smith et al., 2014).

[#] Number of complete lambdoid prophages; please note that "Φ10394.4" is considered an ICE in Table 2, although it does have prophage-like features.

[@] Number of streptococcal phage-like chromosomal islands (SpyCI)

[^] Number of integrative and conjugative elements (ICE)

[+] AGN, acute glomerulonephritis; ARF, acute rheumatic fever; CSF, cerebrospinal fluid; SSTI, skin or soft tissue infection; STSS, streptococcal toxic shock syndrome

[$] Draft genome sizes are calculated as the sum of the length of all contigs ≥500 nt. As such, values can be inaccurate due to the presence of gaps as well as potentially redundant contigs that were not merged due to poor sequence quality, especially at contig edges where sequence read coverage is lower.

ND, not determined

Table 3

Additional draft genomes of GAS that have been published.

Strain	*emm* type or subtype	(predicted) *emm* pattern	Disease/tissue ^	Origin	Accession no. (series)	Reference
M49 591	49	E	skin infection	Germany	AAFV00000000	(Beyer-Sehlmeyer et al., 2005)
HKU30	12	A–C	scarlet fever	Hong Kong	ERS046934	(Tse et al., 2012)
BJCYGAS15	12	A–C	scarlet fever	Beijing	ALKD00000000	(You et al., 2012)
HLJGAS12011	12	A–C	scarlet fever	Heilongjiang (China)	ALKE00000000	(You et al., 2012)
PS001	28.8	E	puerperal sepsis	New South Wales	ERS123195	(Ben Zakour et al., 2012)
PS002	1.40	A–C	puerperal sepsis	New South Wales		(Ben Zakour et al., 2012)
PS003	77.0	E	puerperal sepsis	New South Wales		(Ben Zakour et al., 2012)
PS004	77.0	E	puerperal sepsis	New South Wales		(Ben Zakour et al., 2012)
PS005	28.8	E	puerperal sepsis	New South Wales	ERS123196	(Ben Zakour et al., 2012)
PS006	28.8	E	puerperal sepsis	New South Wales	ERS123197	(Ben Zakour et al., 2012)
PS007	28.8	E	puerperal sepsis	New South Wales	ERS123198	(Ben Zakour et al., 2012)
PS008	28.8	E	puerperal sepsis	New South Wales	ERS123199	(Ben Zakour et al., 2012)
PS009	89.0	E	puerperal sepsis	New South Wales		(Ben Zakour et al., 2012)
PS010	75.0	E	puerperal sepsis	New South Wales		(Ben Zakour et al., 2012)
PS011	89.9	E	puerperal sepsis	New South Wales		(Ben Zakour et al., 2012)
06BA18369	41.2	D	SSTI	Northern Canada	APMZ00000000	(McDonald et al., 2013)
SP1	12	A–C	pharyngitis	Beirut	AYPA00000000	(Tokajian et al., 2014)
SP2	108	D	skin	Beirut	AWOZ00000000	(Tokajian et al., 2014)
SP3	89	E	pharyngitis	Beirut	AWPA00000000	(Tokajian et al., 2014)
SP4	28	E	pharyngitis	Beirut	AWPB00000000	(Tokajian et al., 2014)
SP5	1	A–C	skin	Beirut	AWPC00000000	(Tokajian et al., 2014)
SP6	89	E	pharyngitis	Beirut	AWPD00000000	(Tokajian et al., 2014)
SP7	22	E	pharyngitis	Beirut	AWPE00000000	(Tokajian et al., 2014)
SP8	85	D	pharyngitis	Beirut	AWPF00000000	(Tokajian et al., 2014)
SP10	118	E	pharyngitis	Beirut	AWPG00000000	(Tokajian et al., 2014)

Strain	*emm* type or subtype	(predicted) *emm* pattern	Disease/tissue ^	Origin	Accession no. (series)	Reference
ATCC 10728 *	24.0	A–C	ARF	New York	AEEO00000000	Muzny et al., unpublished
NS88.2	98.1	D	bacteremia	Northern Territory	PRJEA84331	Muzny et al., unpublished

^ARF, acute rheumatic fever; SSTI, skin or soft tissue infection

*Also known as the C98 typing strain, derived from 22RS72

Table 4

Prophages of GAS[&] and their integration sites and associated virulence genes

Target gene	Locus code	Integration side	Associated phage[#]	*emm* type of host cell	Associated virulence genes[^]
ssDNA binding protein *recO*	A	3’	10394.1	6	*sdn*
RNA helicase *snf*	B	3’	8232.1	18	*speA1*
Promoter of hypothetical Spy49_0371	C	5’	NZ131.1	49	none identified
Dipeptidase	D	5’	SF370.1	1	*speC-spd1*
			Man.4	5	*speC-spd1*
			2096.1	12	*speC-spd1*
			9429.1	12	*speC-spd1*
			8232.2	18	*speC-spd1*
			10270.1	2	*speC-spd1*
			10750.1	4	*speC-spd1*
$tRNA_{arg}$	E	3’	10394.2	6	*speA4*
dTDP-glucose-4,6-dehydratase	F	3’	SF370.2	1	*speH-spel*
			Man.3	5	*speH-spel*
			9429.2	12	*speH-spel*
			HKU16.3	12	*speH-spel*
			10270.2	2	*spd3*
			10750.2	4	*spd3*
			NZ131.2	49	*speH*
CRISPR type II system direct repeat sequence	G	5’	315.1	3	none identified
			SPsP6[*]	3	none identified
tmRNA	H	3’	5005.1	1	*speA2*
			A20.1	1	*speA2*
			M1_476.1	1	*speA2*

