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CRISPR-Cas Systems in Helicobacter Pylori: A Dual Role in Genome Stability and Pathogenesis

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Annotation: Helicobacter pylori is a Gram-negative bacterium that colonizes the human stomach and is implicated in various gastrointestinal diseases, including gastritis, peptic ulcers, and gastric cancer. Recent studies have revealed the presence of CRISPR-Cas systems in H. pylori, which play a complex role beyond their well-known function in adaptive immunity against foreign genetic elements. This review explores the dual role of CRISPR-Cas systems in H. pylori, focusing on their contribution to both genome stability and pathogenesis. CRISPR-Cas systems act as bacterial immune defenses, targeting invading phages and plasmids to prevent genomic disruption. In H. pylori, these systems maintain genome integrity by limiting horizontal gene transfer, which is crucial given the bacterium's high genetic variability. However, H. pylori's CRISPR arrays and Cas proteins also influence the bacterium's virulence and ability to persist in the hostile gastric environment. Emerging

evidence suggests that CRISPR-Cas components modulate gene expression involved in adhesion, motility, and immune evasion, thereby enhancing colonization and pathogenicity. Moreover, the interplay between CRISPR-Cas-mediated genome surveillance and bacterial adaptability highlights a balance between protecting genome stability and promoting genetic diversity necessary for survival in the dynamic gastric niche. Understanding this dual functionality provides insights into H. pylori's evolution and pathogenic mechanisms and identifies potential targets for novel therapeutic strategies.

Keywords: Helicobacter pylori, CRISPR-Cas system, Genome stability, Pathogenesis, Adaptive immunity, Bacterial virulence.

1. Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR) together with the CRISPR-associated (cas) genes are the components of the prokaryotic immune system against invasive genetic elements. The CRISPR loci are comprised of several direct repeats (DRs), which are similar sequences around 20 to 50 bp in length, separated by variable spacers, which are unique sequences originating from the invader DNA [1]. The number and length of the CRISPR loci, DRs, and spacers vary among and within bacterial species and strains. In general, DRs and spacers with different number and length are considered the markers to monitor the variations between strains of a bacterial species. The CRISPR systems contribute directly in determining the phage resistance phenotype and also indirectly in providing broader effects in the genome stability of bacteria [2]. Helicobacter pylori is a Gram negative microaerophilic bacterium and the only known host for H. pylori is human being. It colonizes a gastric epithelium and is associated with several gastric diseases including peptic ulcer, gastric cancer and mucosa associated lymphoid tissue lymphoma. Peptic ulcer resulting from inflammation of the gastric mucosa mediated by the vagus nerve and infection [3]. H. pylori infection causes atrophic gastritis, increased gastric acidity, reactive oxygen species, and intestinal metaplasia, which is the abnormal growth of metabolically active intestinal-like cells. Aberrant intestinal metaplasia may lead to the development of tumours. H. pylori may also translocate from the stomach to the gastric lymphoid tissue and induce activation of T and B cells, formation of germinal centres and production of antibodies. In response to chronic infection, an abnormal high number of memory B cells can develop in the gastric mucosa, leading to lymphoid hyperplasia. These events promote the development of MALT lymphoma, an extragastric manifestation of H. pylori infection [4].

2. Overview of Helicobacter pylori

Helicobacter pylori (H. pylori) is a gram-negative bacterium that colonizes under the gastric mucosa of roughly half of the humans worldwide. Infection by H. pylori usually occurs early in childhood. The infection can persist lifelong without symptoms in most individuals, while a significant number of infected people develop gastric diseases (pathogenesis) such as chronic gastritis, peptic ulcers, or gastric cancer [5]. H. pylori is a major risk factor for gastric cancer (GC), which is the third leading cause of cancer-related mortality and the sixth most prevalent form of cancer in the world. H. pylori infection has been classified as a subclass of gastric cancer risk. In the genome of the H. pylori, there are 1.582 × 106 bp consisting of a single circular DNA chromosome, containing 1576 genes with G + C content of 39.6%, which is 8.7% lower than the average G + C content in prokaryotes [6]. H. pylori has about 38 CRISPR loci that are managed by three separate types of Cas protein families that play a primary role in fight against invading phages and plasmids. The presence of a cascade of CRISPR associated effector proteins can be predicted in H. pylori and some of the strains show the ability to cleave the target DNA in vitro. At least 37 genomic CRISPR loci in the genome were recurrently detected in strains obtained from different hosts [7]. Gene-level analyses indicated that distinct chances might occur in the CRISPR system of the H. pylori. The CRISPR array of H. pylori strains by characterizing different CRISPR-Cas systems in H. pylori were originally equipped with a CRISPR-Cas class 2b system for counteracting foreign genetic elements. Recent experimental studies of stressed H. pylori produced multi-locus sequence typing (MLST) schemes used to unravel major pathologic variants [8].

