The Intricate Relationship of G-Quadruplexes and Pathogenicity Islands: A Window into Bacterial Pathogenicity

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Abstract

The dynamic interplay between guanine-quadruplex (G4) structures and pathogenicity islands (PAIs) represents a captivating area of research with implications for understanding the molecular mechanisms underlying pathogenicity. This study conducted a comprehensive analysis of a large-scale dataset from reported 89 pathogenic strains to investigate the potential interactions between G4 structures and PAIs. G4 structures exhibited an uneven and non-random distribution within the PAIs and were consistently conserved within the same pathogenic strains. Additionally, this investigation identified positive correlations between the number and frequency of G4s and the GC content across different genomic features, including the genome, promoters, genes, tRNA, and rRNA regions, indicating a potential relationship between G4 structures and the GC-associated regions of the genome. The observed differences in GC content between PAIs and the core genome further highlight the unique nature of PAIs and underlying factors, such as DNA topology. High-confidence G4 structures within regulatory regions of *Escherichia coli* were identified, modulating the efficiency or specificity of DNA integration events within PAIs. Collectively, these findings pave the way for future research to unravel the intricate molecular mechanisms and functional implications of G4-PAI interactions, thereby advancing our understanding of bacterial pathogenicity and the role of G4 structures in infectious diseases.

Key Words: Guanine quadruplex; Pathogenicity island; Genome; *Escherichia coli*

Introduction

The discovery of the DNA double helix by Watson and Crick in 1953 revolutionized our understanding of genetics and laid the foundation for the modern field of molecular biology (*1*). Nonetheless, the intricate nature of DNA continues to surprise us even today. One such captivating feature is the DNA guanine (G)-quadruplex (G4) structure, a unique arrangement that defies the conventional double helix (*2, 3*). A G4 consists of four guanine bases and stabilized by Hoogsteen hydrogen bonding. These stacked tetrads are interconnected by loop regions, which can vary in length and sequence, adding further complexity to the structure (Fig. 1). It is important to consider the inherent directionality of nucleic acids, with all four strands having the possibility to run in the same 5' to 3' direction, referred to as “parallel”, or alternatively, they can run in different directions, known as “antiparallel”. G4 regions can be very stable *in vitro*, particularly in the presence of K^+ (*4*).

Pathogenicity islands (PAIs) are genomic regions that contribute to the virulence and pathogenic potential of various microorganisms (*5, 6*). PAIs often exhibit close proximity to tRNA genes, suggesting a putative mechanism where tRNA genes act as anchor points for the integration of foreign DNA acquired through horizontal gene transfer (Fig. 1E). One notable feature is their variable GC content, which tends to deviate from the average GC content of the genome in various organisms, such as *Streptomyces* (*7*), *Salmonella* (*8*), and *Yersinia* (*9*). One question raised in PAI is that PAIs often exhibit distinct base composition (G+C contents) compared to the core genome. The underlying reasons for this variation remain unknown, but the

preservation of a genus- or species-specific base composition represents a noteworthy characteristic of bacteria (*5*). Herbert Schmidt et al. (2004) proposed a hypothetical mechanism to explain the observed variation, suggesting that factors such as DNA topology and codon message in the virulence regions present could contribute to the preservation of the distinct base composition (*5*). Hopefully, the availability of genome sequences from pathogenic bacteria and their non-pathogenic counterparts currently presents an exceptional opportunity to explore the intricate structure variance and underlying mechanisms within PAIs.

The possibility of interactions between G4 structures and pathogens has been suggested, although this field of study is still in its nascent phase. Some studies observed that bacterial genomes possess G4-forming sequences within their genome regions (*10, 11*). G4 structures are formed by G-rich DNA sequences, and their stability is influenced by the G+C content and arrangement of G tetrads. Interestingly, PAIs often exhibit an altered GC content, putatively contributing to the propensity of G4 structure formation within these regions. The G4 structures in PAIs might modulate the accessibility of transcription factors, DNA-binding proteins, or RNA polymerase in pathogens, as documented in eukaryotes (*2, 12*), thereby influencing the expression of virulence-associated genes. The formation of G4 structures within PAIs may serve as an additional layer of regulation that fine-tunes the expression of genes critical for pathogenesis. Hence, the investigation of G4 structures within PAIs may open new avenues for the development of therapeutic strategies aimed at disrupting the regulatory mechanisms of pathogenicity-associated genes.

