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Research article

Differences in genetic flux in invasive *Streptococcus pneumoniae* associated with bacteraemia and meningitis☆

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ABSTRACT

Background: Genetic flux, a crucial process of pneumococcal evolution, is an essential aspect of bacterial physiology during human pathogenesis. However, the role of these genetic changes and the selective forces that drive them is not fully understood. Elucidating the underlying selective forces that determine the magnitude and direction (gene gain or loss) of gene transfer is important for better understanding the pathogenesis process, and may also highlight potential therapeutic and diagnostic targets.

Methods: Here, we leveraged data from high throughput genome sequencing and robust probabilistic models to discover the magnitude and likely direction of genetic flux events, but not the source, in 209 multi-lineage invasive pneumococcal genomes generated from blood (n = 147) and CSF (n = 62) isolates, associated with bacteremia and meningitis respectively. The Gain and Loss Mapping Engine (GLOOME) was used to infer gene gain and loss more accurately by taking into account differences in rates of gene gain and loss among gene families, as well as independent evolution within and across lineages.

Results: Our results show the likely extent and direction of gene fluctuations at different niche, during pneumococcal pathogenesis, highlighting that evolutionary dynamics are important for tissue-specific host invasion and survival.

Conclusion: These findings improve insights on evolutionary dynamics during invasive pneumococcal disease, and highlight potential diagnostic and therapeutic targets.

1. Introduction

Bacterial genomes are versatile and consist of a diverse repertoire of genes that enable adaptation to new niche, and progressive changes in the environment and within eukaryotic hosts [1, 2]. Gene acquisition and loss facilitates bacterial genome diversification, and the resulting fluctuations in genome size and content are regulated by positive and purifying selection acting on specific genes [1, 3]. It has been suggested that genetic divergence between ecologically differentiated populations is due to gene-specific rather than genome-wide selective sweeps [4]. These fluctuations are predominantly thought to occur at a few loci within the core genome, resulting in a suite of niche specific genes and alleles associated with colonization of different ecological environments [3, 4].

Bacteria with capsules exhibit higher rates of gene acquisition and loss (genetic flux), and are able to colonize different environments, resist antibiotics and tolerate diverse environmental stresses [5]. The contribution of genetic flux in the emergence and maintenance of ecologically distinct bacterial populations is still unclear.

Here, we examine whether the differences in genetic flux can explain the propensity of *Streptococcus pneumoniae* (pneumococcus), a capsulated Gram-positive bacterium, to cause either of two different severe invasive pneumococcal disease (IPD) outcomes: meningitis (cerebral spinal fluid (CSF) invasion) compared to bacteremia (bloodstream infection).

Pneumococcal meningitis that is commonly preceded by hematogenous invasion often results in host mortality, a dead-end that prevents further pathogen host-to-host transmission [6, 7]. Prior to invasion of normally sterile sites (blood and cerebral spinal fluid (CSF)), multiple strains of pneumococci asymptomatically colonize the nasopharynx where they survive as a biofilm and exchange genetic material both within and outside species [8, 9, 10]. These genetic exchanges enable pneumococcal genomes to rapidly evolve mechanisms to adapt to different host environments, and evade antibiotics and vaccines that

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impose selective pressure, resulting in diverse pneumococcal lineages [8, 9, 10, 11, 12]. Although, differences in alleles and number of genes present or absent are thought to be associated with this difference [2, 13, 14, 15, 16], the core genomes of meningitis- and bacteremia-causing pneumococci are not significantly different in size [2].

