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Article

Distribution of prophages in the *Streptococcus* bacteria genus and their role in increasing host pathogenicity

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SUMMARY

Bacteriophages are a category of viruses that only infect bacteria. Virulent phages cause the cell to produce new phages and lyse, while temperate phages remain in a latent state following phage lysogeny and integration of genetic material into the host genome as prophages. This integration enhances the genetic diversity of the host species, and the new genetic material may offer advantageous traits to aid the host's survival and adaptation to new environments. *Streptococcus* bacteria are pathogens that infect both humans and animals, causing invasive infections and a diverse set of diseases. We hypothesized that strains of *Streptococcus* that have prophages present in their genome are linked to increased pathogenicity or antibiotic resistance. We used PhageWeb to identify prophages found within the 819 strains of *Streptococcus* genus from GenBank and the distribution of prophages across different species within the genus. With the Progressive Mauve software, we compared the sequences of identified prophages to determine similarities and relationships between the different prophages. Ultimately, based on information from gene ontology databases, we found that different prophages were associated with various virulence factors, adherence factors, and antibiotic resistance genes in their respective bacteria. Bacterial strains containing these prophages may have increased pathogenicity and the embedded genes could have a role in bacterial survivability in different environments. This genetic variation and these prophage characteristics shed light on the evolutionary dynamics of these bacteria species and can be applied to phage therapy.

INTRODUCTION

The genus *Streptococcus* contains some of the most invasive bacteria species that inhabit human and animal mucous membranes. They can cause disease, infection, and in extreme cases, death (1). Three of the most common *Streptococcus* species that cause disease in humans are *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Streptococcus agalactiae*. According to the World Health Organization, *S. pneumoniae* is the main factor contributing to the pneumonia-related death of 1.2 million children under 5 years old, most cases of which occur in developing countries (1). Besides infants and children at risk of infection, *S. pneumoniae* also have a severe impact on the elderly and individuals with weakened or compromised immune systems (2). *S. pneumoniae* also causes meningitis and sepsis and has been found to have the capability to acquire both intraspecies and interspecies genetic material from other species inhabiting a similar niche, such as those who are also present within mucosal membranes (3, 4). This adaptability contributes to the high genetic variation of *S. pneumoniae* (3, 4). Another species of invasive *Streptococcus* bacteria is *S. pyogenes*, group A Streptococcus (GAS), which infects around 700 million people every year and is responsible for around 500,000 deaths (5). GAS is responsible for infections such as acute pharyngitis (strep throat), scarlet fever, streptococcal toxic shock syndrome (STSS), rheumatic heart disease, and acute rheumatic fever (6). Furthermore, *S. agalactiae*, group B *Streptococcus* (GBS), causes invasive GBS diseases in infants, including neonatal sepsis, pneumonia, and meningitis, and can also result in miscarriages during pregnancy (7).

In 2014, Gao *et al.* identified two major clusters of *Streptococcus* species; they proposed through a comparative genomic analysis that shared virulence factors contributes to the parallel evolution of virulence factors and bacterial species (8). *Streptococcus* bacteria all possess genes that encode for capsule protection (8). This trait, first identified in *S. pneumoniae* in a study by Hyams *et al.*, protects the bacteria against the host's immune system by evading phagocytosis, the process of a cell engulfing the bacteria to kill it (9). The bacteria without the genes that encode for the capsule are more likely to be eliminated, thus proving that capsule protection is a favorable, preserved trait (9). As such, this association between evolution, survival, and virulence factors stresses the contribution of genetic variation to pathogenicity of *Streptococcus* bacteria. Thus, the infectiousness of these *Streptococcal* bacteria is caused by antibiotic resistant genes and virulence factors, as concluded by prior research. There is a significant body of knowledge surrounding the persistence of *Streptococcus* bacteria among the general population. Despite these insights, little research has been published investigating the relationship between *Streptococcus* bacteria as a whole and the ability of phages to transfer these traits from bacteria to bacteria.