Results and Discussion

Genomic information, PAI patterns, and the presence of G4s in 89 reported pathogenic strains

A dataset of PAIs was compiled from 89 reported pathogenic strains, encompassing 222 distinct types of PAIs. Those pathogens that share closely related PAIs on phylogenetic branches, such as LEE in *E. coli* strains (Fig. 2A), may have acquired these elements through recent common ancestors or horizontal gene transfer events (*5*, *13*, *14*). Additional information, including the genome length (bp), G+C content (%), rRNA density, tRNA density, and PAI length (bp), was present and showed conserved patterns in the same species (Fig. 2A & Table S1). PAIs commonly exhibit mosaic-like patterns, exemplified by the presence of distinct PAIs like FPI in *Francisella tularensis*, SaPIbov in *Staphylococcus aureus*, and Hrp PAI in *Xanthomonas campestris* (Fig. 2B). Many PAIs were present associated with tRNAs, such as the insertions of $tRNA^{Thr}$, $tRNA^{Phe}$, and $tRNA^{Gly}$ in *E. coli* strains (Fig. 2B & Table S2). The presence of PAIs distributes in similar genomic regions across different pathogens or strains, showing non-random patterns and functionally clustered. Employing the G4Hunter search algorithm, the study identified a total of 225,376 putative G4 sequences in these 89 pathogenic genomes (Table S1). The heatpmap showed that the number of G4 structures was diverse in the pathogen genomes (Fig. 2C), similar to previous reports that showed that the G4 structures display uneven

distribution patterns in eukaryotic and prokaryotic genomes and are conserved in evolutionary groups (*15-17*).

Interaction between PAIs and G4s in different genomic features

The analysis of G4 structures across all pathogen species demonstrated a positive correlation between the number of G4s and the GC content in various genomic features, including the whole genome, gene, promoter, rRNA, and tRNA regions (Fig. 2D). Furthermore, the density of G4s, measured as the frequency of predicted G4-forming sequences per 1000 base pairs (bp), also showed a positive correlation with the GC content across the analyzed genomic elements (Fig. 2E). The presence of G4 structures within the promoter, rRNA, and tRNA regions may have functional implications for the regulation of DNA replication, ribosome biogenesis, protein synthesis, and other RNA-related processes (*18-20*). Throughout evolution, there seems to be a greater enrichment of G4 structures in regulatory genes compared to other genes, enabling intricate control of gene expression in cascade signal transduction pathways (*21*).

This study observed that the GC contents in the genome region were significantly higher compared to the corresponding PAIs region that was classified into five parts according to the genome datasets (Fig. 3A-E & Table S1). This suggests that the genomic regions surrounding the PAIs (i.e., core genome) tend to have a higher GC content than PAI regions, which was consistent with the fact that PAIs often exhibit distinct base composition compared with the core genome (*5*). The variation was explained by the presence of G4 sequences within the PAIs, whereas the results were

surprising. This study observed a distinct pattern in the frequency of G4 structures within different regions of the PAIs. This differential distribution of G4 structures suggests that (i) specific segments (e.g., <30% and >60% GC contents) within the PAIs may be more prone to induce G4 formation discrepancy, as these segments showed unstable gene structure; (ii) the variation of base composition between core genome and PAIs is partially correlated with the presence of G4s; (iii) the frequency of G4s in PAIs present stable as the core genome in the most situation; (iv) an alternative hypothesis, other factors, such as i-motif (i.e., the anti-G4 structure) and CpG island, may work synergistically with G4 and potentially contribute the base composition variation (*22*, *23*).