Recent analysis has only confirmed presence of previously well-established antigen genes, for example choline binding protein A (CbpA) that is relevant for IPD, and present in both bacteremia- and meningitis-associated isolates of the same lineage, but no significantly-associated genetic variants for tissue tropism [14, 17]. Pneumolysin is the best-characterized virulent factors, with great promise as a potential vaccine candidate [18]. Pneumolysin induces host cell lysis by forming transmembrane pores in the target membranes. This hinders opsonization, phagocytosis, and elimination by the host immune response [18, 19]. Murine models of colonization, pneumonia, and bacteremia have also highlighted the importance of the pneumococcal pilus as a determinant of adhesion and virulence [20]. Recent reports suggest that pilus adhesin RrgA and pneumolysin synergistically promote the invasion of the meninges, and result in host neuronal cell death [21, 22]. We hypothesized that genetic flux shapes the suite of pneumococcal genes and alleles present, prior to invasion of sterile sites. This later determines pneumococcal propensity to cause meningitis or bacteremia.

Genetic flux events during asymptomatic nasopharyngeal carriage facilitate acquisition of diverse virulence factors that may be responsible for tissue tropism, ultimately determining the nature and course of subsequent invasive pneumococcal disease (IPD) [8, 10, 19, 23, 24, 25]. Despite the large extent of genetic exchanges resulting in diverse pneumococcal lineages, pneumococci maintain a relatively constant genome size (~2.1 Mb) and GC-content (~39.5%), suggesting that gene gain events are counterbalanced by loss of superfluous genes [3, 26].

To establish role of genetic flux in maintenance of ecologically distinct pneumococci, we analyzed a global set of pneumococcal genomes generated from meningitis and bacteremia isolates. We used a stochastic mapping analysis strategy that accurately infers the expectations and probabilities for all gain and loss events [19, 20]. Our analysis strategy takes into account differences in rates of genetic flux among gene families, as well as independent evolution within and across lineages, and similar models have been successfully employed in primates [27] and prokaryotes [28, 29, 30, 31]. We anticipate that our findings will help to identify novel therapeutic targets for invasive pneumococcal disease.

2. Materials and methods

Meningitis (n = 62 genomes) and bacteremia (n = 147 genomes) associated pneumococcal genome datasets were retrieved from the National Center for Biotechnology Information (NCBI) GenBank database [32]. Meningitis and bacteremia genomes were sequenced from isolates collected from human blood and cerebrospinal fluid, respectively (Supplementary able 1). Missing genotypes were inferred using PubMLST [33]. The genomes used represented a global collection of pneumococci from a wide range of genotypes (Supplementary Table 1).

Genomes were analyzed as previously described elsewhere [2, 34]. Briefly, orthologous genes were first identified by clustering annotated genomes using OrthoMCL [35]. Orthologous maps were analyzed using BMX [34] to generate patterns of gene presence and absence (phyletic patterns) as the input for subsequent analysis. The Gain and Loss Mapping Engine (GLOOME) [28], a robust probabilistic framework that evaluates lineage-specific gain/loss dynamics using a mixture-model approach, was used to examine the extent of this genomic flux to infer gene gain and loss events [36]. Previous evolutionary models assumed similarity in the rate of gene gain and loss, and invariable rates for gene families [37]. Recent evidence suggests differing tendencies of gene gain and loss among gene families [28]. GLOOME infers gene gain and loss more accurately by taking into account differences in rates of gene gain and loss among gene families, as well as independent evolution within and across lineages [28, 31]. These models have been successfully employed in analysis of primate [27] and prokaryote [28, 29, 30, 31] datasets.

We quantified the transferability for each gene within the genome to determine likely direction and magnitude of genetic flux in pneumococcal genomes, for each niche under study [28]. We determined the mean magnitude of genetic flux using the Welch two-sample t-test for gain and loss events implemented in R statistical package version 3.5.0 [38].

3. Results

We first defined the size of the core genomes of the meningitis- (n = 2251 genes, 62 genomes) and bacteremia-associated (n = 2428 genes, 147 genomes) datasets, and their associated accessory genes (n = 38 and n = 124), respectively. To quantify the magnitude of genetic flux for each disease outcome, we then determined the cumulative number of gene gain and loss events within the entire set, core and accessory genomes of bacteremia- and meningitis-associated datasets (Figure 1). Our results suggest that cumulatively there is a larger magnitude of loss compared gain events (Figures 1A, 1B, 1C & 1D), except in the accessory genes (Figures 1E & 1F) where the opposite was true. Overall, genetic flux was more pronounced in bacteremia dataset (Figure 1).