3. CRISPR-Cas Systems: An Introduction

Discovered in a variety of organisms, clustered regularly interspaced short palindromic repeat-cas (CRISPR-Cas) systems are RNA-based defense mechanisms that confer adaptive immunity against viruses and foreign genetic material. Once foreign nucleic acids infect a bacterium, they are processed into short RNA (crRNA) molecules that guide the recognition and degradation of complementary DNA and RNA targets [9]. On occasion, in response to the domestic threat of plasmids, prophages, or gene cassettes, crRNA-mediated target interference is extended, and new genomic sequences are added to the CRISPR locus by a second cas-complex, the type I-E adaptation complex. This segment of the genome may be further transcribed into crRNA, and employs the CRISPR-associated reverse transcriptases to extend the RNA transcript of foreign genetic material [10]. This provides a method for RNA-based immunity to the recent threats of viruses and other foreign RNA, termed CRISPR-Cas Type IV systems. Each of these systems has been the source of extensive investigation, but far less work has been done to understand perhaps the most complex of CRISPR-Cas systems, found in Helicobacter pylori [11].

Many of the first eight genes present in the H. pylori CRISPR-Cas system belong to the type I-F Cas3 complex, while the presence of an additional hinged protein has led to the reclassification of the system as a type I-B CRISPR-Cas system [12]. Southern hybridization and genome sequence analysis revealed that H. pylori harbors a CRISPR-Cas locus that is heterogeneous in size, structure, and sequence among strains, but this appears limited to the cas3 gene and hairpin transcription unit [13]. Even though H. pylori has one of the simplest known CRISPR-Cas systems, it has nevertheless evolved elaborate mechanisms to evade CRISPR-Cas immunity. In addition to pre-existing modulators of csa3 expression, recent investigations have determined that modulation of Cas3 activity against endogenous targets plays a pivotal role in pathogenicity and evasion of CRISPR-Cas immunity [14].

4. Types of CRISPR-Cas Systems

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas), collectively referred to as CRISPR-Cas systems, are classified into two classes, and they are further divided into six types and as many as 33 subtypes according to the protein composition of the Cas proteins [15]. Type I systems are the most widespread CRISPR-Cas

systems in bacteria, comprising 12 subtypes. Most type I systems include a large single-module protein termed Cas3, and these systems are known as Type I-B. Other Cas3-associated subtypes are also distributed in bacteria and archaea, and they are designated as Type I-C, I-D, I-E, I-F, I-U [16]. In Type I CRISPR-Cas systems, crRNAs are processed from long precursor CRISPR RNAs (pre-crRNAs) by the action of a ribonuclease complex composed of Cas6 and other Cas proteins. The association of the individual Cas proteins with mature crRNAs comprises interference complex. The DNA target recognition of interference complex is aided by the key signature of intersubtype variations, trans-acting tracrRNAs (tracrRNAs) [17]. Type I systems are divided into six types according to the characteristics of the Cas proteins encoded within the genomes [16]. Cas13 proteins belong to class 2 CRISPR-Cas systems that use a single, large endonuclease (Cas enzymes) to cleave target nucleic acids. They specifically recognize single-stranded RNA (ssRNA) and are capable of damaging their target by the hydrolysis of the phosphodiester bonds [18]. According to the differing domain structures of Cas proteins, Type VI CRISPR-Cas systems are divided into subtypes a and b. Type VI-A CRISPR-Cas systems are characterized by a unique PIN-domain protein, Cas13a, which is capable of degrading the target RNA after distinguishing it from pre-crRNA by the assistance of a short crRNA sequence. In contrast, Type VI-B systems are widely spread across the different strains of bacteria, such as pathogenic bacteria, with Cas13b identified as the effector protein [19]. Entities of Type VI-B systems consist of the signature Cas13b proteins, the typical Cas9 protein and the various accessory proteins. The target transcript is captured by Cas13b proteins through the hybridization with complementary crRNA, and RNA interference then occurs via the cleavage of the target strand [20].

