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BRIEF ARTICLE

Predicting a novel pathogenicity island in *Helicobacter pylori* by genomic barcoding

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Abstract

AIM: To apply a new, integrated technique for visualizing bacterial genomes to identify novel pathogenicity islands in *Helicobacter pylori* (*H. pylori*).

METHODS: A genomic barcode imaging method (converting frequency matrices to grey-scale levels) was designed to visually distinguish origin-specific genomic regions in *H. pylori*. The complete genome sequences of the six *H. pylori* strains published in the National Center for Biotechnological Information prokaryotic genome database were scanned, and compared to the genome barcodes of *Escherichia coli* (*E. coli*) O157:H7 strain EDL933 and a random nucleotide sequence. The following criteria were applied to identify potential pathogenicity islands (PAIs): (1) barcode distance distinct from that of the general background; (2) length greater than 10000 continuous base pairs; and (3) containing genes with known virulence-related functions (as determined by PfamScan and Blast2GO).

RESULTS: Comparison of the barcode images generated for the 26695, HPAG1, J99, Shi470, G27 and P12 *H. pylori* genomes with those for the *E. coli* and random sequence controls revealed that *H. pylori* genomes contained fewer anomalous regions. Among the *H. pylori*-specific continuous anomalous regions (longer than 20 kbp in each strain's genome), two fit the criteria for identifying candidate PAIs. The bioinformatic-based functional analyses revealed that one of the two anomalous regions was the known pathogenicity island *cag*-PAI, this finding also served as proof-of-principle for the utility of the genomic barcoding approach for identifying PAIs, and characterized the other as a novel PAI, which was designated as *tfs3*-PAI. Furthermore, the *cag*-PAI and *tfs3*-PAI harbored genes encoding type IV secretion system proteins and were predicted to have potential for functional synergy.

CONCLUSION: Genomic barcode imaging represents an effective bioinformatic-based approach for scanning bacterial genomes, such as *H. pylori*, to identify candidate PAIs.

Key words: *Helicobacter pylori*; Genome analysis; Pathogenicity islands; Genomic bar coding

Core tip: The genomic barcoding technology was recently developed to increase the accuracy of genome analysis, and has facilitated the identification of origin-

specific genomic regions of both eukaryotic and prokaryotic lifeforms. In this study, we applied the genomic barcode imaging approach to screen for pathogenicity islands (PAIs) in *Helicobacter pylori* using the six strains for which the complete genome sequences have been published and performing comparison to a common *Enterobacter* species. Bioinformatic-based functional analysis not only provided proof-of-principle (identifying the known *cag*-PAI) but also identified a novel PAI (designated as *tsf3*-PAI).

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative pathogen that colonizes the stomachs of over half the world's population[1,2]. Despite being one of the most common chronic infections among humans, it often remains undiagnosed until an unknown trigger causes manifestation of gastric diseases (*e.g.*, gastritis[3], ulcers[4], and gastric carcinoma[5]) with varying degrees of symptom severity and outcome. Extensive research efforts have been dedicated to understanding the molecular mechanisms of *H. pylori* pathogenesis, and have identified several (*bona fide* and putative) classes of virulence factors, including adhesins[6,7], cytotoxins[8], and lipopolysaccharide (LPS)[9]. While LPS has received the majority of research attention in the *H. pylori* field, due to its prevalence among pathogenic bacteria and its well-characterized interactions with the Toll-like receptor 4 of the host innate immune system, systematic investigations of the cytotoxins have also elucidated the host-pathogen signaling interactions leading to pathogenic changes in the infected tissues. For example, the vacuolating cytotoxin (VacA) has been shown to induce apoptosis in epithelial cells, and the cytotoxin-associated antigen (CagA) has been shown to counteract the VacA-induced apoptosis to promote survival of infected host cells and facilitate stomach colonization[10].

Recent evidence has suggested that pathogenicity islands (PAIs) in the bacterial genome play an important role in pathogenesis[11,12]. PAIs are defined as large DNA fragments that have been acquired through horizontal transfer and which bear multiple genes encoding bacterial factors with virulence functions[13]. The genes located on each PAI serve as molecular markers for clinical testing to diagnose bacterial pathogens, estimate their pathogenic potential, and predict treatment response (*i.e.*, antibiotic resistance)[14]. Therefore, genomic scanning to determine the PAI profile of *H. pylori* will not only provide insights into the molecular evolution and pathogenic mechanisms of this important human pathogen but also identify putative targets for effective molecular therapies.