However, some specific genes may have a larger magnitude of genetic flux that could influence the overall direction of gene gain or loss, when only considering cumulative number of events. We therefore determined the mean magnitude of genetic flux using the Welch two-sample t-test for gain and loss events to establish the overall direction of genetic flux (Figure 2). To avoid confounding due to differences in dataset sizes we randomly selected two subsets (n = 62 genomes) from the entire bacteremia dataset (n = 147 genomes), and compared them with the meningitis dataset (n = 62 genomes). We found that there were significantly more gene loss compared to gain events in the meningitis dataset (p = 0). The opposite was true for the bacteremia dataset (p = 0) (Figures 2A, 2B & 2C). To determine whether it is the core or accessory genome component that drives our observation, we quantified genetic flux in these components for the meningitis and bacteremia datasets. There was significant gene loss compared to gain events in the meningitis core-genome (p = 0) (Figure 2F). There were no significant differences in the bacteremia core-genomes (Figures 2D & 2E). There were significant gene gain compared to loss events in the bacteremia accessory genes (p = 0) (Figures 2G and 2H), and no significant differences in meningitis dataset (Figure 2I). Taken together, these findings suggest that genetic flux occurs more significantly in the core genome for meningitis-, and accessory genes for bacteremia-associated genomes.

We examined established antigens (Figure 3) to determine whether genetic flux influences a suite of genes that are solely responsible for the two different disease outcomes. We examined the prevalence of the RrgA pilus subunit pilus that has been implicated in the pathogenesis of meningitis. We observed that only 30% (20 of the 62 isolates) of the meningitis associated pneumococcus had the RgrA pilus gene in the global collection of genomes under analysis. The number of genomes with the RgrA genes detected had the following geographic distribution: 5 (25%) from USA; 3 (15%) from South Africa; Netherlands, Russia, Germany, and UK each had 2 (10%); and Taiwan, Sweden, India and Malawi each had a single genome (5%); for a total 20 (100%) genomes (Supplementary Table 2). Pneumolysin did not exhibit high expectation values for gene loss or gain events in both the meningitis and bacteremia datasets (Figure 3). We found that peptidase C39, competence stimulating peptidase (comC), and pneumococcal histidine triad D (phtD) had high expectation values for gene loss events in the meningitis dataset. Immunity proteins pncB, pncF, and pncK had a higher expectation of gene loss events in the bacteremia dataset (Figure 3).

4. Discussion

The genome of *Streptococcus pneumoniae* rapidly evolves to adapt to the host environments within a single infection [12]. Pneumococcal gene