Putative origin, transfer mechanisms, and functions of G4s in PAIs

To understand the origin of G4 structures within PAIs, the study hypothesized that these G4 sequences could be acquired through three types of horizontal gene transfer mechanisms: conjugation, transformation, and transduction (Fig. 3F). These mechanisms serve as means for genetic material exchange between different organisms. Considering the presence of G4 sequences within the PAIs, it is plausible that these sequences are transferred along with the PAIs through these horizontal gene transfer mechanisms. Later, the study used *E. coli as an example* to investigate the potential regulatory role and function of genes covered by G4 structures in PAIs. *E. coli contains at least ten types of* PAI in different strains, and one of the well-known PAI is LEE (Fig. 3G), harboring genes responsible for causing attaching and effacing lesions (*14*, *24*). One stable G4 structure with a G4hunter score of 1.6 was identified

at position 37,085 in the LEE PAI of *E. coli* str. O103:H2 12009 (Fig. 3H), located between an IS element and a tRNA insertion site. The tRNA region generally contains a higher G4 frequency compared with transfer-messenger RNA (tmRNA) and rRNA regions in the bacterial genome (*16*). Interestingly, this G4 structure found in *E. coli* str. O103:H2 12009 was present in close proximity to a tRNA region, suggesting a potential regulatory role of G4s in the tRNA gene, or upstream- and downstream-genes that are responsible for LEE virulence. Additionally, another stable G4 sequence with a score of 1.381 was discovered at position 12,457 in the *E. coli str. CFT073* PAI II to provide one more evidence of G4 in PAI regions (Fig. 3I).

Functional enrichment analysis was conducted to explore the putative functions of G4-covered genes in two *E. coli* strains (Table S3&S4). The results revealed that the genes covered by G4 structures were predominantly involved in genetic information processes, including DNA binding, DNA integration, and nucleic acid metabolism processes (Fig. 3J & K). This suggests that G4 structures may play a regulatory role in these essential cellular processes, especially gene expression and DNA-related functions. For instance, G4 structures in the promoter regions of certain transcription factors may influence their binding affinity to DNA and subsequently affect downstream gene expression patterns (*25, 26*). These elements frequently utilize DNA integration mechanisms mediated by integrases, recombinases, or transposases to transfer or incorporate genetic material into the bacterial genome (*27, 28*).

Overall, the conserved evolutionary relatedness of PAIs, the detection of stable G4 structures in specific genomic positions, and the enrichment of G4-covered genes in genetic information processes collectively support the hypothesis that G4 structures may have regulatory functions in key biological processes in pathogens.

Methods

Selection and extraction of DNA Sequences

A total of 89 genomes corresponding to the identified pathogens from the Pathogenicity Island Database (PAIDB) were included in the study. The complete bacterial genomic DNA sequences and their corresponding annotation files in .gff and .fna formats were obtained from the Genome database of the National Center for Biotechnology Information (NCBI). To ensure the reliability and completeness of the dataset, only completely assembled genomes were included in the analysis. To avoid redundancy and incomplete sequences, one representative genome was selected for each species or strain. The selection of representative genomes was based on a careful examination of the supplementary material (Table S1) accompanying the study. The study employed TBtools (https://cj-chen.github.io/tbtools), a versatile bioinformatics tool, to investigate the presence of G-quadruplex (G4) sequences in specific genomic features. The gene regions, promoters (2 kb upstream of the genes), tRNA regions, and rRNA regions were extracted from the selected genomes using TBtools. PAI regions were downloaded following previously documented information in PAIDB

(Table S1&S2). Default thresholds and parameters were applied during extraction to maintain consistency across all genomes.

Data process and detection of G4s in genomic features

The G4Hunter algorithm, a widely used tool for G4 prediction, was employed for the identification of G4 motifs in the genomic sequences. The G4Hunter parameters were set to a window size of "25" and a G4 score threshold of 1.2, which ensured the identification of potential G4 sequences. The analysis was performed for each species group, where the genomic DNA size and the number of putative G4 sequences found were determined. The study quantified the predicted number of putative G4-forming sequences within different genomic features, including the whole genome, gene, promoter, tRNA, rRNA, and PAI regions. The density of G4 motifs was determined by dividing the number of G4 sequences by the total length of the genome, while the length ratio of G4 motifs was calculated by dividing the total length of the G4 sequences by the total length of the genome.

