Molecular Evolution of Clinical Pathogenic Streptococci

Dongdong Li[1] and Chuanmin Tao[1]*

[1]Division of Clinical microbiology, Department of Laboratory Medicine, West China Hospital, Sichuan University, Chengdu, China.
*Corresponding Author: taocm@scu.edu.cn

Abstract

The genus *Streptococcus* comprises a wide variety of pathogenic and commensal gram-positive bacteria, many of which the pathogenic species cause severe, invasive infections that account for a high burden of morbidity and mortality. Here, we reviewed the evolution of representative virulence factors, capsule in *Streptococcus pneumoniae*, M protein in *Streptococcus pyogenes* (GAS), biofilm in *Streptococcus agalactiae* (GBS) and some oral *Streptococcus*, as well as the effect caused by evolution, antibiotic resistance and vaccine escape. Thanks to the rapid development of whole genome sequence (WGS) data, the impact of genetic recombination to the *Streptococcus* evolution has been proved. As to adaptive evolution caused by antibiotics, vaccine and so on, continuous surveillance is an essential to monitor evolution of *Streptococcus* causing disease. This knowledge is invaluable to the development of preventative and control strategies against this important pathogen.

Introduction

The genus *Streptococcus* comprises a wide variety of pathogenic and commensal gram-positive bacteria, many of which the pathogenic species cause severe, invasive infections that account for a high burden of morbidity and mortality. Among them, *S. pyogenes* (commonly as an alternative to group A streptococci, GAS), *S. agalactiae* (group B streptococci, GBS) and *S. pneumoniae* are the three most important streptococcal human pathogens, opportunistic pathogens which colonizes the skin, digestive and genitourinary tract (Donati et al., 2015; Edwards, 2010). Apart from these bacteria, the streptococci also include the viridans group streptococci (VGS = anginosus, mitis, mutans and salivarius groups), *Streptococcus suis* (Kohler, 2007; Olson et al., 2013) and so on. These streptococci exist oral unique environment, forming a characteristic microbiome. The streptococcal commensal flora intimately interacts and communicates with other bacterial species and the human host to support colonization. Being generally commensal colonizers of humans and other animals, streptococci express a wealth of colonization factors that allow them to adhere to epithelium, including capsule, pili and matrix-interacting proteins, and of virulence-related proteins (Nobbs et al., 2015). Streptococcal virulence factors usually participate in multiple events such as colonization, internalization, invasion of the host tissues and dissemination, systemic toxicity, and, evasion of the host defense (Sitkiewicz, 2017). The understanding of recent advances in descriptions of molecular mechanisms of streptococcal virulence, coupled with massive sequencing efforts to isolate genomes, have allowed the field to better understand the molecular and evolutionary changes leading to pandemic strains (Brown et al., 2012; Wilkening and Federle, 2017).

Clinical interventions-vaccine and/or antibiotics generated a selection pressure that drove the evolution of vaccine-escape mutants and strains that were highly resistant against antibiotics (Straume et al., 2015), as a result of gene transfer intra- and inter-species gene transfer involving *Streptococcus* (Donati et al.,

2015). For *Streptococcus* to survive, rapid adaptation to clinical interventions and its human immune responses is essential (Croucher et al., 2011). The plasticity of bacterial genomes ensures rapid adaptation to changing environmental conditions and enables bacteria to colonize new niches successfully. Molecular mechanisms contributing to genome plasticity include point mutations, genome rearrangements, mobile genetic elements, homologous or non-homologous recombination (Darmon and Leach, 2014). The rapid influx of whole genome sequence (WGS) data and the advent of novel analysis methods and powerful computational tools for population genetics and evolution studies has transformed our understanding of how genetic recombination drives streptococci adaptation and evolution. As we all know, evolutionary studies have highlighted the mechanisms that arises phenotypes changements. In this paper we present an overview of the latest results on the population structure, genomics and mechanisms of genome evolution of a selection of species from the genus *Streptococcus*. The species discussed include three major pathogens, *S. pneumonia*, *S. agalactiae* and *S. pyogenes.* Oral *Streptococcus* is also considered.

Genome of *Streptococcus* and evolution

Bacteria are haploid in naturally, possessing a single copy of most genes. The *Streptococcus* genomes can be best described as consisting of core and accessory genomes. The core genome in pneumococcus represents those genes that are common to all human pneumococcal isolates. These include key colonization (adhesions, matrix-interacting proteins and pili) and virulence genes (M protein, proteases and so on) as well as genes that enable adaptation and regulation of metabolism in response to environment alterations, and anaerobic conditions (Nobbs et al., 2015). The core genome size is dependent on the number of isolates used during the analysis, and this gradually decreases with the addition of new genomes until a plateau is reached (Tettelin et al., 2005). The accessory genome represents the set of genes specific to a isolate, which