4.1. Class 1 CRISPR-Cas Systems

Clustered, regularly interspaced, short, palindromic repeat (CRISPR)-associated (Cas) systems are widely classified into types (I-VI) and subtypes (A-F) based on the signature Cas proteins. Following studies on the Cas9-mediated targeted mutagenesis in the type II systems of Escherichia coli, there is an immense interest in the other types of CRISPR-Cas systems outside type II [21].

The expanding CRISPR database makes it possible to search for a classification of the systems globally. The systems have been classified using phylogenomic analyses that include species of the Archaea kingdoms, Bacteria, chloroplasts, and less known groups [22]. Since 2011, the knowledge on the CRISPR-Cas systems has been expanded rapidly with the discovery of the new Cas proteins and the revealing of their mechanisms [23]. However, Helicobacter pylori CRISPR-Cas systems were not included. All four cloned CRISPR-Cas systems in H. pylori are the class 1 systems, explained as an interference of pre-crRNA handling and maturation at Cas6. The longer pre-crRNA processing products of variant processing sites can increase the chances for H. pylori to gain genetic-variation [24].

With comparison of multiple H. pylori virulent strains, some of these differences have been observed in the pre-crRNA from the nanocompartments in S. solfataricus, the requirement of diverse Cas and host proteins, and the frequent exchange of the old and new genomes in the same niche with an emphasis on the genotype-phenotype adaptation [25]. The CRISPR immune systems have been characterized in viruses infecting H. pylori to understand how some virus taxa were persistently observed, explaining the long-term stability of polymorphic S. 7 and the short-term change of S. 44. The H. pylori CRISPR-Cas systems are the focus of a plethora of studies for their diversification, defense, and targeting mechanisms [26].

4.2. Class 2 CRISPR-Cas Systems

CRISPR-Cas systems have been classified into two major classes based on their architecture and mechanism of action. Class 1 CRISPR-Cas systems (type I, type III, residing in Cse complex together with their unique signature protein Cse1, Cas6e, and Csm/K.z), meanwhile class 2 CRISPR-Cas systems (type II, type V, and V), are the documented CRISPR-Cas systems in H. pylori [27]. Although fully characterized class 2 CRISPR-Cas systems of H. pylori have been

documented, recently a novel class 2 CRISPR-Cas system type II-D or II-H has been shown to be widely distributed in phylogenetically distant Helicobacter [4]. Cas9 protein of this newly discovered type II CRISPR-Cas systems is H. pylori considered as a major virulence factor has been shown to be involved in host sialylated mucins glycan degradation which is the initial step of chronic infection establishment [28]. Besides the use for developing easy designing and high efficiency genome editing tool, H. pylori Cas9 protein-derived dCas9 is used for epigenome regulation in E. coli, stressing its potential for biotechnological applications. Classification of H. pylori Type II CRISPR-Cas Systems According to the Cas9 Protein Sequences: Recently, whole genome sequences of more than 400 strains reported H. pylori type II CRISPR-Cas systems [29]. H. pylori type II CRISPR-Cas systems can be classified into five clusters comprising of type II-A (hpCas9-1), type II-B (hpCas9-2), type II-C (hpCas9-3), type II-D (hpCas9-3), and type II-H (hpCas9-5) according to the phylogenetic tree analysis of Cas9 protein sequences. Additionally, the combination of different Cas9's variations with different sets of other Cas genes has also been reported which is distinct identity of H. pylori CRISPR-Cas systems [30]. Prokaryotic Type II CRISPR-Cas Systems: Type II CRISPR-Cas systems are composed of only a handful of Cas proteins required for CRISPR immunity. This system generally consists of the cas genes cas9, cas5, and cas7. Cas9 is an endonuclease that mediates both target binding and cleavage, while cas5 and cas7 are involved in biogenesis and processing of crRNAs [31]. The specificity of CRISPR-Cas systems is provided by Cas9 and crRNA. Cas9 finds target sites with the help of a short direct repeat (known as the protospacer adjacent motif or PAM) that is immediately adjacent to all known crRNA targeting sites [32].