Relationship between G4s and PAIs

The heatmap was used to show the distribution of G4 motifs in the genome divided by ten parts as PAI regions using R package “pheatmap”. The correlation between the number of G4 structures and the GC content was analyzed across various genomic elements, including the whole genome, gene, promoter, rRNA, and tRNA regions. The analysis utilized the *R*-squared value (R^2) to determine the strength of the correlation. The significance of the correlation was assessed using *p*-values. The GC content in the genome regions and corresponding PAI regions was compared and

classified into different ranges to explore the variation in base composition. GraphPad Prism (V.5.02, GraphPad Software, Inc) was employed to conduct Normality and Lognormality Tests. The K-S test and F-test were used to assess normal distribution and variances, and Student's t-test was used to identify significant difference.

Phylogenetic Tree Construction

The exact Taxonomy ID (taxid) for each analyzed group was obtained from the NCBI Taxonomy Database using the Taxonomy Browser. The Neighbor-Joining (NJ) method was employed to construct the phylogenetic trees for the analyzed groups. The phylogenetic trees were generated using MEGA11 software (www.megasoftware.net), which offers robust algorithms and comprehensive tools for phylogenetic analysis. To assess the reliability and statistical support of the phylogenetic tree branches, bootstrap analysis was performed. One thousand bootstrap replicates were used to estimate the confidence levels of the branching patterns in the phylogenetic trees. The phylogenetic trees, along with the bootstrap support values, were displayed and visualized using the Interactive Tree Of Life (ITOL) platform (https://itol.embl.de/).

Gene functional annotation

The gene sequences covered by G4 structures within PAIs were subjected to gene ontology annotation. The gene sequences were translated into protein sequences using the Expasy online toolkit (https://web.expasy.org/translate/). This tool performs the translation based on the standard genetic code, converting the DNA nucleotide sequence into its corresponding amino acid sequence. The GO annotation database

assigned GO terms to the protein sequences based on their predicted functions and known biological process (BP), molecular function (MF), and cellular component (CC). Fisher's exact test was employed to determine the statistical significance of the enrichment results. The obtained *p*-values indicated the overrepresentation of specific GO terms, with lower p-values suggesting higher significance.

Statistics and reproducibility

All genomic data utilized in this study, including the species-specific datasets, were obtained from publicly available sources. Statistical analyses, such as the Kruskal-Wallis test, F test, Student's t-test, Wilcoxon test, and chi-square test, were performed using GraphPad Prism software. The samples used in the statistical analyses corresponded to the genomic data, PAIs, or specific genes under investigation.

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Competing Interests

The author declares no competing interests.

Funding information

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Credit author statement

Bo Lyu: writing original draft, data analysis, and visualization.

Ethical approval

Ethical review and approval were not required for this study.

Availability of data and materials

The original reported PAIs datasets analyzed in this study are available from the publication with the DOI: 10.1093/nar/gku985. Additionally, supplementary materials accompanying this article provide further PAIs data analyzed in the study. These supplementary materials contain detailed information and datasets that support the findings and conclusions presented in this research. Researchers and interested individuals can access supplementary materials to explore and validate the results obtained in this study.

Consent for publication

Not applicable.