are not shared in their entirety across the whole pneumococcal population. In other words, the accessory genome does not contain genes essential to cell survival however it plays an important role in bacterial pathogen evolution (Jackson et al., 2011). For example, antibiotic resistance is largely due to the acquisition of mobile genetic elements that harbor antibiotic resistance determinants (Jackson et al., 2011). From the complete genomes, the basic information of GAS genome is as follows: the genome is composed of a single chromosome that is approximately 1.8-Mbp long with a GC content of 38.5 ± 0.1% on average, and includes 1,826 ± 92 protein-coding regions, 5 or 6 rRNA operons, and from 57 to 67 tRNA encoding genes (Maruyama et al., 2016). Pneumococci possess a 2.1 megabases (Mb) pair circular genome that consists of over 2000 predicted protein coding regions and approximately 5% insertion elements. On average the core genome of *S. pneumoniae* consists of 1647 predicted coding sequences. Variation in the pneumococcal core genome is predominantly introduced by random mutations and homologous recombination that involves both short and long stretches of DNA, whilst recombination involving unrelated loci is more restricted to the accessory genome. The accessory genome does not contain genes essential to cell survival however it plays an important role in bacterial pathogen evolution. This is largely due to the acquisition of mobile genetic elements that harbor antibiotic resistance determinants and virulence factors. Comparative genome analysis among organisms of the same species not only shows a high degree of similarity in gene content and organization, but also a high degree of sequence heterogeneity as evidenced by the large number of single nucleotide polymorphisms present. Comparative genome analysis between the species showed that *S. pyogenes* was more closely related to *S. agalactiae* than with *S. pneumoniae* or *Streptococcus mutans* (Ferretti et al., 2004).

Molecular Mechanism of Streptococcus Evolution

Molecular mechanisms contributing to genome plasticity include point mutations,

genome rearrangements, mobile genetic elements and horizontal gene transfer (Darmon and Leach, 2014). Genetic recombination is the exchange of genetic material between different organisms which leads to production of offspring with combinations of traits that differ from those found in either parent recombination occurs between similar molecules of DNA. Horizontal gene transfer (HGT) is the sharing of genetic material among organisms other than by the DNA transmission from parent to offspring, which enable organisms to acquire new genes and phenotypes. HGT, a fundamental process in the genome evolution of bacteria (Gogarten and Townsend, 2005), can accelerate genome innovation and evolution (Jain et al., 2003) and spreads genetic diversity by moving genes across species boundaries. Genes are preferentially exchanged among organisms sharing similar genome size, genome G+C composition, carbon utilization, and oxygen tolerance. It enables bacteria to evolve rapidly through the acquisition of novel genetic determinants, or genetic determinants that are homologous to existing DNA, which were not previously resident within the recipient's genome (Nobbs et al., 2015). In bacteria, uptake of exogenous DNA is achieved via three main processes: transformation, conjugation and transduction. Of the three known HGT mechanism operating in bacteria, natural genetic transformation has been studied in most detail in *S. pneumonia*. Bacterial conjugation occurs when there is direct cell-to-cell contact of the bacteria exchanging DNA via a sex-pili that protrudes from one cell into the other. During transduction, DNA is transferred from one bacterium to another by viruses that infect bacteria called bacteriophages. Bacterial transformation involves the acquisition of exogenous DNA from the bacterial surroundings followed by the integration of the acquired DNA into the host cell genome.

Recombination events vary in size, which identified in the pneumococci range from very small fragments to thousands of base pairs (bp) (Croucher et al., 2013; Mostowy et al., 2014; Wyres et al., 2013). Two classes of recombination have been proposed based on the sizes of the recombination events: 1)

micro-recombination which involve single and short stretches of DNA occur more frequently. 2) macro-recombination, which are usually rare and consists of multi-fragment replacements of DNA (Andam and Hanage, 2015; Mostowy et al., 2014). Mostowy et al proposed several mathematical models to describe the rate and size of recombination in the evolutionary history of two very distinct pneumococcal lineages and concluded that macro-recombination is comparatively rarely observed in nature (Mostowy et al., 2014). But Cowley et al proved that macro-recombination is likely is more likely to occur in environments that permit cell-to-cell contact (Cowley et al., 2018). Macro-recombination was associated with major phenotypic changes, including serotype-switching events, and thus was a major driver of the diversification of the pathogen (Mostowy et al., 2014). A single nucleotide change in a Streptococcal housekeeping gene is at least 50-fold more likely to change as a result of recombination than as a result of mutation (Feil et al., 2000; Feil et al., 1999). Thanks to the whole genome sequencing of a large panel of isolates and comparative genomics, it has recently become clear that HGT both within and between species is the major driver of genome evolution of these organisms, allowing them to quickly evolve their genomic content in response to environmental stimuli and share a large number of genes important for virulence (Chewapreecha et al., 2014). For example, large imports of genes from *Streptococcus mitis* and other *anginosus*, and s*alivarius* group streptococci to *S. pneumoniae* have been reported to contribute to the evolution and structural diversity of the pneumococcal capsule (Kilian et al., 2014). Phylogenomics of GAS and closely related species revealed that interspecific HGT events among species of the pyogenic division are well-documented. Its closest genetic relatives are *Streptococcus canis* and *Streptococcus dysgalactiae* subspecies *equisimilis*. Richards et al (Richards et al., 2011) provided strong evidence for two cases of interspecies HGT within the shared bovine environment: bovine *S. agalactiae* with *Streptococcus uberis* (nisin U operon) and *Streptococcus dysgalactiae* subsp *dysgalactiae* (lactose operon). They also found evidence for HGT, involving the salivaricin operon,

between the bovine *S. agalactiae* strain and either *S. pyogenes* or *Streptococcus salivarius*. Also, we know that frequency of recombination varies by niche for example higher levels of recombination have been observed in carriage than during septic infection.