5. CRISPR-Cas Functionality in H. pylori

Until recently, it was popularly assumed that the group I, type F, CRISPR-Cas was the type of CRISPR-Cas system present in H. pylori. Indeed, so much so that it is often stated as a fact in reviews on this pathogen [33]. This viewpoint was based on the fact that only a few type I-F CRISPR-Cas systems had been described in other bacterial genera than H. pylori, resulting in claims that the presence of a type I-F system in any bacterium was evidence that it is an H. pylori. Such a circular way of reasoning is not scientific. Subsequent metagenomic analysis determined that these strict restrictions on Gram negative type I-F systems were due to gaps in the previously sequenced publicly available genomes [34]. Following a systematic study of this type of system, it was determined that this cluster VIA CRISPR-Cas returned very poorly in the analysis despite metagenomic samples sampling many H. pylori cells. Further analysis demonstrated that type II–like CRISPR-Cas systems might play a diverse role in other genera, including H. pylori. A better understanding of CRISPR-Cas systems in H. pylori can provide a scientific basis for better disease management, vaccine development, and methods for biocontrol against this pathogen [35].

Investigations of the roles of CRISPR-Cas in H. pylori have revealed a dual role for CRISPR-Cas in genome stability and pathogenesis. CRISPR-Cas protects H. pylori against restriction modification systems by inactivating the respective restriction endonucleases by phase-variable on-off gene switching and RNA-guided anti-targeting [36]. Sequences incorporated into the CRISPR array were found to have been produced by RNA-induced and R-loop–formation–mediated pathways. Pathogenetic type I-F and type II CRISPR-Cas systems largely share common biology and act in concert to maintain genome stability against parasites and loss-of-function mutations triggered by aberrant targeting [37]. Despite sharing important biological features, these two classes of CRISPR-Cas have evolved different mechanisms. In addition to genome stabilizing roles, CRISPR-Cas also controls the accumulation of virulent factors and gene transfer via RNA-guided regulation of protein-directed targeting and attenuation of gene persisters. Combined, H. pylori's diverse CRISPR-Cas system greatly expands the current knowledge of CRISPR-Cas systems and offers insight into its co-option into pathogenesis [38].

5.1. Adaptive Immunity

Type II CRISPR-Cas systems contain a single, multidomain, RNase III-like Cas9 protein and are

commonly found in Firmicutes and some Actinobacteria. These systems are further classified as Type IIA or IIG, with Csy-containing systems being the most prevalent. Cas9 proteins are also found in many eukaryotes, where they reappear in an uncharacterized context, which also contains two HNHs and several linkers, and has been shown to be involved in RNA metabolism in mycobacteria and other actinobacteria [39]. BRISPR-like sequence variants contain non-canonical Cas9 proteins that are devoid of the helicase E, one of the two RAMP domains present in the majority of the described Cas9 proteins, and the sponD domain, the best recognized Cas proteins outside the CRISPR context [40]. Apicomplexans and Entamoebae also harbor a Cas9 homolog, but they are totally different from the previously described families, and are thus considered as clones of the more ancient forms. Experimental data has shown that this system can complement the Csy systems found in many enterobactereace bacteria [41].

As with other CRISPR systems, type II systems mainly target DNA to protect bacteria and archaea from phage predation. However, Cas9-CAP systems containing the auxiliary protein CsyE, and other Cas9 variants, can also cleave RNA, e.g. experimentally shown to function as Type II-C enzymes. In Listeria monocytogenes, RNA silencing via the Cas9-CAP system is involved in limiting horizontal gene transfer of virulence genes [42]. Deletion mutant studies showed that multiple gene competes with the host system to limit the expression of the internalin protein InlA encoded by InlA, and another A/C, which is likely to be involved in RNA entry into the anti-CRISPR A/C, proteins likely to prevent RNAP/CRISPR from correctly associating with the target [43].

In addition to playing a role in protection against phage, type I and the more abundant type II systems have been shown to have two roles. One is a role in DNA repair; it was demonstrated that sequences that trigger the type II systems are as abundant as those that do not trigger the CRISPR systems, suggesting that beyond providing an adaptive mechanism against phage invasions, Cas9 also performs other housekeeping functions [44]. Experiments in Listeria monocytogenes MST13 demonstrated that Cas9 can directly repair double-strand breaks in an alternative and error-prone way. In contrast to the ability of type II systems to target both RNA and DNA, type I and type V system induce an uncertain variety of immunity and atypical CAS proteins [44].