Phages, also known as bacteriophages, are viruses that specifically infect bacteria without harming human or animal cells (10). They are the most abundant, and likely the most diverse, group of organisms on the planet (11). Phage DNA is successfully integrated into the bacterial genome by the process of Horizontal Gene Transfer (HGT) and transduction (12). Through transduction, phages can inadvertently perform HGT between bacteria (13). Phages move genetic information from the donor bacterium when infecting other bacteria, a process that is non-genealogical and can even occur across different species (14). HGT increases genomic

variance among bacteria, and ensuing natural selection preserves favorable characteristics, including virulence factors and antibiotic resistance (15). Due to large population sizes, rapid growth, relatively short doubling time, and non-membrane-bound genetic information, bacterial acquisition of these traits occurs rather frequently and quickly (16). Through phage-mediated recombination and transduction, the genetic diversity of *Streptococcus* bacteria is increased and favorable traits for optimal survival under selection pressures are obtained (17). Consequently, HGT is involved with bacterial environmental adaptation and contributes to bacterial genome evolution and structure (16, 18).

Virulent phages, through lysis, force the host to use its own nucleotides and amino acids to produce structure for the phage. Then, the host cell lyses and ejects the new phages. Temperate phages, through both lysogeny and lysis, integrate their DNA into the host's genome during lysogeny and remain there while the bacterium replicates, resulting in bacterial daughter cells with temperate phage DNA. Replication occurs until the cells enter the lytic cycle (19). Lysis often follows environmental factors, such as exposure to ultraviolet light or certain chemicals, or spontaneous prophage induction (20). Dispersed host and viral genes are subsequently available for uptake by nearby bacteria (21). Phages may become "grounded" and are unable to induce cell lysis when a mutation occurs in the location of phage integration into the host genome or in the genes that encode for the particular recombinase that removes the prophage for lysis (22, 23). Ultimately, temperate phages remodel the individual bacterium's genome by lysogeny and impact bacterial populations by lysis (15). Prophages are temperate phages that have undergone lysogeny and are latent as they do not actively harm the host bacteria (24). They play a crucial role in the evolution of pathogenic bacteria via HGT, diversifying bacterial genome architecture (25, 26). Using microarray analysis on mRNA expression patterns, Smoot *et al.* concluded that prophages play a major role in cell physiology through gene regulation (27, 28).

To further understand the mechanisms behind virulence and antibiotic resistance within the *Streptococcus* species, we have identified the distribution of prophages within all current *Streptococcus* strains and the relations between the prophages. We hypothesized that the prophages found within the most strains of *Streptococcus* bacteria would be associated with virulence and antibiotic resistance. In total, we identified 879 prophages the genomes of 819 *Streptococcus* strains available on the National Center for Biotechnology Information (NCBI) Genome Database. Due to some prophages being found across different *Streptococcus* species and some *Streptococcus* species possessing multiple instances of the same prophage sequence, we concluded that there have been many instances of HGT and that prophages aren't fixed to one specific *Streptococcus* bacteria. Consequently, we deduced that HGT with prophages provide advantageous traits to the bacterial host. The goal of this research was to interpret the role of prophages across the *Streptococcus* genus to pinpoint commonalities to be used as potential targets for wide-ranging translational medicine.

RESULTS

To determine the type of prophage present, how frequently the prophage appeared within one strain, and the prevalence of the prophage across the entire *Streptococcus* genus, we input the accession numbers of the *Streptococcus* strain genomes input into PhageWeb. We subsequently utilized Progressive Mauve to align the prophage sequences identified in each of six species with the most total strains. In total, we analyzed 819 *Streptococcus* strains and identified 879 prophages. While most of the strains contained at least one prophage, 32% of all strains (267 out of 819) did not possess prophages within their genome. There were more total prophages than total bacterial strains that contain prophages, indicating that some strains contain more than one type of prophage in its genome (**Table 1**).

Total Prophages	879
Total Bacterial Strains	819
Bacterial strains without phages	267

***Streptococcus* Phage Name**	**Number of Phages Identified**	**Total Species with Phage**
20617	165	5
phiD12	147	14
T12	136	6
P9	110	6
JX01	77	7
phi3396	43	5
LYGO9	33	5
A25	20	3
SpSL1	18	7
phi20c	17	1
Spn1	12	4
phiARI0131-2	10	2

Table 1. Occurrence of prophages in species and strains of *Streptococcus* identified by PhageWeb. The phages are ordered by prevalence. The total prophages are greater than the total strains because some *Streptococcus* strains had more than one phage present.