Natural genetic transformation

Natural genetic transformation has been studied in most detail in *S. pneumoniae* (Straume et al., 2015). *S. pneumoniae* is competent for natural genetic transformation, a property that enables the pneumococcus to acquire new traits by taking up naked DNA from the environment and incorporating it into its genome through homologous recombination. Four distinct steps (Straume et al., 2015) in *S. pneumoniae*, the transformation processes were described: regulation of competence induction, DNA-uptake and homologous recombination, the fratricide mechanism and termination of the competence period. This process involved many genes. The proteins required for this process are conserved between species and are produced during a specific physiological state known as competence. The review of competence regulation has revealed several cues regarding its physiological function in a species (Fontaine et al., 2015). Recent findings regarding competence regulation by the ComCDE and ComRS cell–cell signaling pathways and the Clp proteolytic system are specifically highlighted (Fontaine et al., 2015). The comX-inducing peptide (XIP) of *Streptococcus mutans* is a key regulatory element in the activation of genetic competence, which allows cells to take up extracellular DNA (Kaspar et al., 2017; Shanker et al., 2016). Flechard et al investigated on a large scale if loci coding for early competence factors (ComX and the two pheromone-dependent signalling systems ComCDE and ComRS) of streptococci are especially targeted by transposable elements. Transformation is even more common among the streptococci than has been recognized (Morrison et al., 2013). At the other extreme, no species of the *S. pyogenes* has been reported to transform in laboratory culture (Mashburn-Warren et al., 2012).

When transformation involves DNA exchange from closely related loci, it is known as homologous recombination. When transformation occurs between unrelated loci, it is known as non-homologous recombination. Homologous recombination also occurs between mobile genetic elements (MGE) such as insertion sequences (IS), integrons, bacteriophages, plasmids and transposons, considered being part of the accessory genome (non-core genome). The evolutionary function of natural competence remains controversial, as imported DNA can act as a source of substrates or can be integrated into the genome. Exogenous homologous DNA can also be used for genome repair. Veening and Blokesch (Veening and Blokesch, 2017) proposed that predation of non-related neighboring bacteria coupled with competence regulation might function as an active strategy for DNA acquisition, and argued that the forced release of DNA from killed bacteria and the transfer of non-clonal genetic material have important roles in bacterial evolution. Clinical interventions as drivers of genome evolution in *S. pneumoniae* demonstrate that natural genetic transformation plays a key role in these processes. Johnston et al (Johnston et al., 2013) showed that transformation produces a population containing many different merodiploid cells. Merodiploidy provides opportunities for evolution of new genetic traits via alteration of duplicated genes, unrestricted by functional selective pressure such as antibiotic treatment in *S. pneumoniae*, reinforces the plasticity potential of this bacterium and transformable species generally.

The role of mobile genetic elements (MGEs)

Mobile genetic elements (transposable elements, integrons, plasmids and bacteriophages) play an important role in pneumococcal evolution, both as an additional means of mobilizing genetic material. Integrons are genetic elements that can help bacteria to enhance their ability of adaptation and evolution through the acquisition, stockpiling, excision and differential expression of new genes. The genes are usually contained in a gene structure called cassette. In Streptococci, *S. pneumoniae* was proved to carry class 1 integron. There are

two key advantages owning by the integron system for the genomic innovation. First, the new cassette will not disturb the existing genes. Second, the newly integrated cassettes is ready for the natural selection (Gillings, 2014). The Tn5253 and Tn916 ICE families are commonly found in pneumococci and are important vehicles for the dissemination of antibiotic resistance in pneumococci and between *Streptococcus* species (Roberts and Mullany, 2011). These transposons are particularly known for carrying and transferring the tetracycline resistance determinant *tet* (M) and chloramphenicol acetyltransferase (*cat*) resistance gene. Some Tn916-like elements also carry other resistance genes, such as *erm* (B)-mediated erythromycin resistance, and genes associated with other accessory functions (Cochetti et al., 2007). The contributions of ICEs and phages to pneumococcal biology and pathogenicity further highlight the remarkable ability of these bacteria to exchange genes through different transfer mechanisms (transformation, transduction and conjugation). However, the diversity of these pneumococcal mobile elements and phages has not been fully explored and remain under-appreciated. Croucher et al (Croucher et al., 2014) showed that prophage content is highly variable even within pneumococcal lineages, suggesting frequent horizontal transmission that would necessitate rapidly diversifying antiphage mechanisms to prevent these viruses sweeping through populations. But recently, Brueggemman et al (Brueggemann et al., 2017) proved that some prophages persisted over long time periods. Pneumococcal prophages are likely to play a more important role in pneumococcal biology and evolution than previously recognized, but the vast array of prophage DNA within these pneumococcal genomes warrants a detailed exploration. We anticipate that genome sequencing of pneumococcal samples from diverse geographical origins will provide additional insight into the extent of the genetic diversity of ICEs and phages, and how these combines with other loci to make up the accessory genome of pneumococci.

HGT is a pervasive mechanism of diversification in many microbial species, but

its primary evolutionary role remains controversial. Much recent research has emphasized the adaptive benefit of acquiring novel DNA. But Croucher et al (Croucher et al., 2016) argued intragenomic conflict provides a coherent framework for understanding the evolutionary origins of HGT. The competence for transformation was found to provide an effective defense against parasitic MGEs. MGEs inhibit transformation through integrative disruption of genes encoding the competence machinery across many species and through secretion of DNases to reduce the concentration of extracellular DNA. Therefore, this framework is able to explain both common properties of MGEs, and the seemingly paradoxical bacterial behaviors of transformation and cell-cell killing within clonally related populations, as the consequences of intragenomic conflict between self-replicating chromosomes and parasitic MGEs. The antagonistic nature of the different mechanisms of HGT over short timescales means their contribution to bacterial evolution is likely to be substantially greater than previously appreciated. Regarded as a common genetic element responsible for horizontal gene transfer, integrons are widely distributed in various pathogens considered as a determinant in the acquisition and evolution of antibiotic resistance. Li et al (Li et al., 2017) summarized the occurrence, pathogenicity and virulence mediated by integrons in typical Gram-positive microorganisms (*Staphylococcus*, *Enterococcus*, *Corynebacterium* and *Streptococcus*) and the role of integrons in antibiotic resistance.