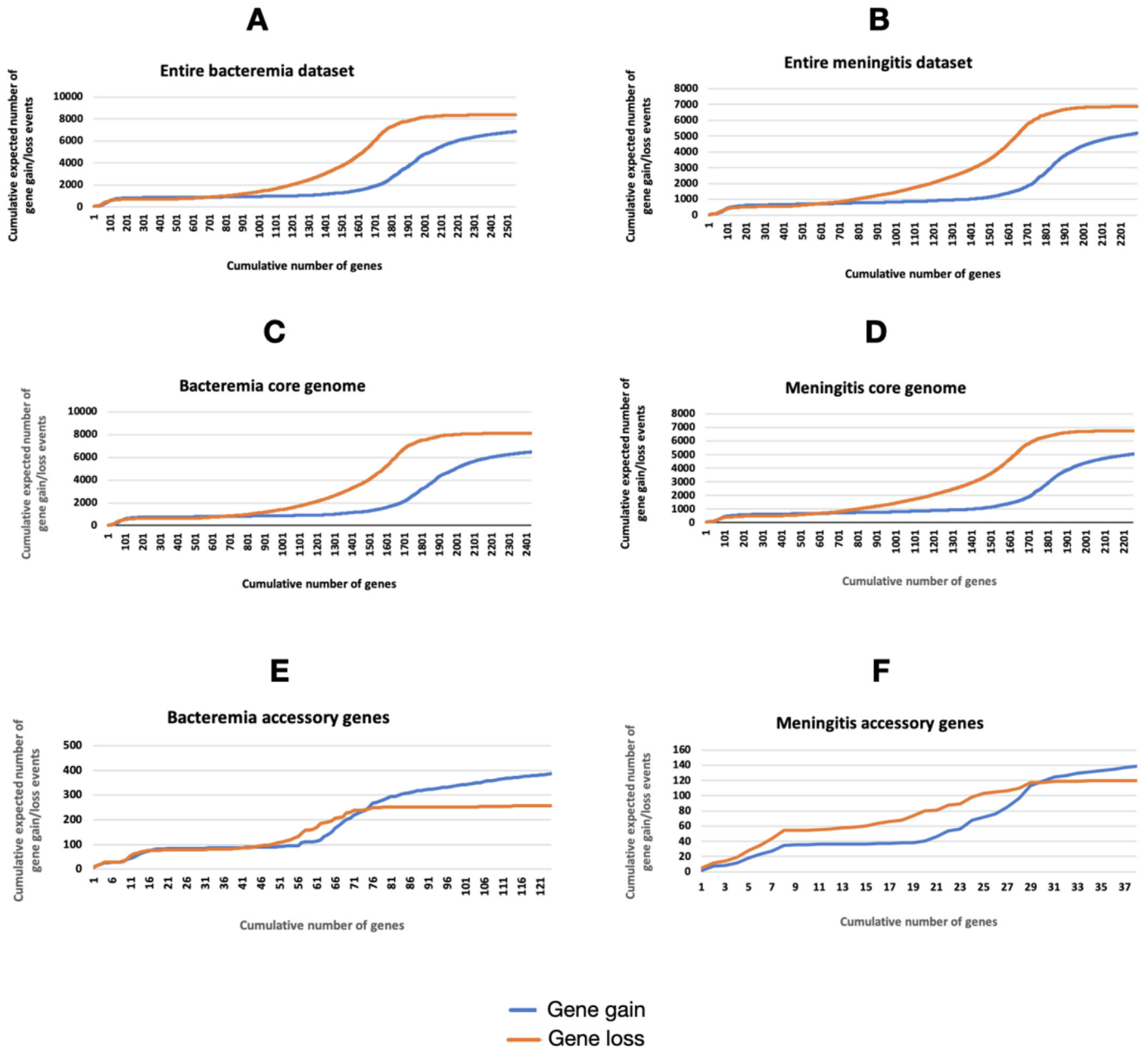

Figure 1. Cumulative expected number of gene gain and loss events in the bacteremia and meningitis datasets. (A) Cumulative expected number of genetic flux event in the entire bacteremia genomes dataset, (B) entire meningitis genomes dataset, (C) bacteremia core genome, (D) meningitis core genome, (E) bacteremia accessory genes, and (F) meningitis accessory genes. Gene gain events are shown in blue, and loss events in red.

families are acquired and lost by recombination and sequence evolution processes, resulting in chromosomal reorganization. Gene gain and loss events make significant contributions to the genetic diversity associated with niche adaptation, colonization, and other alterations in bacterial lifestyle [23, 29]. However, the role of gene gain and loss in the emergence and maintenance of ecologically differentiated bacterial populations has remained unclear [3].

The use of probabilistic models in this study provides accurate inference of gene gain and loss events highlighting pneumococcal gene content fluctuations, and adaptation to different niche. Understanding the likelihood, extent and overall direction of gene fluctuations is an important element of understanding genome evolution and proteome functionality in clinically relevant bacteria. Quantification of transferability allows close examination of evolutionary forces that determine the tendency of a gene to get fixed along the genome. Information on the probable direction of genomic flux is invaluable in understanding niche adaptation mechanisms employed during disease progression in pathogenic bacteria, as well as highlighting important potential therapeutic targets.