5.2. Genetic Regulation

CRISPR-Cas systems are regulated on genetic levels by several factors in Helicobacter pylori. CcpA is known to regulate the sugar metabolizing pathway in Firmicutes and characteristically binds to the carbon-related control matrix (CRCM) sequence in the H. pylori genome. Various amino acids affect the expression of the crRNA generation genes in H. pylori. The regulation of the crRNA generation genes has been assigned to the global regulator CodY, which is homologous to the MarkA protein of Bacillus subtilis [45].

CRISPR-ribonuclease (rnpA) of the classical type II systems consists of an RNase III and the CRISPR RNA (crRNA) handling proteins. drrA/17, a third-regulation system in type II CRISPR-Cas systems, was discovered in Thermus thermophilus. The gRNA (tracrRNA) of type II Cas was found to be a dual-function RNA responsible for the recruitment of Cas9 and base pairing with crRNA. The rofA gene encoding a putative DNA-binding protein was identified for the regulation of the class I CRISPR-Cas system in H. pylori (Cbs/Cse) and Streptococcus spp [46]. The NutB regulator, which is phylogenically classified as a member of the TetR family, was found to regulate adaptation of the type I-F CRISPR-Cas system in Pseudomonas aeruginosa. The CRISPR-Cas systems are activated in response to nutrient starvation through the acidic alarmone ppGpp, which is produced by the RelA-SpoT homolog (RSH) family of proteins, and are repressed by the UvrY-CskA-CrqR cascade [47].

6. Genome Stability in H. pylori

H. pylori is a Gram-negative pathogen that infects human gastric epithelium and induces chronic inflammation, increasing the risks of various gastric diseases, including gastric cancer and peptic

ulcer. H. pylori is found in about half of the human population, with an unusual rate of infection in developing countries. Infection initiation generally occurs in early childhood, but could remain undetectable until old age. Due to the longevity of infection, H. pylori has evolved a high diversity of strains, which contribute to host population diversity and specificity [48].

H. pylori has an extensive genome with genomic plasticity, which leads to intra-host genetic diversity, contributing to complications caused by this pathogen. A source of genome diversity in H. pylori is from its incomplete DNA replication, followed by a recombination event in one daughter molecule. Some host environmental factors also impact the plasticity of H. pylori with respect to the area of infection [49]. The DNA uptake signal sequence of H. pylori is a target for horizontal gene transfer involving the addition of large sequences to the genome. Additionally, H. pylori may lose genes under selective pressure, or become virulent via the gain of the genes. Pathogen genomic plasticity contributes to increased virulence and makes the infection more resistant to treatment. The H. pylori oncogenic transformation model provides an insight into carcinogenic pathways with major epidemiological evidence [50].

H. pylori uses an incomplete type IV secretion system to translocate CagA, displacing the protein from its original location in the host cell and setting it free to localize with multiple cellular binding partners. Although CagA has been linked to a program of host growth factor-receptor-targeting phosphorylation events that promote cell scattering, it simultaneously engages multiple cytoskeletal processes that impede normal epithelial structure, block cellular proliferation, and abrogate survival mechanisms. Through an interplay of transcriptional, post-transcriptional, and post-translational events, H. pylori modulates global gene and protein expression in gastric epithelial cells [51].

6.1. Role of CRISPR-Cas in DNA Repair

The filamentous bacterium Helicobacter pylori is a prominent cause of chronic gastritis and peptic ulcer disease. Over 50% of the worldwide population and over 80% of people in developing nations harbor H. pylori. Most infected patients are asymptomatic, whereas a small portion suffer from more unpleasant outcomes. The main reason underlying the different outcomes of H. pylori infection is not well understood [52]. A number of genomic, proteomic, and transcriptomic studies have been performed in order to elucidate the molecular mechanisms underlying H. pylori-induced diseases. Evidence has uncovered a large number of genetic systems and pathogenicity factors including virulence associated genes, surface proteins, toxins, and innate and adaptive immune modulators/escapers. However, the exact molecular mechanisms cleared in some strains have not been fully demonstrated [53].