Mosaic genes

Interspecies recombination results in mosaicism, i.e. genes that are composed of alternate blocks of nucleotides derived from a donor and its recipient. These blocks may be the result of small fragments that have recombined within a gene or the result of almost the whole gene being replaced by incoming genetic material (Dowson et al., 1989). Whichever outcome, the new mosaic usually encodes a protein with a different activity to that of the original recipient and possibly to that of the donor. Following integration of advantageous mosaics,

such as penicillin resistance conferred by alterations within genes encoding penicillin-binding proteins, these become fixed within the population (Coffey et al., 1999). Once fixed, these successful mosaics can then readily spread laterally from species to species. For example, the DNA segments encoding the transpeptidase domains of PBP1a, PBP2x and PBP2b in highly resistant clinical isolates have a mosaic structure that have been generated by several successive recombination events. It is difficult to trace the sources of the various mosaic blocks. However, at least in some cases it has been shown that they originate from other species, namely *Streptococcus mitis* and *Streptococcus oralis* (Chi et al., 2007; Dowson et al., 1993; Sibold et al., 1994).

Crosstalk between vertical and horizontal gene transfer

Both intrinsic (vertical) and acquired mechanisms (HGT) affect susceptibility to a large variety of antibiotics (El Moujaber et al., 2017). Horizontal gene transfer is a key process in the evolution of bacteria and also represents a source of genetic variation in eukaryotes. Meanwhile, the vertical gene transfer was not talked much here. Among elements participating in gene transfer, thousands of small (<10 kb) mobile bacterial plasmids that replicate by the rolling circle mechanism represent a driving force in the spread of antibiotic resistances. In general, these plasmids are built as genetic modules that encode a replicase, an antibiotic-resistance determinant, and a relaxase that participates in their conjugative mobilization. Lorenzo-Diaz et al (Lorenzo-Diaz et al., 2017) reported that the MobM conjugative relaxase encoded by the promiscuous plasmid pMV158 participates in regulation of the plasmid copy number by transcriptional repression of the antisense RNA, thus increasing the number of plasmid molecules ready to be horizontally transferred (mobilization) and/or vertically inherited (replication), which is the first report of a type of crosstalk between genetic modules involved in vertical and horizontal gene flow. Much more work involved in vertical and horizontal gene transfer is need in future.

Virulence factors and evolution

Pathogenicity implies that a bacterium can be harmful to the host and virulence indicates the degree to which this pathogenicity is expressed. Pathogenicity and virulence depend on multiple factors such as capsule, M protein, biofilm, and resistance to immune responses, vaccine, antibiotics and other defensive mechanisms of the host, competitiveness, inflammation, invasion, translocation, toxin production and cytotoxicity.

Serotype replacement and capsular switching

In pneumococci, genetic exchange at the capsular locus is greatly facilitated by the syntenic organization and homology of genes in the capsular locus. This process of substituting the genes encoding one type of capsule with genes encoding for another is referred to as serotype or capsular switching , as a strategy to increase pneumococcal virulence (Sabharwal et al., 2014). This population level change in the serotype distribution, most often involving pre-existing clones and serotypes that were already in circulation before vaccine implementation, is known as serotype replacement. In Casablanca, the leading serotypes of penicillin non-susceptible *S. pneumoniae* (PNSP) were 14 (33 vs. 57%) and 19A (18 vs. 14%) before and after vaccination among children. For adults, serotypes 19A (53%) and 23F (24%) were the dominant serotypes in the pre-vaccination period, while serotype 14 (22%) was the most prevalent after vaccination (Diawara et al., 2017). Although there was evidence of natural capsule switching in the absence of vaccine induced selection pressure, most of the highly prevalent capsule-switched isolates were associated with acquisition of vaccine-targeted capsules (Chaguza et al., 2017). Changes in serotype prevalence among pneumococcal populations result from both serotype replacement and serotype (capsular) switching. This can be attributed to the phenomena of “serotype replacement”, the expansion of preexisting NVT pneumococci, and/or “serotype switching”, and a change of serotype of a single clone by alteration or exchange of its cps locus. The capsular polysaccharide

(CPS) the main virulence factor of *S. pneumonia*, of which there are more than 90 known serotypes protects against phagocytosis during invasive pneumococcal disease and may also prevent clearance during nasopharyngeal colonization. A historical perspective study (Wyres et al., 2013) of seven decades showed that capsular switching events were presumably the result of nucleotide substitution and/or deletion, and recombination-imports of various lengths, inserted at different points around the cps locus, with or without the adjacent *pbp* sequences. Sabharwal et al (Sabharwal et al., 2014) proved that capsular switch events can result in *S. pneumoniae* strains of enhanced virulence for respiratory tract infection. Mostowy et al (Mostowy et al., 2017) found that found cps to be an evolutionary hotspot with elevated substitution and recombination rates to generate novel serotypes by recombination. Chiba et al (Chiba et al., 2017) reported a penicillin-resistant *S. pneumoniae* (PRSP) isolate from an adult patient, characterized as a recombinant strain derived from PRSP of serotype 23F with the cps locus (20.3 kb) replaced by that of a penicillin-susceptible strain of serotype 3. Much of serotype replacement is due to clonal expansion of serotype variants extant prior to vaccination and not those that arise through capsule switching. This should be clear because it may be erroneously interested that capsule switching is a major driver for serotype replacement. Moreover, switching was much more likely to exchange one serotype for another within the same serogroup than expected by chance (Croucher et al., 2015). Nontypeable pneumococci (NTPn) fall into several categories and reviewed (Andam and Hanage, 2015): those that do not possess capsule genes, pneumococci that are phenotypically non-encapsulated but possess down-regulated or defective capsule genes, and a population of atypical non-encapsulated isolates that are genetically divergent from most pneumococci. Sporadic or random switching between encapsulated and nonencapsulated states may play an important role in pneumococcal population dynamics and antibiotic resistance. Andam et al (Andam and Hanage, 2015) illustrated a possible model that there is a switching between encapsulated and

non-encapsulated states in a closely related group of pneumococci. One strain loses its ability to produce a capsule through gene loss, deletion or insertion of a stop codon within the capsule locus. The strain may eventually regain its original capsule type or another capsule of a different serotype, which may also correlate with increased resistance due to recombination.