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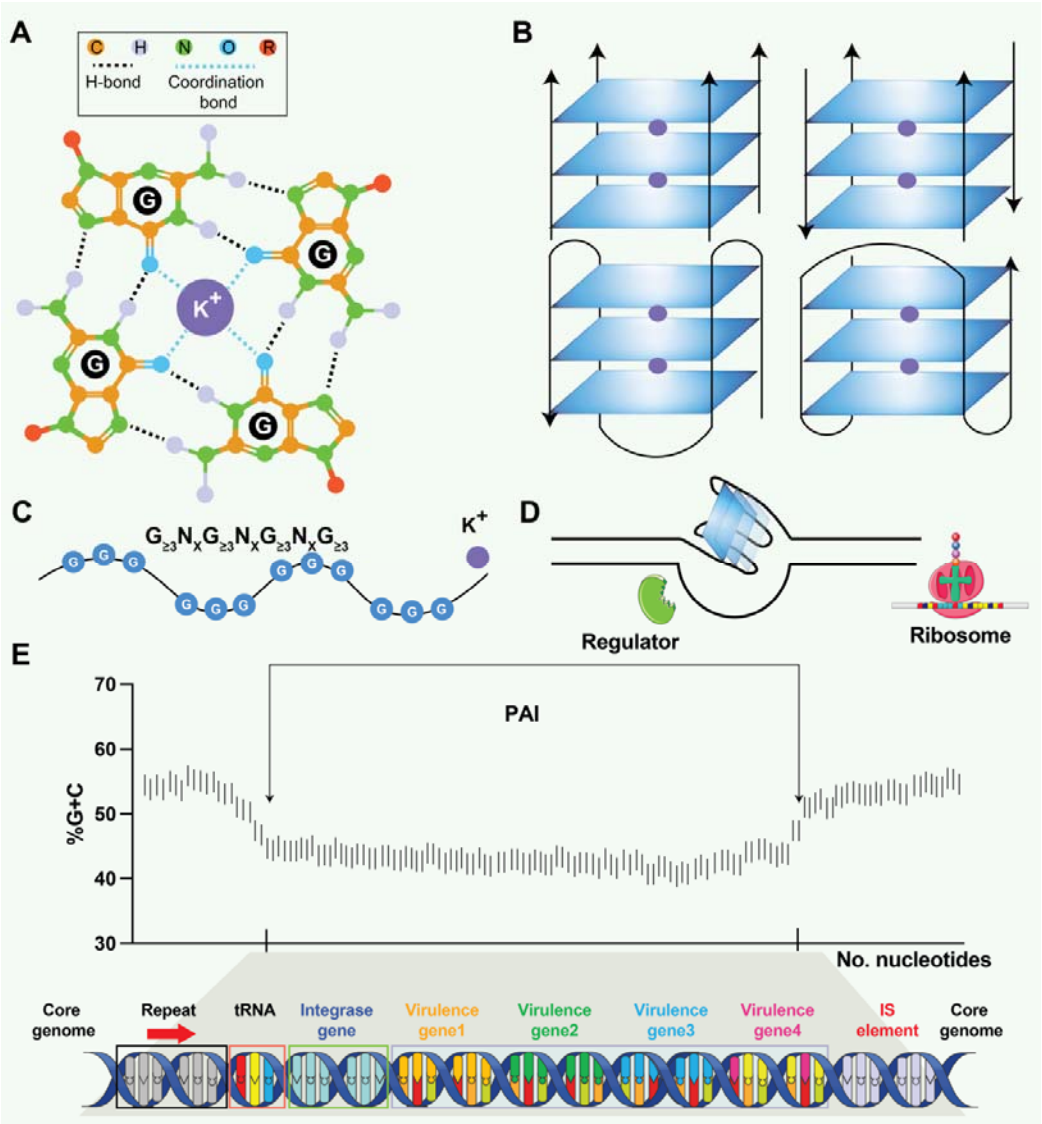


Figure 1. Structural and Functional Aspects of G-quadruplex (G4) Structures and Pathogenicity Islands (PAIs). (A) Schematic representation of a guanine tetrad stabilized by Hoogsten base pairing and a positively charged central ion, illustrating the key elements of G4 structures. (B) Structural heterogeneity of G4s. G-quadruplex structures exhibit polymorphism and can be categorized into different families, such as parallel or antiparallel, based on the orientation of the DNA strands. They can fold either intramolecularly or intermolecularly, leading to diverse structural