Target gene	Locus code	Integration side	Associated phage[#]	*emm* type of host cell	Associated virulence genes[^]
			Alab49.3	53	*spd3*
putative gamma-glutamyl kinase	M	5’	315.6	3	*sdn*
			SPsP1	3	*sdn*
$tRNA_{ser}$	N	3’	5005.3	1	*sda*
			A20.3	1	*sdaD2*
			M1 476.3	1	*sdaD2*
			HKU16.2	12	*sdaD2*
			2096.2	12	*sda*
			9429.3	12	*sda*
HAD-like hydrolase	O	5’	8232.5	18	*sda*
Excinuclease subunit *uvrA*	P	5’	10394.7	6	*spd3*
			HKU16.1	12	*speC*
			HSC5.3	14	*spd3*
Conserved hypothetical protein Spy49_1532	Q	5’	NZ131.3	49	*spd3*
SSU ribosomal protein S4P	R	3’	10270.5	2	none identified
			6180.4	28	none identified

[&] Analysis includes the 20 completed and assembled GAS genomes listed in Table 2

[*] SPsP1 through SPsP6 are the prophages in strain SSI-1

[^] Gene abbreviations: *speA, speC, speH, speI, speK, speL, speM* and *ssa* and their alleles encode superantigens; *sda, spn* and *spd* alleles encode DNases; *sla* alleles encode phospholipase; *mefA* encodes a macrolide efflux pump.

[#] The phage numbering scheme for many strains follows the assignments as defined in (Beres and Musser, 2007). Skips in numerical sequence reflect those elements that are presently defined as SpyCIs.

Table 5

Biologically important GAS genes having a strong correlation with *emm* pattern group

Gene	AGR	Function [#]	Lineages [^]	% pattern A–C	% pattern D	% pattern E	Significant differences ($p < 0.05$) [*]	No. *emm* types in study	Reference
rofA	2/2X	transcriptional regulator	*rofA;* ***nra***	15	84	7	D versus A–C or E	112	(Bessen et al., 2005; Kratovac et al., 2007)
msmR	2/2X	transcriptional regulator	N/A	50	91	68	A–C versus D	95	(Bessen et al., 2011)
prtF1	2/2X	FnBP	N/A	75	21	93	D versus A–C or E	112	(Kratovac et al., 2007)
prtF2	2/2X	FnBP	N/A	50	92	69	A–C versus D	112	(Kratovac et al., 2007)
cpa	2/2X	collagen-binding protein	N/A	25	92	55	A–C versus D or E	112	(Kratovac et al., 2007)
spn/nga	3	NAD^+ glycohydrolase	**active**; inactive	10	14	94	E versus A–C or D	110	(Riddle et al., 2010)
ralp3	9	transcriptional regulator	N/A	15	6	34	D versus E	95	(Bessen et al., 2011)
srtT	13	lantibiotic (streptin) biosynthesis (ICE/RD)	N/A	45	24	82	E versus A–C or D	95	(Bessen et al., 2011)
grab	16A/B	α2-macroglobulin binding protein	***grab***; AGR-16B	85	94	55	D versus E	95	(Bessen et al., 2011)
sse	18A/B	esterase	***sse-A****; sse-B*	95	97	66	D versus E	95	(Bessen et al., 2011)
mga	21/21X	transcriptional regulator	***mga-1****; mga-2*	95	0	0	A–C versus D or E	112	(Bessen et al., 2005; Kratovac et al., 2007)
sof	21/21X	serum opacity factor; FnBP	N/A	5	11	100	E versus A–C or D	112	(Kratovac et al., 2007)
sfbX	21/21X	FnBP	N/A	10	12	93	E versus A–C or D	95	(Bessen et al., 2011)
fbaA	21/21X	FnBP; antiphagocytic	N/A	25	100	77	D versus A–C or E	95	(Bessen et al., 2011)
ska	21/21X	streptokinase; plasminogen activator	*ska-1; ska-2a;* ***ska-2b***	0	67	4	D versus A–C or E	67	(Kalia and Bessen, 2004)

[#] FnBP, fibronectin-binding protein

[^] The lineage highlighted in bold corresponds to data for % distribution among *emm* pattern A–C, D and E groupings

[*] Fisher exact test (2-tailed) with Benjamini-Hochberg corrected *p*-values

N/A, not applicable