M protein in GAS

The M protein, first described in 1927 by Rebecca Lancefield, who demonstrated it to be a primary source of strain-specific immunity (Metzgar and Zampolli, 2011) coats the surface of GAS. M protein inhibits phagocytosis, promotes adhesion to epithelial cells, and helps the bacteria evade innate immune defenses (Smeesters et al., 2010). There are >200 different serotypes of M protein (i.e., M6, M12, M18, M24, and so on), an individual may become infected by more than one group A streptococcal type during a lifetime (Pinto et al., 2016). The M protein is hyper variable and has long served as the primary target for epidemiological typing of GAS. More than 80 distinct M types were identified, and 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. 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 (Bessen et al., 2015). 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 (Bessen et al., 2015). Comparative analyses of the genomes of GAS clearly demonstrated the

importance of MGEs, LGT, and recombination in generating diversity between *emm*-types. Most GAS genomes possess multiple bacteriophages, which in turn often carry virulence genes predicted to change the virulence of a lineage (McMillan et al., 2013). Integrative conjugative elements (ICE) and remnant MGEs are also common. The *S. pyogenes* genome is remarkable for its content of prophages, streptococcal phage-like chromosomal islands (SpyCIs), and other mobile genetic elements (MGEs), such as integrative and conjugative elements (ICEs) (Maruyama et al., 2016). In GAS, prophage regions have been shown to encode virulence factors such as exotoxins. In addition, it has been reported that genome rearrangements can generate a new prophage in an M3 strain (Maruyama et al., 2016). Generalized transduction occurs in GAS and is mediated by bacteriophages (McShan and Nguyen, 2016). Generally, the susceptibility of cells to phage-mediated transduction probably varies by growth state and genetic background, both of which could influence horizontal transfer (McShan and Nguyen, 2016). The majority of the genome prophages (72%) are found to be integrated into genes encoded on the lagging strand. No prophages have been found to target genes in the hypervariable regions, which include the M-protein (*emm*) or the streptococcal pilus. Lysogenic bacteriophages of GAS are capable of mediating transfer of antibiotic resistance by transduction. Strains with bacteriophage T12-like prophages can produce transducing lysates that are capable of transferring resistance to tetracycline, chloramphenicol, macrolides, lincomycin, and clindamycin, following lysogen induction. Generalized transduction transfer of erythromycin and streptomycin resistance, following mitomycin C treatment of endogenous prophages, has also been observed (McShan and Nguyen, 2016). Horizontal transfer of serotype-related genes and the emergence of new strains/clones may be a result of selective pressures conferred by the host immune response (Rouge et al., 2016). Studies suggest that the acquisition of new MGEs, resulting in the elaboration of novel virulence gene repertoire is an important facet underlying the changes in virulence (McMillan et al., 2013). GAS has a rich evolutionary history of horizontal transfer

among its core genes (Bessen et al., 2011). In countries where streptococcal disease is endemic, and *emm* type diversity is high, the conditions for LGT of the *emm*-gene is much greater than non-endemic countries (McMillan et al., 2013).

Streptococcus population evolution

Establishing the contribution of co-adaptation and competition in the maintenance of discrete lineages is important since the outcome of certain interventions, such as vaccination depends crucially on these underlying determinants of population structure. Populations of *S. pneumoniae* are typically structured into groups of closely related organisms or lineages, but it is not clear whether they are maintained by selection or neutral processes. Camilli et al (Camilli et al., 2017) showed the impact of pneumococcal conjugate vaccine (PCV7 and PCV13) on pneumococcal invasive diseases in Italian children and insight into evolution of pneumococcal population structure. Recombination events (Duvvuri et al., 2016) were observed by whole genome sequencing among non-PCV 13 serotypes (22F, 15A and 8) populations after the in introduction of pneumococcal conjugate vaccines. Serotype 22F (ST433) has emerged into two sub-populations, with 28% (7/25) exhibiting recombination events, and five also acquiring macrolide resistance as a result of recombination. Lourenco et al (Lourenco et al., 2017) indicated that lineages evolved through immune selection on the groEL chaperone protein. The groEL protein is part of the groESL operon and enables a large range of proteins to fold correctly within the physical environment of the nasopharynx, thereby explaining why lineage structure is so stable within *S. pneumoniae* despite high levels of genetic transfer (Lourenco et al., 2017). Hence, vaccine strategies based on groESL variants would target entire lineages instead, including all uncommon serotypes within and thereby preventing their expansion. Both pre- and post-genomic studies have revealed that HGT and recombination occurs between these two organisms and plays a major role in shaping the population structure (McNeilly and McMillan, 2014). *S. pneumoniae* from invasive (IPD) and noninvasive