We demonstrate extensive genetic flux and show that this accrues in both the core and accessory genome. We highlight that these genetic flux events for two different invasive pneumococcal disease outcomes, meningitis and bacteremia, are more pronounced in the core genome and accessory genes, respectively. Adaptation to different niches may involve selection at a small proportion of genome loci creating barriers to stockpiling of neutral diversity [39, 40]. This supports previous evidence of extensive genetic flux in parasitic bacterial lineages capable of switching from commensal to obligate host-associated bacteria [41]. Bacteria rely on specific sets of virulence factors appropriate for different niche invasion and colonization [42, 43, 44, 45]. This suggests that

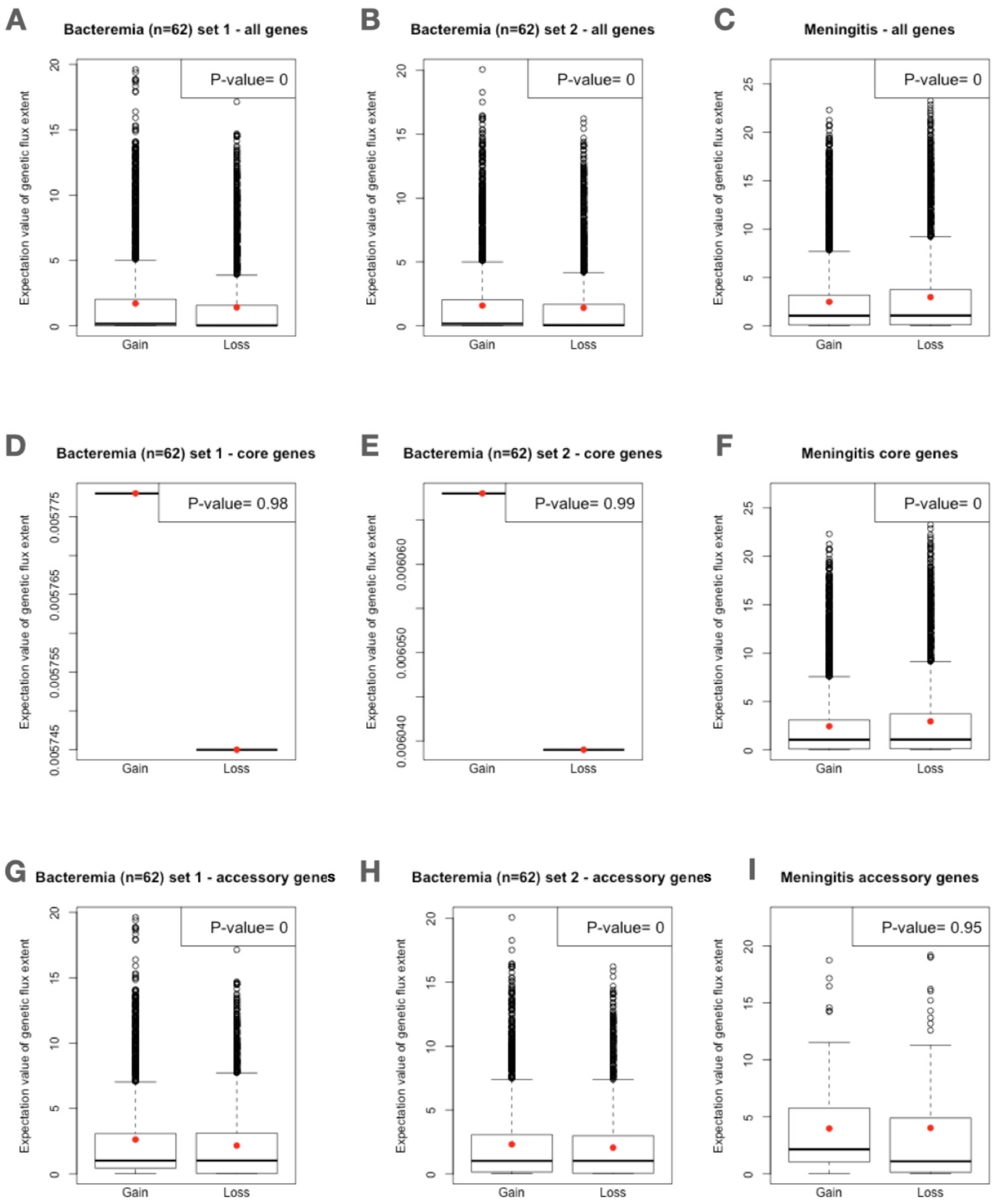

Figure 2. The mean magnitude of genetic flux. mean magnitude of genetic flux using the Welch two-sample t-test for gain and loss events to establish the overall direction of genetic flux in (A & B) the entire set of the bacteremia subsets (n-62), and (C) meningitis dataset. The core genomes of the bacteremia and meningitis sets (D,E, &F), and the accessory genes (G,H, &I).