We studied a total of 56 species of Streptococci, and the five species with more than 50 strains were selected for further analysis. Each of these five species possessed multiple strains that had at least one prophage present, with as many as eight prophages present within three different strains of *S. pyogenes* (**Table 2**). Furthermore, the phage that was most prevalent across all species of *Streptococcus* was prophage phiD12. It was also the most prevalent among the five *Streptococcus* species present in all except for *S. pneumoniae* (**Figure 1, Table A1**). Although there were more appearances of prophage 20617 (18.8%, 165 out of 879 phages) than prophage phiD12 (16.7%, 147 out of 879 phages), the majority of prophage 20617 were found in strains of *Streptococcus thermophilus* (88.5%, 146 out of 165 phages)

Streptococcus Species	Strains with at least one prophage	Number of Prophages per Strain							
		1	2	3	4	5	6	7	8
S. pyogenes	179	41	45	33	25	9	19	4	3
S. thermophilus	60	30	3	2	8	10	3	3	1
S. agalactiae	56	25	18	11	0	1	1		
S. suis	55	24	12	13	5	1			
S. pneumoniae	28	21	5	2					

Table 2. Distribution of prophages per strain for the top five *Streptococcus* species.

(**Figure 2, Figure A1**). Therefore, prophage 20617 seemed to be unique to *S. thermophilus*, while prophage phiD12 was shared between several bacteria species. Overall, *S. pyogenes* had the most prophages present (26.9%), likely due to having the most strains in the analysis (220 out of 819 strains) (**Figure 1, Table A1**).

The third most common phage among all strains of *Streptococcus* studied was *Streptococcus* phage T12 and was identified in six Streptococcus species. Of these six species, *S. pyogenes* contained the most strains that possessed the phage (**Table 1**). Overall, *Streptococcus* phage T12 was found in 136 strains of the *Streptococcus* genus (16.6%, 136 out of 819 strains), and 84.6% (115 out of 136 strains) were identified as *S. pyogenes* (**Figure 1, Figure A1**). Following *Streptococcus* phage T12, *Streptococcus* phage P9 is the fourth most common phage found in the *Streptococcus* genus (12.5%, 110 out of 879 phages), and is also most commonly found in *S. pyogenes* (84.5%, 93 out of 110 strains) (**Figure 1, Figure A1**). This trend may likely be due to *S. pyogenes* having the most strains (317 strains) compared to other species of *Streptococci*. Furthermore, prior research indicates that *Streptococcus* prophages phiD12, 20617, T12, and P9 all have been found to be associated with pathogenicity.

As the G+C content, or the amount of guanine-cytosine base pairs, of the *Streptococcus* genus is low compared to other bacteria genera, averaging about 39.25%, the G+C content of the prophages in *Streptococcus* species could also be classified as low (**Table 3**) (8). There is evidence of an association between the G+C content of the host bacterial genome and that of the bacteriophage due to a need to remain integrated in the host genome. Thus, prophage G+C content also corresponded to the G+C content of the host and this similarity allows the host to determine if the inserted DNA is compatible with their own genetic material (29, 30). Moreover, the prophage will continue to be passed down to subsequent daughter cells, and ultimately increase the chances of HGT between different strains of *Streptococcus* bacteria once it enters the lytic cycle due to environmental stressors (31).

With Progressive Mauve Multiple Genome Alignment, we identified locally collinear blocks (LCBs) from the complete genomes of all prophages present in *S. pyogenes* and *Streptococcus suis* (**Figure 2**). LCBs are homologous sequences without genomic rearrangements shared by selected genomes under study. These similarities are represented through color-coded sections and lines that link the LCBs. In the synteny map for prophages in *S. pyogenes*, similarity between genomes was relatively lower than in *S. suis*. Moreover, the LCBs, and consequently the genes, were embedded within the prophage genomes in a random manner (**Figure 2a**). This was likely due to the variety of strains of the species and prophages present within the genomes (**Figure 1, Table A1**). *S. suis* showed the most LCBs, which were