pneumococcal disease (NIPD) were studied by serotype and multilocus sequence typing (MLST) for population structure characteristics (Zhou et al., 2017). The seven predominant STs were ST271, ST320, ST876, ST3173, ST236, ST81 and ST342, which were mainly associated with serotypes 19F, 19A, 14, 6A, 19F, 1, and 1/23F, respectively. The colonization rate of GBS in postmenopausal women was 17.8%. Capsular type III was predominant (34.6%), followed by type V (22.4%). The most frequent sequence type (ST) was 19 (23.3%), followed by 23 (18.7%), 1 (16.8%) and 17 (12.1%). Isolates were assembled into three phylogenetic groups from ST-19, ST-23 and ST-17 founders. All isolates were susceptible to penicillin, whereas resistance to erythromycin and clindamycin was recorded in 23.4% and 20.6% of isolates, respectively. The population structure of GBS is highly diverse and contains different STs (Molto-Garcia et al., 2016). Athey et al, hypothesized that *Streptococcus suis* serotype 2 strains of the ST25 lineage are genetically heterogeneous, and proved that the importance of lateral gene transfer and recombination as drivers of diversity in *Streptococcus suis* (Athey et al., 2016). More attention should pay to MLST genotyping for *Streptococcus* and evolution relationship between different ST types, as well as the relationship of ST types to virulence and antibiotic resistance profile. The sequences of seven housekeeping loci used for *Streptococcus* MLST genotyping are stored in the databases hosted on PubMLST (https://pubmlst.org/).

Evolution in *Streptococcus* biofilms

Biofilms are defined as structured communities of microorganisms that are attached to a surface and enmeshed in an extracellular polymeric matrix (Flemming et al., 2016; Hobley et al., 2015). Dental caries (tooth decay) is a polymicrobial biofilm disease driven by the diet and microbiota–matrix interactions that occur on a solid surface. Early studies prove that microorganisms residing within biofilms are embedded in a matrix containing extracellular polymeric substances (EPS) (Hobley et al., 2015; Lebeaux et al.,

2014). Obviously, polymicrobial interactions and the local biofilm microenvironment play instrumental roles in modulating health and disease conditions (Dewhirst, 2016; Koo et al., 2013; Mira et al., 2017). Coevolution of *S. mutans* with the increased sugar consumption largely explains how well adapted *S. mutans* is to colonize and thrive on teeth when its human host ingests sugars. *S. mutans* can utilize a wide variety of carbohydrates to produce EPS and acids for adaptation of stress resistance and bacterial competence (Bowen and Koo, 2011). Furthermore, pathological process of the dental caries relies on the milieu within which the organisms interact, and acids accumulate. Thus, *S. mutans* can contribute to the biofilm matrix assembly as the main producer of insoluble glucans among oral bacteria and reset the microenvironment for other aciduric-cariogenic bacteria to thrive and become established (Johansson et al., 2016; Simon-Soro and Mira, 2015). If the commensal environment is supplied with sugar, *S. mutans* can work synergistically with other aciduric species and cause ecological changes to shape the biofilm community, structure, and metabolism conducive to caries development. Carriage of the pneumococcus in the nasopharynx is thought to be mediated by biofilm formation, an environment where isogenic populations frequently give rise to morphological colony variants, including small colony variant (SCV) phenotypes. Churton et al (Churton et al., 2016) revealed that SCVs exhibit reduced growth rates, reduced capsule expression, altered metabolic profiles, and increased biofilm formation, and all SCVs studied had mutations within the DNA-directed RNA polymerase delta subunit (RpoE). Furthermore, recurrent mutation of the pneumococcal *rpoE* gene presents an unprecedented level of parallel evolution in pneumococcal biofilm development. The formation of GBS biofilm can be strongly influenced by environmental conditions like PH and glucose concentration (D'Urzo et al., 2014), as well as endogenous factors including pili and bacterial surface adhesin of GBS (BsaB/ FbsC) (Buscetta et al., 2014; Jiang and Wessels, 2014). No matter what the specific GBS or host factor is, the evolutionarily conserved interaction between GBS factor and host surface molecular always allow GBS to survival

and multiply to an enough community, and then cause harm to the host with virulence element. Obviously, biofilm formation enhances fomite survival of *S. pneumoniae* and *S. pyogenes* (Marks et al., 2014).

Evolution in immune escape

The host has developed multiple immune responses to fight the pathogens. Simultaneously, these bacterial also evolved complex mechanisms to survive in host internal environment. To avoid immune clearance, GBS can degrade host-derived intermediate immunological molecules to inactive form which cannot stimulate host immune response pathway. Simultaneously, these bacterial also evolved complex mechanisms to survive in host internal environment. To avoid immune clearance, GBS can degrade host-derived intermediate immunological molecules to inactive form which cannot stimulate host immune response pathway. Hyaluronan (HA), known to have an important role in immune surveillance, can be synthesized by cervical fibroblasts, epithelial cells and immune cells (Akgul et al., 2014). In the condition of infectious or non-infectious tissue injury, HA will be degraded by host hyaluronidases into HA fragments which can interact with TLR2/4 (Toll-like receptor) to elicit inflammatory responses. It was believed that most clinical GBS strains can secrete hyaluronidase. With the help of hyaluronidase, GBS can degrade HA fragments produced by host to non-stimulatory disaccharide subunits and then facilitate immune evasion. Furthermore, the HA disaccharides can inhibit TLR2/4 pathways activated by HA fragments or other TLR2/4 ligands. Then the immune recognition and production of pro-inflammatory cytokine will be blocked, and the GBS pathogens can escape the host immune detection (Kolar et al., 2015). Induction of type I interferon (IFN) is an important host immune response to GBS infection. The production of type I IFN can be activated by the sensing of bacterial nucleic acids present in host cytoplasm (Monroe et al., 2010). The cyclic GMP-AMP synthase (cGAS), as a host DNA receptor, can be activated by GBS double-stranded DNA and produce a cyclic dinucleotide (cdN). These GBS