configurations. (C) General sequence formula for G4s, highlighting the repeated occurrence of guanine-rich sequences that form G-quartets. (D) Regulatory roles of G-quadruplexes in transcription. G4 structures can regulate transcription by blocking RNA polymerase from binding to promoter sequences or aiding in single-stranded DNA (ssDNA) formation, thereby enhancing transcription. (E) General structure of PAI. PAIs are characteristic regions of DNA found within the genomes of pathogenic bacteria, distinguishing them from nonpathogenic strains of the same or related species. Repeat sequences are DNA segments duplicated within the PAI and can serve as recognition sites for various enzymes involved in the integration and excision of the PAI from the bacterial chromosome. tRNA genes act as anchor points for the insertion of foreign DNA acquired through horizontal gene transfer. Virulence genes encode proteins or factors that play crucial roles in the virulence and pathogenicity of the bacterium, contributing to adhesion, invasion, immune evasion, toxin production, or other pathogenic mechanisms. Insertion elements include transposons, bacteriophages, or plasmids, enabling the PAI to be transferred between bacterial cells and potentially disseminated to different strains or species.

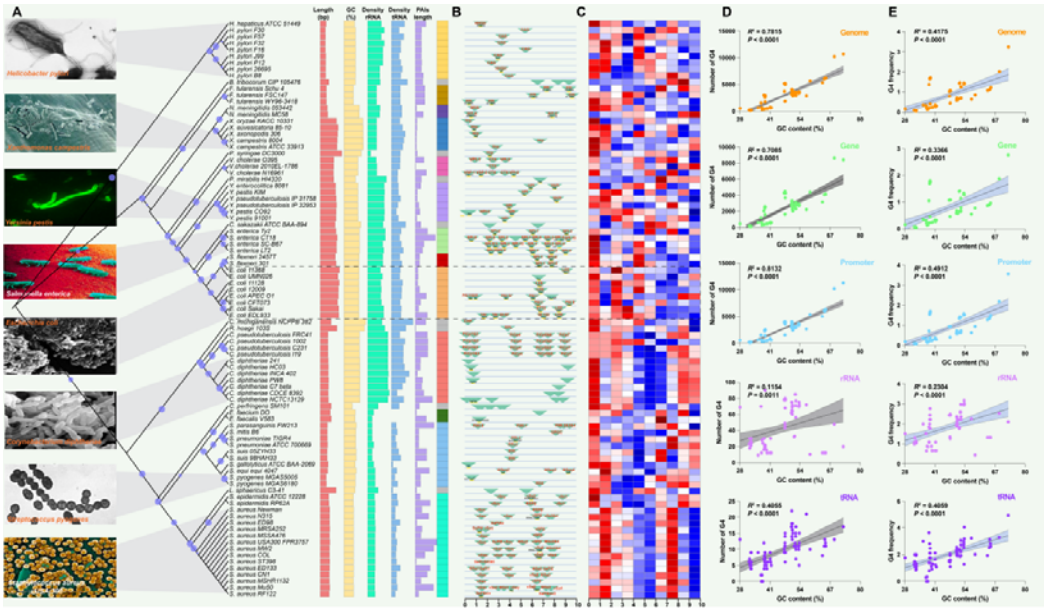


Figure 2. Analysis of Pathogenicity Islands (PAIs) and G-quadruplexes (G4s) in Pathogen Genomes. (A) Phylogenetic analysis of pathogen genomes based on 89 bacterial strains, showing the evolutionary relationships among species. Additional genomic information, including genome size, GC content, rRNA density, tRNA density, and PAI length, is provided. The same color indicates the same species. (B) Genomic location of specific PAIs in bacterial genomes, divided into ten regions. PAIs are represented by green triangles, and their names are indicated. The tRNA insertion sites are also marked. (c) Heatmap illustrating the relative abundance of G4s in bacterial genomes, divided into ten regions. Red indicates a higher relative abundance, while blue indicates a lower relative abundance. (D & E) Correlation analysis between the number of G4s, the frequency of G4s and GC content in various genomic features, including the whole genome, genes, promoters, rRNA, and tRNA.