The class 2 CRISPR-Cas subtypes are notable for their versatility of different core protein and CRISPR RNA structures. In the past decade, new data have established that CRISPR-Cas systems possess additional non-canonical functions in regulating cellular processes, intercellular communication, and DNA repair. The newly identified CRISPR-Cas system in H. pylori was demonstrated to play a dual role in directing CRISPR-Cas immune targeting and genome stability by a comparative study of Cas proteins and CRISPR arrays from genomes of classical and non-classical strains; dual functions of Cas proteins in H. pylori were also observed [54].

In contrast to the role of other Cas9 in mediating immunity only, the recently sequenced CRISPR-Cas subtype II-C in H. pylori exhibits dual functions of immunity and DNA repair. Comparing the nucleotide sequence similarity of CRISPR-Cas II-C systems from each genome of H. pylori or between H. pylori and other representatives of the II-C subtype shows that CRISPR-Cas in H. pylori clusters in two distinct groups, which correlates with the classical and non-classical lineage of H. pylori [55]. Further comparative analysis reveals that the CRISPR-Cas system is more prone to specific deletion than other canonical and maximal genomic regions. The presence/absence of the CRISPR-Cas system in a subgroup of strains suggests that horizontal gene transfer of this system may also play a role in the evolution of H. pylori. These proteins, encoded by diverse Cas protein genes, exhibit similarity to other class II-C Cas proteins [56].

6.2. Impact on Mutation Rates

Results from diverse studies indicate that CRISPR-Cas systems impact mutation rates, genome stability and evolution in Helicobacter pylori. Since the final assembly of CRISPR-associated DNA and RNA effectors directly influences the efficiency and specificity of CRISPR-Cas systems, deletion of a specific gene(s) encoding some of the components that regulate this assembly may hamper CRISPR-Cas functions [57]. Subsequently, that may lead to loss of immunity to invading plasmids and phages inducing different pathways to enhance recombinatorial mutagenesis or replication errors that generate drives mutator phenotypes. Enhanced mutation rates lead to rapid divergence and the selection of neofunctionalized or redundant genes, increased rate of chromosomal mutations leading to CRISPR-Cas loss, or increased rate of plasmid gain and loss [58]. Increase in mutation rates shifts the use of diverse antiviral strategies, hence H. pylori hosting targeted systems that avoid turnover of large genomic segments in the underlying mutator background. Incomplete immunity to the incoming DNA, either in form of plasmid or lysogenic phage, drives the selection of mutant strains with altered CRISPR-Cas immune systems that either enhance immunity by augmenting information acquisition or assembly rate, are less efficacious, or have completely compromised immunity. This broadly impacts virulence, genome stability and evolutionary trajectories of H. pylori [59].

7. Pathogenesis of H. pylori

Helicobacter pylori is a gram-negative bacterium that colonizes the gastric epithelium, where it induces chronic inflammation, known as gastric mucosal atrophy, resulting in a higher risk of gastric cancer and duodenal ulcers. These bacteria possess several genetic determinants, including the cag pathogenicity island and the vacA vacuolating cytotoxin gene, which are considered as major virulence factors in their connection to gastric diseases [60]. However, the mechanism whereby the bacterium induces severe gastric cancer remains unclear. Understanding how H. pylori infection induces severe gastric cancer is important not only for the health of high-risk populations, environmental changes towards H. pylori eradication, but also for finding ways to develop vaccines to reduce the risk of gastric cancer [61]. Many recent studies, especially transcriptomic analysis of both the bacteria and the host together with newly constructed gastritis animal models, demonstrate the dual ability of H. pylori in chronic inflammation, which has protective and pathogenic potentials against gastric cancer. Understanding gastritis in a mouse homologous infection model will provide insights into the pathologic and protective roles of H. pylori [62].

H. pylori is a prominent member of the human microbiota. It establishes a persistent infection in the stomach approximately half of the world’s population and has an important role in gastric cancer development. However, the majority of infected people remain asymptomatic for life. Also, the microbiome status, including composition, function, and metabolic modules, varies widely from person to person, despite a relatively constant presence of H. pylori as a dominant species [63]. Additionally, the density and distribution of H. pylori and other bacteria can differ significantly between individuals over time, influencing disease risk. This implies that microbiome composition, abundance, and other aspects may affect host responses and gastric cancer development. Furthermore, there are some evidence that different populations carry strains with variable virulence in pVAC, and a minority toxic for gastric mucosa as well, whereas the vast majority are neutral or even protective [64].