derived cdNs can bind stimulator of IFN genes (STING) to activate type I IFN production and promote inflammation. Andrade et al reported an ectonucleotidase CdnP expressed by GBS can hydrolyze extracellular cyclic dinucleotides, block the stimulation of STING and inhibit the production of type I IFN, ultimately leading to immune escape (Andrade et al., 2016). The histidine triad proteins (HTPs), also known as Pht proteins in *S. pneumoniae*, constitute a family of surface-exposed proteins that exist in many pathogenic streptococcal species. Although many studies have revealed the importance of HTPs in streptococcal physiology and pathogenicity, little is known about their origin and evolution. In previous study (Shao et al., 2013), several major findings were made. First, *htp* genes originated earlier than the *Streptococcu*s genus and gene-loss events have occurred among three streptococcal groups, resulting in the absence of the *htp* gene in the bovis, mutans and salivarius group streptococci. Second, the copy number of htp genes in other groups of *Streptococcus* is variable, ranging from one to four functional copies. Third, both phylogenetic evidence and domain structure analyses support the division of two htp subfamilies, designated as htp I and htp II. Although present mainly in pyogenic group and in *Streptococcus suis*, htp II members are distinct from htp I due to the presence of an additional leucine-rich-repeat domain at the C-terminus. Finally, htp genes exhibit a faster nucleotide substitution rate than do housekeeping genes. Specifically, the regions outside the HTP domains are under strong positive selection. This distinct evolutionary pattern likely helped *Streptococcus* to easily escape from recognition by host immunity.

Evolution associated with antibiotics and vaccine

Evolution drive drug resistance

Genetic recombination plays an important role in the development of antibiotic resistance in pneumococci. Antibiotic induced stress is known to induce competence in pneumococci; during the competence phase, the pneumococci acquire exogenous DNA, which may include genes that confer resistance to

antibiotics (Slager et al., 2014). It has been reported that recombination replacements are responsible for the mosaic structure typically observed in penicillin binding proteins (PBP) genes in *S. pneumoniae* (Dowson et al., 1989). Mutations in PBP genes confer resistance to β-lactam antibiotics including penicillin, amoxicillin and cefotaxime (Hakenbeck et al., 1999; Li et al., 2016). Hakenbeck et al (Hakenbeck et al., 2012) showed that clinical isolates display a mosaic structure of the affected PBP genes, the result of interspecies gene transfer and recombination events. Fan et al (Fan et al., 2009) proved that both the ComD and ComC are involved in the drug resistance of *S. pneumoniae* to cefotaxime. Recombination also mediates the dissemination of transposon and integrative conjugative elements (ICEs) that carry an array of antibiotic resistance determinants, throughout the pneumococcal population. Such mobile genetic elements include the Tn916-likeMGEs, Tn5251 (a Tn916-like element) (Provvedi et al., 1996), Tn5252 (Provvedi et al., 1996), Tn5253 (a composite of Tn5251 and Tn5252 transposons) (Provvedi et al., 1996) and many others. The Tn916, Tn5251, Tn5252, Tn5253 and Tn1545 transposons carry tetM gene, which confers resistance to tetracycline (Chaguza et al., 2015).

The ability to effectively treat pneumococcal infection has been compromised due to the acquisition of antibiotic resistance, particularly to β-lactam drugs (Appelbaum, 2002). Resistance to β-lactam antibiotics in pneumococci is due to alterations in PBPs, especially PBPs 2X, 1A and 2B (Coffey et al., 1991). Recently, two studies have used whole-genome sequencing to analyze isolates from the PMEN1 genetic lineage. The first study (Croucher et al., 2011) focused solely on the PMEN1 lineage and described considerable genomic diversity believed to have originated by horizontal gene transfer in response to antimicrobial and vaccine selective pressures. The second study (Kong et al., 2013) focused on understanding the evolution of penicillin resistance among pneumococci and revealed a surprising directional transmission of penicillin-resistance genes and other genes associated with virulence and

antibiotic resistance from the PMEN1 clone to several genetically unrelated clones. All the isolates were resistant to penicillin, cotrimoxazole and chloramphenicol, and shared the same pbp1a allele, whereas multiple alleles of *pbp2b, pbp2x, pspA* and *pspC* were detected. Of the isolates, 89.7% were tetracycline resistant and 60.3% were macrolide resistant, and resistance was associated with different Tn916-like transposons. Genetic variability was observed among PMEN1 isolates collected in our area over the past 20 years (Domenech et al., 2014).

Generally, the proportions of PNSP in Casablanca, Morocco between pre- and post-vaccination periods (PCV-13 introduced in 2010 and replaced by PCV-10) were 31 and 13%. There were 21 *pbp* genotypes in the pre-vaccination period vs. 12 for post-vaccination period. PFGE clustering showed six clusters of PNSP grouped into three clusters specific to pre-vaccination period (clusters I, II and III), two clusters specific to post-period (clusters V and VI) and a cluster (IV) that contained clones belonging to the two periods of vaccination. It was demonstrated a high degree of genetic diversity among PNSP. PFGE clustering combined with *pbp* genotyping revealed that vaccination can change the population structure of PNSP (Diawara et al., 2017).