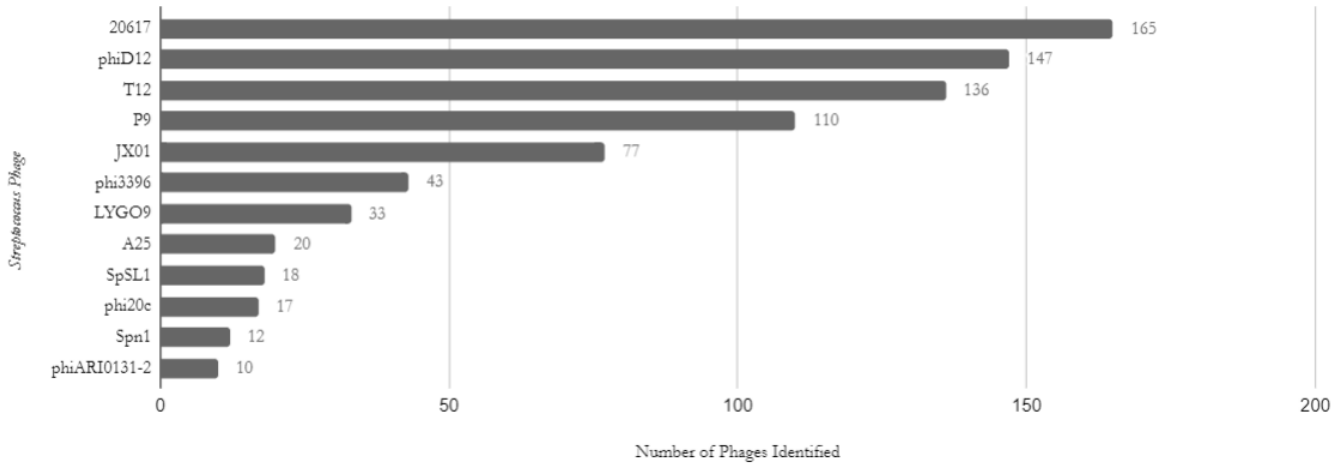

Figure 1. Distribution of *Streptococcus* phages across 819 *Streptococcus* strains with a frequency of 10 or greater. After identifying the prophages in all 819 strains of *Streptococcus* bacteria with PhageWeb, we compiled the total number of prophages found in each strain. Only the Streptococcus species with 10 or more strains were selected.

Streptococcus Species	Average G+C%	Minimum G+C%	Maximum G+C%
S. agalactiae	35.74	34.69	37.17
S. pneumoniae	40.33	38.59	42.34
S. pyogenes	38.45	37.31	40.59
S. suis	41.08	39.60	42.21
S. thermophilus	38.78	37.89	40.32
Total	38.88	34.69	42.34

Table 3. G+C content of prophages found in the top five *Streptococcus* species.

arranged in a more predictable manner than those found in *S. pyogenes* (**Figure 2b**). The prophage genomes of both *S. suis and S. pyogenes* showed very similar sequences, indicating that the groups of prophages found in both species likely underwent multiple instances of the same HGT (20). Prophages found in strains of *S. suis*, prophages phiSS12, phi7917, phi891591, phi5218, phi20c, and SMP were the most similar to one another due to many LCBs with high similarity plots (**Figure 2b**). In addition, prophages phiST1, phiD12, and SpSL1, were the most similar to one another. The order of the LCBs was highly variable from prophage to prophage, creating a mosaic structure.

DISCUSSION

We identified 879 prophages present within the 819 strains of *Streptococcus* bacteria, with 67.4% of the bacterial strains containing prophage. The most common prophage found in streptococcal bacteria strains is *Streptococcus* phage phiD12, found most frequently in *S. suis*. Prophages that are genetically similar to phiD12 contain genes that are possibly involved in GBS pathogenesis and increased lysogeny (32). Consequently, its presence in 14 of 56 species in the *Streptococcus* genus indicates the potential transfer of similar traits into other bacteria. Prior research indicates that some genes located within phage phiD12 encode defense mechanisms against HGT and phage attacks, as well as the ability to rapidly adapt to hostile environments. This also includes cell division, biofilm formation, and bacterial persistence (33).

The phage that is most prevalent throughout the *S. pyogenes* strains is *Streptococcus* phage T12. This phage contains the *speA* gene that encodes erythrogenic toxin A, a toxin that potentially causes scarlet fever and STSS when incorporated into *S. pyogenes* genome as a prophage (34). Scarlet fever was on a decline, yet the last decade revealed major outbreaks in countries including England, Vietnam, and China, as well as minor outbreaks in North America (35). On the other hand, STSS cases are very rare and are generally sporadic in nature as they only occur within closed environments (36).