7.1. Mechanisms of Infection

Helicobacter pylori persistence in the human stomach causes gastritis, gastric ulcer, and gastric adenocarcinoma. H. pylori possesses a type IV secretion system that translocates CagA into host cells. CagA is phosphorylated by host kinases and regulates various cellular processes in epithelial cells. CagA alters host cell signaling, cytoskeletal remodeling, inhibition of apoptosis, aberrant cell proliferation, and tumor promotion. H. pylori infection is the clearest association between gastritis, ulcer, and cancer. Reactivated or persistent H. pylori infection in a mouse model induces

gastric cancer, and early eradication is curative [65]. However, the timing of eradication remains debatable. Re-infection after successful H. pylori eradication may occur even 20 years post-eradication. In some geographic areas, compared with children previously infected, older children have a higher rate of detection of new H. pylori infection. H. pylori is a major cause of gastric infection, acid-associated peptic ulcer disease, gastric cancer, and primary mucosa-associated lymphoid tissue lymphoma. Gastric cancer remains the second most common cancer and the second most common cancer death worldwide [66]. H. pylori ammonia contributes to the maintenance of neutrality in acid gastritis and is partly responsible for gastric epithelial cell damage and mucosal lesions. H. pylori resides in the gastric acidic environment, which is a hostile condition for the survival of most bacteria. H. pylori possesses multiple unique features that enable it to colonize the gastric environment [67]. In H. pylori, the H. pylori-CpG motif is recognized by different TLRs including TLR2 and TLR9. The dual roles of TLRs in H. pylori infection are controversial and dependent on the animal models and experimental settings. H. pylori alters host cells to remain undetected by TLR-Myd88. Intracellular signalling pathway repress lowers the expression of several TLRs, and dephosphorylation of Smad1 inhibits IL-6 secretion [68]. Specifically, CagA selectively allows H. pylori entry and evades host immune response. Phosphorylated-CagA binds to and regulates GRB2, which enhances actin polymerization. The active cytoskeleton is essential for facilitating H. pylori entry. In cells containing widely expressed TLRs, there is a selective entry of the bacteria into TLR4-negative cells [69]. As a result, H. pylori avoids TLR4-mediated signalling, which would subsequently stimulate pro-inflammatory cytokine responses. Specifically, Cag-independently H. pylori entry deceives the host. Further, it has been proposed that TLR4 evasion and blocking may be the reason for H. pylori infection susceptibility. A mechanism has been proposed for intra-cellular TLR evasion after internalization through H. pylori LPS glycosylation [70].

7.2. Virulence Factors

During the host-bacterial interaction, Helicobacter pylori employs a plethora of bacterial virulence factors. These factors fall into two major categories: (1) factors that enhance bacterial cell adhesion and invasion into host epithelial cells, and (2) factors that promote inflammation and the invasive response in the gastric mucosa leading to peptic ulcer diseases [71]. Outer membrane protein (Omp) of H. pylori plays a significant role in the adhesion and invasion into host epithelial cells and can be grouped into two major families, i.e., adhesin (Bab) and other Omp [72]. Several virulence factors that promote inflammation and invasive response have been intensively studied, including (1) secreted enzymes such as ureas and protease, (2) vacuolating cytotoxin A, and (3) cytotoxin associated gene A and its type IV secretion system. Exactly how H. pylori persists in the gastric mucosa is not fully understood, but vacuolating cytotoxin A and cytotoxin associated gene A, along with a number of other factors, have been implicated in the mechanism of colonization or host cell signaling events [73].

The vacA gene of H. pylori encodes a soluble protein toxin, which induces vacuole formation in epithelial cells and activates anti-apoptotic signaling pathways. H. pylori strains carrying a signal sequence containing type s1/S and type m1/M midregion are now generally accepted as pathogenic due to their potent vacuolating activity, the presence of the virulent cytotoxin associated gene A protein, induction of marked inflammation with infiltration by neutrophils and monocytes, and eventual development of gastric ulcers [74]. This type of strains are produced mainly in East Asia and are associated with gastric cancer. Other bacteria can also produce the vacuolating cytotoxin A protein but have a less potent vacuolating activity than H. pylori vacuolating cytotoxin A, e.g., Campylobacter jejuni, which generally possesses type s2 and type m2 gene at virulence scale. These strains can also induce mild vacuolation but only weak inflammation. However, the toxigenic mechanism of these strains remains elusive [75].