The advent of high-throughput sequencing technologies has allowed for the complete genome sequences of a large number of prokaryotes; in conjunction with the rapid accumulation of such minable data in publicly available databases, various *in silico* methods have been developed to detect PAIs[15,16]. Most of these methods depend on finding aberrant G + C content and/or bias in codon usage[17] among various genera and species. Yet, this approach produces a high frequency of false negative results due to post-transfer changes that naturally accumulate in the transferred fragments over the course of evolution in a new environment.

In our previous studies, we addressed the limitations of the *in silico* methods. It was found that when genome scanning was performed using a fixed window size of at least 1000 bp, the frequency of each κ-nucleotide sequence ($2 < \kappa < 7$) was highly stable across a whole genome[18]. As a result, we represented the κ-nucleotide sequence frequency distributions across a whole genome as a 2-D barcode-like image, which was designated as a genomic barcode. By visualizing the barcodes of each genome, we were able to easily identify those sequences of foreign origin, such as horizontally transferred genes[18].

In the current study, we applied the genomic barcode imaging technique to scan the *H. pylori* genome for PAIs. Both known (serving as a proof-of-principle finding) and novel PAIs were detected.

MATERIALS AND METHODS

Genome sequence data

Complete genomes of the 26695, HPAG1, J99, Shi470, G27 and P12 strains of *H. pylori*, as well as those of *Escherichia coli* (*E. coli*) O157:H7 strain EDL933 (serving as a control for comparative analysis), were downloaded from the National Center for Biotechnological Information FTP server (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/) in January 2012. In addition, a random nucleotide sequence was generated by a K-order Markov chain model for use as an additional control.

Generation of genomic barcode images

Each genome was partitioned into non-overlapping fragments of 1000 bp and a 4-nucleotide-based barcode was calculated for each genome. Specifically, the barcode for each genome is a matrix of N (4) columns and genome length/M rows, so that N (4) = 136, with the i^{th} value being the combined frequency of the i^{th} 4-nucleotide and its reverse complement in this fragment. The κ-nucleotide frequencies were then converted to grey-scale levels to visualize the overall barcode image profile for the whole genome. Darker grey levels represent lower frequencies.

Identification of PAIs

The following criteria were applied to identify potential PAIs: (1) barcode distance distinct from that of the general background; (2) length greater than 10000 continu-

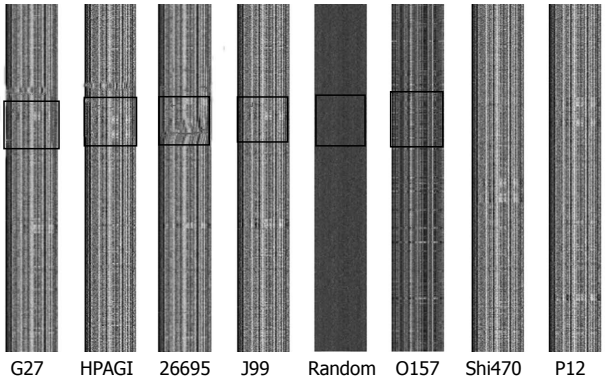

Figure 1 2-D barcode images of genomes of *Helicobacter pylori* strains J99, G27, 26695, HPAG1, P12, and Shi470, *Escherichia coli* O157:H7 strain EDL933, and a random sequence. The y-axis represents the genome axis from top-down, with each pixel representing a fragment of n = 1000 bp; the x-axis represents the 4-nucletide frequencies. The abnormal barcode regions are demarcated by a rectangle.

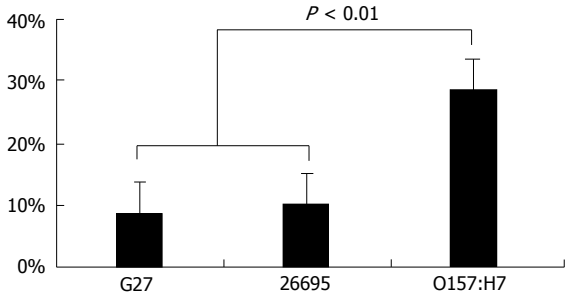

Figure 2 Fraction of anomalous fragments detected by genomic barcode imaging of *Helicobacter pylori* strains G27 and 26695, and *Escherichia coli* O157:H7 strain EDL933.

ous base pairs; and (3) containing genes with known virulence-related functions (as determined by PfamScan[19] and Blast2GO[20]).