Another prophage that is most commonly found in *S. pyogenes* and *Streptococcus equi* is *Streptococcus* phage P9. In *S. equi*, specifically *S. equi subsp. Zooepidemicus*, prior research has determined it is similar to other phages in *S. pyogenes*, indicating that phage P9 has hosts across different species (37). In addition, the phage contains many virulence factors, including mitogens, hyaluronidase, and streptodornase, and disrupts DNA recombination and repair (37). Defense and adaptation genes were also located in phage P9 (29). However, many of the prophages identified in the *Streptococcus* genus have unknown functions, which limits identifying the role they play in the bacteria.

Previous studies of prophages have uncovered their association with pathogenicity and ability to improve the survival fitness of the lysogen, in addition to increasing antibiotic resistance among bacteria, as they rely on the host for survival (38). A study by Javan *et al.* showed that the presence of prophages can increase host pathogenicity

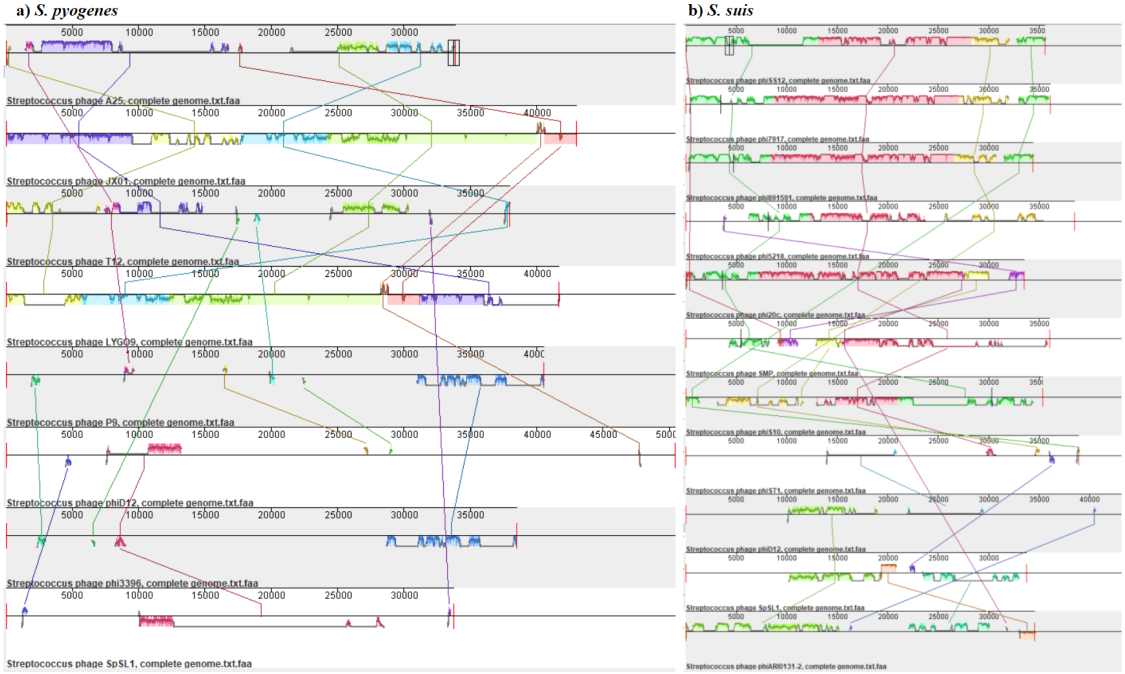

Figure 2. Progressive Mauve alignment of prophages present in S. pyogenes and S. suis. The color-coded regions represent homologous sequences shared between the phages. **a)** *S. pyogenes*. **b)** *S. suis*.

(39). These useful genes transferred by prophages provide a selective advantage to the host by increasing immunity and protection. Overall, prophages increase the genetic variability between different strains of the same bacterial species (13, 40).