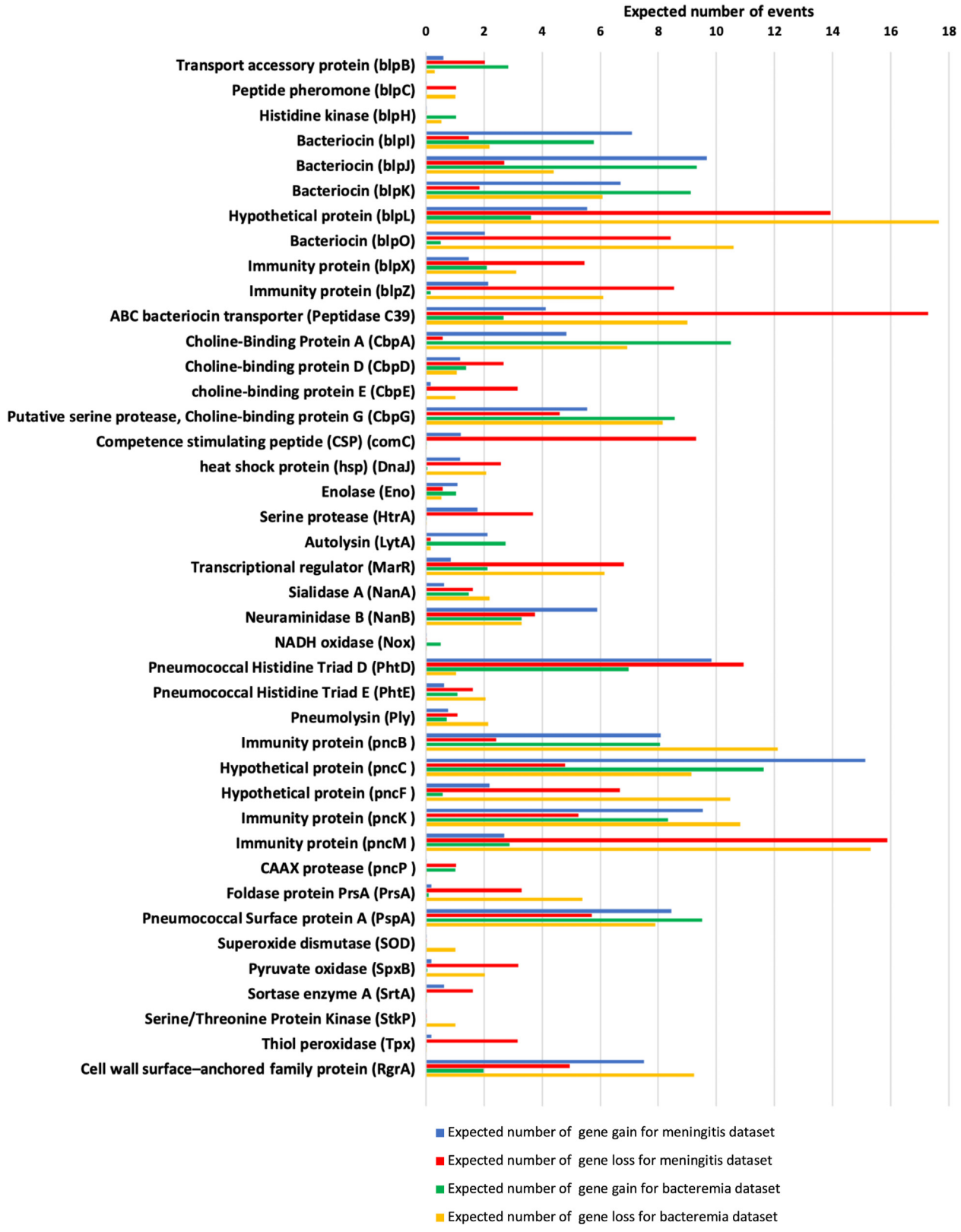

Figure 3. The number of genetic flux events in established antigens in the bacteremia and meningitis datasets.

pneumococcal evolution, may be characterized by constant recombinant sampling of specific genes, at different rates, until they arrive at a combination which provides the requisite gene complement to support invasion and survival in a new niche (blood or CSF) [23].

Despite the high extents of genetic flux within lineages and across groups, it is less pronounced in the meningitis dataset. This validates previous findings using tissue culture and animal models demonstrating the importance of specific gene sets required for tissue-specific invasion

and survival [27, 43, 45, 46]. This implies more considerable transfer of genes among the bacteraemia-associated genomes compared to those associated with meningitis, supporting previous evidence on tissue-specific roles of pneumococcal virulence factors during pathogenesis [43, 44, 45, 46, 47].

It signals the possible requirement of a relatively more conserved gene set to be able to evade immune responses and survive in the CSF. For example, *phtD* facilitates better attachment to respiratory epithelial cells and inhibition of complement deposition, which occurs during pneumococcal invasion of the respiratory system that often occurs prior to entry into the blood to cause bacteremia [48, 49, 50]. Peptidase C39 and competence stimulating peptidase (*comC*) are both important for pneumococcal cell-to-cell communication, and ensure genetic diversity and community-level synchronization to generate phenotypes capable of adaptation to the fluctuations of the host environment [51]. We detected the *RrgA* pilus subunit in a small proportion of the pneumococcal genomes (15%), which is consistent previous findings that detected a low prevalence (9%) of type 1 subunit genes sets, from an pneumococcal datasets from Africa [20]. Although, pneumococci are naturally competent and exhibit no sequence