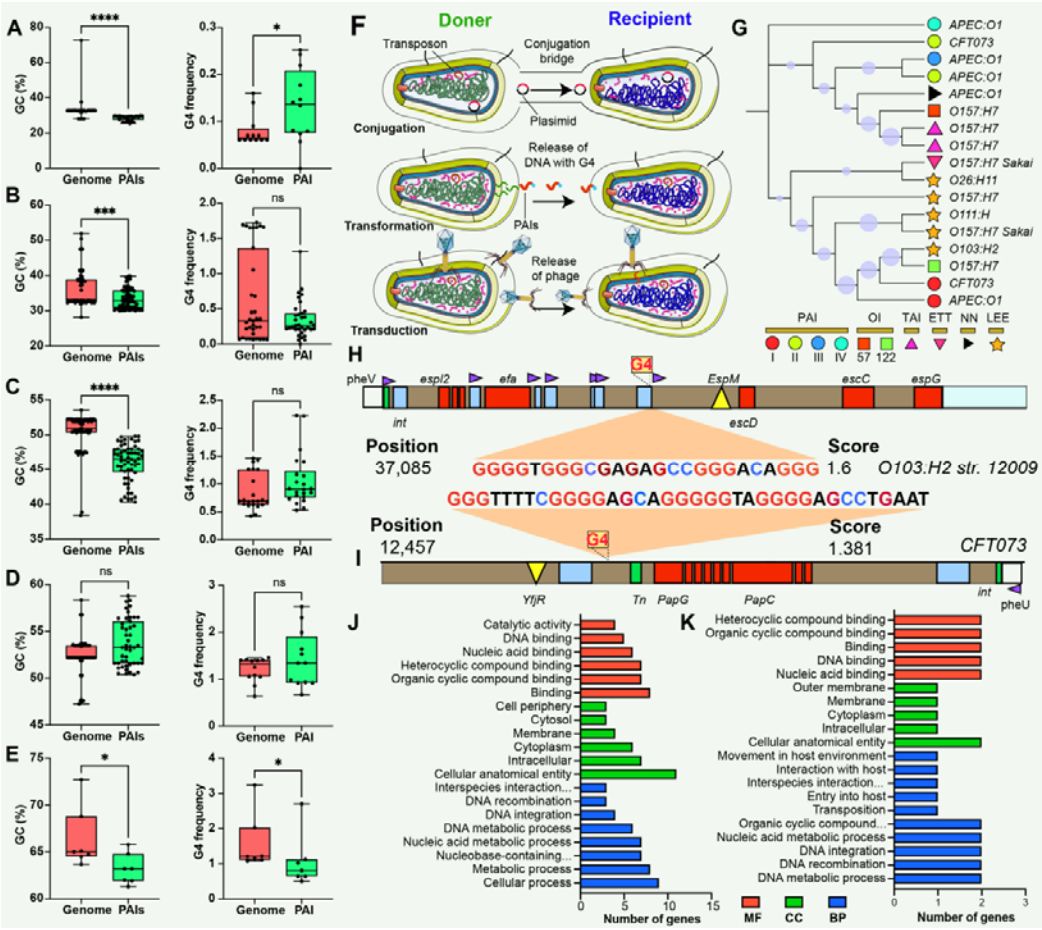
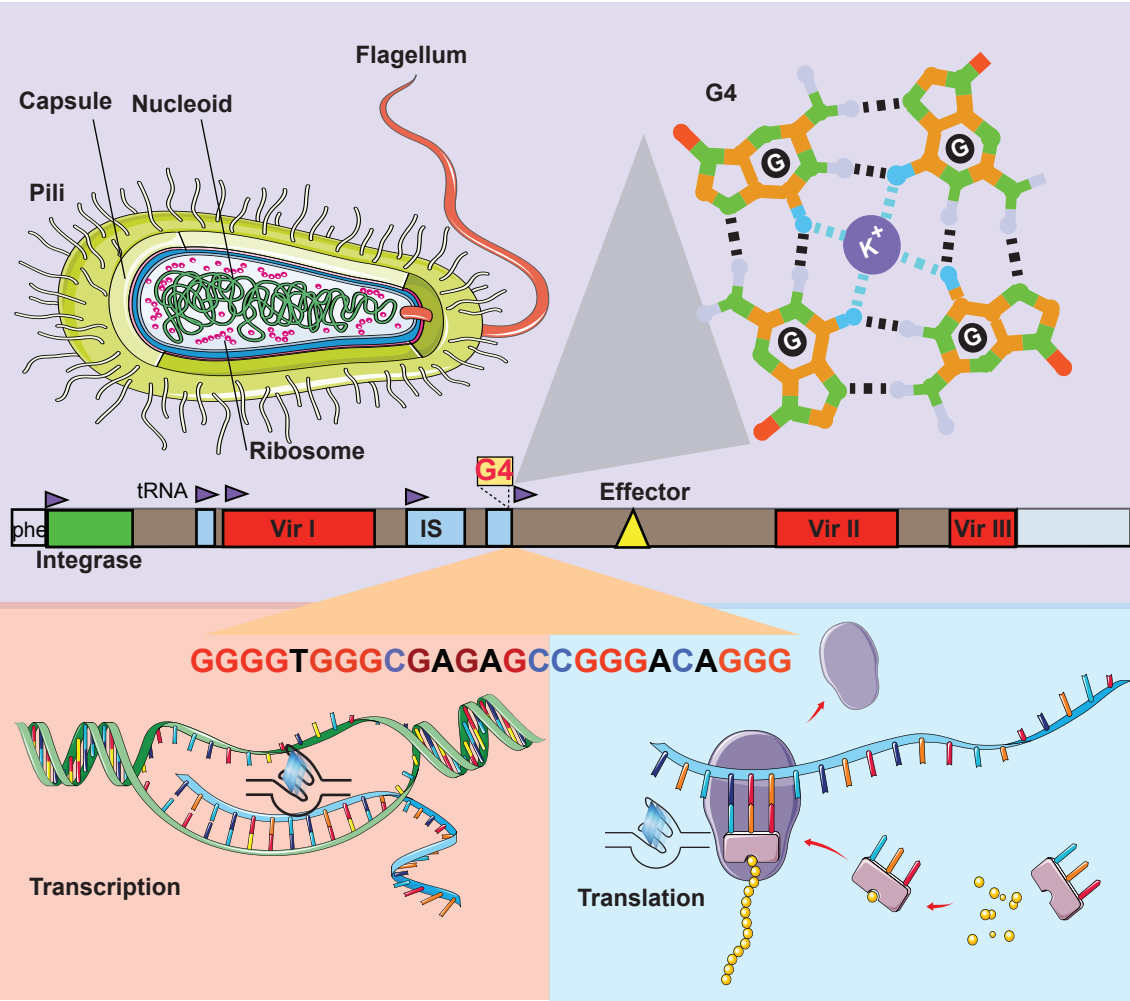