Our research is limited by the number of bacterial sequences that are available in online databases. As the rate of genomic sequencing increases, we will get an even clearer picture of the distribution of prophages in different bacterial species. Future research may delve into identifying certain genes with vast distribution in bacterial genomes and correlating it with their specific functions and phenotypes based on wet-lab techniques. Identifying the distribution of prophages is an important first step in understanding their role in the bacteria.

Phages may be manipulated for phage therapy by taking advantage of their broad host range in bacteria that infect both livestock and humans (31). Phage therapy utilizes bacteriophages that inject themselves into the target bacteria to induce the lytic cycle and halt bacterial growth. (31). This is a promising alternative to antibiotics due to an increase in antibiotic-resistant bacteria, and phage therapy may also be applied in biosensors to detect pathogenic bacteria (41, 42). Identifying the types of prophages present within the *Streptococcus* bacteria, as well as their frequency and distribution, will allow us to further understand the role and host specificity of prophages within the bacterial genome. Pathogenic bacteria contain numerous lysogenic prophages in their DNA, which includes virulent and temperate phages in a latent state, yet certain conditions may initiate prophage induction and the beginning of the lytic cycle (43). Identification of phage-inducing factors and detection of toxins like hydrogen peroxide and streptococcal pyrogenic exotoxin C in bacterial genomes and prophages may serve to further the lytic nature of phages used in phage therapy (44). Whole-genome sequencing of human bacterial pathogens and further bioinformatics analyses should be done to clarify the pattern and timing of gene expression of prophages. This may help future CRISPR/Cas9 genome editing usage to genetically modify these phages for viable implementation of phage therapy.

In conclusion, our results point to several instances of the same prophage being found across different species of *Streptococcus*, which indicates that bacteriophage-mediated HGT could be occurring at a high frequency throughout the genus. Moreover, we propose that bacteria with the most individual prophages are likely to have many different types of prophages embedded within its genome and may confer virulence factors to aid the host's pathogenicity. These findings align with prior research on *S. suis* and GAS by Tang *et al.* and Banks *et al.* emphasizing the association between the presence of prophages and virulence factors (44, 45). These prophages may have a larger influence on the host genome than currently understood. Our results demonstrate that prophages are not always host specific, aiding their ability to survive (46). Their wide host range may be advantageous for phage therapy, with implications that a single phage can combat infection from multiple bacterial species.

MATERIALS AND METHODS

Accessing Genomic Data

Accession numbers of 819 strain genomes from 56 *Streptococcus* bacteria species were retrieved from U.S. National Institutes of Health NCBI Genome Database (47). Following retrieval, the species were ordered alphabetically. The *Streptococcus* species with the most total strains were focused on, specifically those with 50 or more strains. These species were *S. agalactiae* (127 strains), *S. pneumoniae* (83 strains), *S. pyogenes* (220 strains), *S. suis* (67 strains), and *S. thermophilus* (65 strains). These strains were picked because they were the most abundant in the NCBI Genome Database.

Identifying Prophages

All prophages were identified with the software PhageWeb (43). PhageWeb utilizes the changes in G+C content and transfer RNA prediction sites, and also compares genetic sequences to a reference phage genome within online databases, specifically the NCBI database and the European Bioinformatics Institute database (48). The parameters were set to 80% identity for Basic Local Alignment Search Tool Options, at least 6 coding sequences for Prophage Identification, and at least 80% Genome Integrity as a cutoff parameter in PhageWeb, which is the standard cutoff set by the website. When analyzing the results, we looked at all strains, regardless of complete or incomplete integrity. Finally, only the *Streptococcus* species that had an associated accession number was input into PhageWeb.

One limitation of the PhageWeb is that when we input some *S. pyogenes* strains, the software returned *S. pyogenes* as an output, although *S. pyogenes* itself is not a phage. This is most likely due to a misclassification within the NCBI database from which the data was retrieved, hence we have excluded *S. pyogenes* as a result from the data set of phages identified in strains of *Streptococcus*.

Aligning Prophage Genomes and Synteny Comparison

The genome sequence of the prophages was retrieved from NCBI GenBank. Afterwards, the sequences were grouped together according to their presence in the *Streptococcus* species with the most total strains and total phages mentioned above. The sequences were aligned with Progressive Mauve: Multiple Genome Alignment tool (version 2.4.0) and subsequently ordered based on similarity (49).

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