preference, and are therefore hyper-recombinant [23], they possess mismatch repair mechanisms that detect dissimilarities between donor and recipient DNA [23, 40]. A difference in flux could be a result of inadequate mechanisms in the case of bacteraemia or conversely very effectual one in the meningitis group. Our findings suggest that the pneumolysin gene does not have extensive genetic flux, emphasizing its pivotal role in both bacteraemia and meningitis disease outcomes. Differences in flux suggest enhanced competence in the bacteraemia group, thereby increasing uptake of genetic material from the surrounding environment prior to pathogenic invasion [52, 53]. High expectation of gene loss of bacteriocin immunity proteins, pncB, pncF, and pncK, in the bacteraemia dataset also suggests reduced pneumococcal inhibition of other closely related bacteria in the same environment, thereby promoting horizontal gene transfer [54].

These findings highlight the likely direction and magnitude of genetic flux in the core and accessory genes of bacteremia- and meningitis-associated pneumococcal genomes, thereby improving the understanding of the role of selection pressure on pathogenesis at different niche. They validate previous findings using tissue culture and animal models demonstrating the importance of specific gene sets required for tissue-specific invasion and survival.

Declarations

Author contribution statement

BK conceived and designed the experiments. TM performed the experiments. TM and BK analyzed and interpreted the data. TM and BK wrote the paper. All authors read and approved the final manuscript.

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Data availability statement

The datasets used in this study are publicly available in GenBank, and their accession numbers and metadata are provided in the supplementary tables (Supplementary Table 1 and 2).

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e12229.

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