Statistical analysis

The distance between two barcodes was calculated as the Euclidean distance between the corresponding 136-dimensional vectors. The distance database was built using Microsoft Excel spreadsheet software, and SPSS 13.0 statistical software was employed for analysis of the data using descriptive methods and the χ^2 test.

RESULTS

Visualization of H. pylori genomes based on genomic barcode images

Each genome was partitioned into a series of non-overlapping fragments of 1000 bp, and the combined frequencies of each 4-nucleotide/reverse complement were calculated. The frequency matrices converted to grey-scale are shown in Figure 1. The unique barcode image for each of these microbial genomes represents the underlying base composition. The 2-D barcode images of the *H. pylori* strains were similar to one another but distinct from that

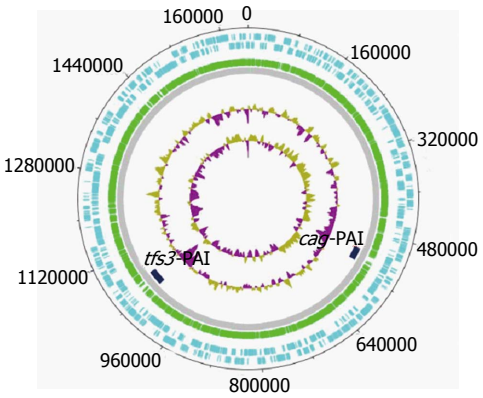

Figure 3 Circular representation of the *Helicobacter pylori* 26695 chromosome. The outermost (first) concentric circle denotes the predicted coding regions on the plus strand. The second concentric circle denotes the predicted coding regions on the minus strand. The third concentric circle denotes the predicted coding regions on both strands. The fourth concentric circle denotes the buffer zone. The fifth concentric circle denotes the predicted pathogenicity island (PAI) candidates. The sixth concentric circle denotes the guanine and cytosine (GC) content. The seventh concentric circle denotes the GC content. The figure was created using GenVision from DNASTAR.

of *E. coli*, demonstrating the close relationship of strains from the *H. pylori* species. It should be noted that no barcode structure was able to be produced for the random nucleotide sequence, indicating that the genomic barcode is an inherent property of the microbial genome.

Identification of H. pylori-specific genomic regions

While the genomes of different *H. pylori* strains possessed the conserved κ-nucleotide frequency producing the visual barcode, some regions appeared to have an abnormal structure. As shown in Figure 1, an abnormal band was apparent across the barcode image of the corresponding genome. In principle, these regions may have been acquired through horizontal gene transfer or derived from phage-mediated gene conversion.

The percentage of the anomalous regions in each genome are shown in Figure 2. As expected, the *H. pylori* strains contain fewer anomalous regions than *E. coli* ($P < 0.01$).

Identification of PAIs in H. pylori

We collected continuous anomalous fragments, longer than 20 kbp in each genome, and kept only those specific for most *H. pylori* genomes. In addition, some anomalous fragments found only in some *H. pylori* genomes, but subdivided into a number of discrete smaller segments in another *H. pylori* genome, were excluded from further analysis since such fragments may have resulted from frequent recombination events[21,22]. As a result of this procedure, two specific genome regions were selected as potential PAI candidates. Figure 3 and Table 1 show the position of these two candidate PAIs in *H. pylori*.

The bioinformatic-based functional analyses revealed that one of the two anomalous regions was the known pathogenicity island *cag*PAI, this finding also served as proof-of-principle for the utility of the genomic barcoding approach for identifying PAIs, and characterized the

Table 1 Pathogenicity island candidates in sequenced *Helicobacter pylori* genomes

	Size	GC	Barcodedistance	ORF
Wholegenome	1.5-1.7 Mbp	38.0% ± 0.2%	114.3 ± 14.9	-
cag-PAI	35 kbp	35.4% ± 0.8%	134.6 ± 20.1	20.0 ± 0.6
tfs3-PAI	30 kbp	33.0% ± 0.8%	138.0 ± 20.0	17.0 ± 3.0

PAI: Pathogenicity island; ORF: Open reading frame; GC: Guanine and cytosine.

other as a novel PAI, which was designated as *tfs3*-PAI and was located at the 3' end of the *Ser*-tRNA gene.