Figure 3. Putative Origin and Functional Annotation of G-quadruplexes (G4s) within Pathogenicity Islands (PAIs) in *Escherichia coli*. (A-E) Comparison of GC content (left panel) and GC frequency (right panel) between the genome and pathogenicity islands (PAIs), categorized into five regions (20%-30%, 30%-40%, 40%-50%, 50%-60%, and 60%-70%). */**/***/**** indicates significant difference (p < 0.05/0.01/0.001/0.0001). (F) Hypotheses on the origin of G4 structures within PAIs, involving gene horizontal transfer mechanisms (conjugation, transduction, and transformation). (G) Evolutionary relatedness of 10 types of PAIs (categorized into six main categories) in E. coli strains. (H & I) Examples of G4 structures within PAIs in E. coli strains. The grey bar represents the virulence region, the red box indicates a

virulence gene, the blue box represents an insertion site region or repeat, the green box denotes an integrase, the purple triangle indicates a tRNA insertion site, and the yellow triangle indicates an effector. (J &K) Functional annotation analysis of G4-covered genes within PAIs in two E. coli strains, including biological process (BP), cellular component (CC), and molecular function (MF) categories.


Flagellum
Capsule
Nucleoid
Pili
Ribosome
G4
G
K+
tRNA
G4
Effector
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Vir I
IS
Vir II
Vir III
Integrase
GGGGTGGGCGAGAGCCGGGACAGGG
Transcription
Translation