The prevalence of fluoroquinolone-resistant *S. pneumoniae* (FQRSP) decreased from the initial survey to the second survey (PPV23). Prevalence increased in the third survey (fluoroquinolone restriction) (Ben-David et al., 2014). Fluoroquinolone nonsusceptibility among pneumococci results mainly from point mutations in the QRDR topoisomerase genes (Ben-David et al., 2014; Richter et al., 2005). In the current study, identical mutations were found in different serotypes among the strains in adults. This finding suggests that the mutation occurred before capsular switch. Olivieri et al (Olivieri et al., 2015) evaluated temporal fluctuations in macrolide resistance rates, analyzing genetic determinants of resistance and clonal evolution in a population of 2744 *S.*

pyogenes isolates collected in an area of central Italy, which suggested that changes in bacterial population structure, rather than horizontal transfer of resistance determinants, plays a major epidemiological role in *S. pyogenes*. Zhou et al (Zhou et al., 2014) held a study to identify the mechanism responsible for the horizontal transfer of transposon Tn2010 in *S. pneumoniae*, and the genomic alterations introduced by the transfer process Tn2010 tended to be transferred by transformation rather than conjugation in *S. pneumoniae*, and the spread of Tn2010 could have a profound effect on the evolution of the genome. Many other recombinations were scattered throughout the genome of the transformants in addition to transposon Tn2010. The acquisition of Tn2010 with negligible fitness cost may facilitate spread of the transposon.

Understanding how changes in antibiotic consumption affect the prevalence of antibiotic resistance in bacterial pathogens is important for public health. In a number of bacterial species, including *S. pneumoniae*, the prevalence of resistance has remained relatively stable despite prolonged selection pressure from antibiotics (Lehtinen et al., 2017). The evolutionary processes allowing the robust coexistence of antibiotic sensitive and resistant strains are not fully understood. Lehtinen et al (Lehtinen et al., 2017) suggested that heterogeneity in duration of carriage is a partial explanation for the coexistence of sensitive and resistant strains and that factors determining bacterial duration of carriage will also affect the prevalence of resistance.

Evolution drive vaccine escape

Capsule switching is a natural process that occurs when different pneumococcal serotypes ‘swap’ their capsular polysaccharide locus through alteration of the capsule biosynthesis locus via mutations (single base changes, insertions or deletions) or genetic recombination. PCVs directly work against a set of pneumococcal serotypes whose capsular polysaccharides have been targeted in the vaccine formulation. The introduction of PCVs has led to the emergence of

the capsule-switch variants arising due to the vaccine derived selective pressures. Current vaccine strategies that target a selection of capsular serotypes can lead to the expansion of non-vaccine serotypes (Chaguza et al., 2017; Di Pasquale et al., 2017; Latasa Zamalloa et al., 2017). An increase in the prevalence of these capsule-switched variants can result in serotype replacement, which is the increase of NVT associated pneumococcal clones that follows the decrease in VT associated clones (Wyres et al., 2013). Apart from capsule switch, serotype replacement that occurs after introduction of PCVs is mainly caused by 'serotype unmasking' (Lipsitch, 1999). Unmasking is the process where less prevalent or 'masked' NVT serotypes rise in prevalence to occupy the ecological niche vacated by the 'more' competitive VT serotypes after vaccination (Lipsitch, 1999). However, genetic variation may not always result in phenotypic differences which produce novel serotypes. With the introduction of high throughput whole genome sequencing, discovery of novel genotypic variants is not unexpected. Kapatai et al (Kapatai et al., 2017) found a novel variant of the serotype 23B CPS operon, characterized as a novel genotypic subtype (23B1) with 70% homology to the published 23B CPS sequence. Although the 23B1 variant appears to have no phenotypic impact and cannot be considered as novel serotype, it appears to have led to a genetic restructuring of the UK serotype 23B population. Another work using whole genome sequencing finds a robust and unexpected pattern of serotype switching in a sample of bacteria collected following the introduction of routine anti-pneumococcal vaccination: switching was much more likely to exchange one serotype for another within the same serogroup than expected by chance (Croucher et al., 2015). However, higher rates of within-serogroup switching could not be fully explained by either more frequent, shorter recombinations, or by genetic linkage to genes involved in β–lactam resistance. This suggested the observed pattern was a consequence of selection for preserving serogroup. Several hypotheses are presented and tested to explain this pattern, including limitations of genetic recombination, interactions between the genes that

determine serotype and the rest of the genome, and the constraints imposed by bacterial metabolism.

Conclusion and future directions

The impact of genetic recombination to the *Streptococcus* evolution has been proved. Horizontal gene transfer is a key process in the evolution of bacteria and also represents a source of genetic variation in Streptococci. Thanks to the rapid influx of whole genome sequence (WGS) data and the advent of novel analysis methods, future studies should aim to identify the functional roles of certain recombination events in order to provide further insights into pathogenesis, carriage dynamics and strain transmission. Long term evolution-population genomics and tissue tropism, as well as short term evolution focusing comparative genomics in epidemics and outbreaks should be gain much more attention. Furthermore, crosstalks between different recombination mechanisms also should be focused. Here, we reviewed the evolution of representative virulence factors, capsule in *S. pneumoniae*, M protein in GAS, biofilm in GBS and some oral *Streptococcus*, as well as the effect caused by evolution, antibiotic resistance and vaccine escape. Multiply phenotype change, for example capsular switch and multiple antimicrobial nonsusceptibility mutations, can occur in some clone. As to adaptive evolution caused by antibiotics, vaccine and so on, continuous surveillance is an essential to monitor evolution of *S. pneumoniae* causing disease. In order to better survey, control and prevent the emergence of drug-resistant or vaccine escape strains, antimicrobial stewardship, national surveillance and public awareness programs should be developed.

Acknowledgements

We thank Doctor Yanhua Zhao, Shu Feng and Zhonghao Wang for help with references annotation and management, and page proof in this work.

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