Identification of genes in cag-PAI and tfs3-PAI and prediction of the pathogenic role for each

We verified that the genes located in *cag*-PAI encode components of the type Ⅳ secretion system (T4SS), as characterized by previous studies[22-24].

Compared with *cag*-PAI, *tfs3*-PAI displayed some sequence variability due to rearrangements. The *tfs3*-PAI consisted of three distinct domains separated by mobile genetic elements. The first module contained the largest number of genes and encoded mobile sequence elements including a transposase (IS605), which is an essential element for a PAI. The second module encoded homologous genes of *tfs3* gene clusters, which formed a T4SS. The function of the *tfs3* gene cluster is not yet known, but it may play a role in bacterial conjugation and host cell signaling complementary to that of the *cag*-PAI-encoded system, which indicates a functional synergy. Most genes of the third module encoded hypothetical genes; as these genes have no orthologs in the databases, it is not clear at this point how many of them are in fact pseudogenes. It worth noting that *tfs3*-PAI consists of 17 open reading frames, six of which encode homologous genes of the T4SS. Therefore, this region may be related to pathogenesis in gastroduodenal diseases, and may represent a useful target for new vaccines and antibiotics.

DISCUSSION

The first potential PAI was *cag*-PAI, a well-known pathogenicity island in *H. Pylori*[25]. This approximately 35 kbp cluster of genes was acquired through horizontal transfer from an unknown extraneous source and integrated into the *H. pylori* chromosome. It is known that, compared to Enterobacteriaceae, *H. pylori* has less opportunity to obtain foreign genes by horizontal transfer since only a few bacterial species colonize human stomachs. Indeed, a previous microarray-based study of a larger strain collection suggested that up to 10% of all genes in an individual isolate may be accessory genes[26], which corroborates our finding.

T4SS is one of at least six specialized secretion systems characterized in bacteria. Usually consisting of 12 components, T4SS plays various functions in transporting a wide range of components, from single protein to protein-protein complexes and protein-DNA complexes. Moreover, T4SS facilitates injection of bacteria-encoded effectors into host cells during the infection process. The *cag*-PAI-encoded secretion systems have been implicated in modulation of bacteria-host interactions, interference with host signal-transduction pathways, and promotion of apoptosis, to name a few[22-24].

Pathogenesis of *H. pylori* is a multi-stage process. It is likely that multiple bacterial and host mechanisms are involved; however, a long-standing dogma of infectious biology claims that PAIs of *H. pylori* are stable entities and could be robustly correlated with disease progression or outcome. Screening and functional analysis of PAIs in *H. pylori*, as developed and demonstrated in this study, will aid in the development of more accurate and timely diagnosis and improved control of this common pathogen.

COMMENTS

Background

Recent evidence suggests that pathogenicity islands (PAIs) play an important role in bacterial pathogenesis. Scanning of PAIs in the *Helicobacter pylori* (*H. pylori*) genome will provide insights into the molecular evolution and pathogenic mechanisms of this important human pathogen but also identify putative targets for effective molecular therapies.

Research frontiers

Auhtors have applied the genomic barcode imaging technique to scan PAIs in *H. pylori*. Bioinformatic-based functional analysis not only provided proof-of-principle (identifying the known *cag*PAI) but also identified a novel PAI (designated as *tsf3*-PAI).

Innovations and breakthroughs

A novel PAI, *tsf3*-PAI, was detected in *H. pylori* using the genomic barcode imaging technique. Bioinformatic-based functional analysis revealed that *tsf3*-PAI encodes a type Ⅳ secretion system (T4SS) which may functionally synergize with the T4SS encoded by *cag*-PAI.

Applications

The genomic barcode imaging technique is useful for identifying known and novel PAIs in bacterial genomes. The PAIs identified in this study may be related to the manifestation of *H. pylori*-induced gastroduodenal diseases, and may represent useful targets of new molecular therapies or vaccines.

Terminology

The genomic barcode is generated by measuring the κ-nucleotide sequence frequency distributions across a whole genome using a fixed window size of at least 1000 bp. The 2-D barcode-like image is generated by converting the frequency matrices to grey-scale levels.

Peer review

This manuscript applied genomic barcodes to screen for PAIs in *H. Pylori*, which showed that genomic barcode technique is more usefulness and accuracy tool for genome analysis so far. The proof-of-principle work showed that one known and one novel PAI could be detected using this technique.

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