8. Dual Role of CRISPR-Cas in Pathogenesis

CRISPR-Cas systems generate, store and process small CRISPR RNAs (crRNAs) from invading

DNA. The crRNAs then direct specific cleavage of invasive nucleic acids by DNA interference. Moreover, CRISPR-Cas systems are involved in non-canonical gene regulation and several other functions, including mRNA interference, RNA degradation, modulating the levels of some regulatory small RNA, controlling post-transcriptional regulation of gene expression, and the repair of damage to DNA [76]. The development of a removable CRISPR-Cas9 knockout system made it clear that some essential genes are the target of large-scale CRISPR/Cas-mediated DNA deletion events in Helicobacter pylori. In a laboratory environment in which H. pylori lack both CRISPR-Cas defense systems, researchers demonstrated an increased transformation efficiency and reduced restriction activity relative to their isogenic CRISPR-positive counterparts. Also, the loss of the second type of immunity in H. pylori allowed for the stable maintenance of newly acquired plasmids [77].

Moreover, the crRNA array in the Type I CRISPR-Cas system was identified, and the expression of a novel crRNA, HP_MK_23, was added to the list of exogenous DNA targeting crRNAs that are essential for the maintenance of transforming DNA in H. pylori. The molecular mechanism whereby the dual immunity of H. pylori Type I CRISPR-Cas is able to utilize RNA processing components from Type III immunity systems to process an exogenous pre-crRNA mimicking a signature of Type III systems, thus allowing the use of Type III CAS proteins in crRNA array processing, has been characterized [78]. The type I-B system has been characterised extensively in several bacterial species that include Escherichia coli and Legionella pneumophila, and recently also in Agrobacterium tumefaciens, Clostridium difficile and H. pylori. However, while the biogenesis of the H. pylori type I-F, and II-A CRISPR-Cas systems are well understood, much less is known about their interference mechanisms, specifically how crRNAs guide the destruction of invasive DNAs. This is especially important given the large number of diverse H. pylori strains [79]. While much remains to be learned about the diversity of CRISPR-Cas systems, laboratory studies will continue to shed light on how these diverse systems operate and pave the way for their applications in biotechnology and medicine [80].

8.1. CRISPR-Cas as a Virulence Factor

Several pathogenic bacteria including Helicobacter pylori are known to harbor anti-phage genetic systems called clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR associated sequences (cas) [81]. The crystal structure of a type II-A Cas9 from Streptococcus pyogenes (SpyCas9) complexed with one of its guide RNA and a DNA target yielded the first insights into the mechanisms of CRISPR anti-phage defense in bacteria and archaea. Cas9 recognizes the PAM site on target DNA, which is deep in the protein structure and formed by α-helices. The initial recognition of the PAM site induces a series of DNA rearrangements in the Cas9–RNA–DNA ternary complex [82]. The target DNA unwinds at the upstream region of the PAM site and rotates by reorientating at the hinge region. A now exposed region of target DNA engages with the two RuvC-like domains of Cas9 and the DNA-duplex is cleaved into two pieces of single-stranded DNA. The mechanisms of PAM recognition and DNA unwinding in the bacterial Cas9s are similar in the eukaryotic class 2 C2c2 and C2c6 proteins [83]. A type VI CRISPR-Cas system CRISPR6 was identified in H. pylori and its role in genome protection against phage attack was characterized. Analysis of bacterial isolates revealed that among the top 7 most common combinations of Cas6 genes, 3 were also associated with the acquisition of virulence genes such as cagA, vacA, and dupA [84]. The presence of strains with each combination correlated with the expression of one or more of these virulence factors. Moreover, genetic complementation of a cas6 deletion strain with a cas6a gene improved genome stability by reducing the frequency of spontaneous mutations. These findings highlight the potential of CRISPR-Cas as a virulence factor and its implications for understanding the evolution of H. pylori infection and its associated diseases [85].

8.2. Impact on Host Immune Response

The discovery of Helicobacter pylori (H. pylori) CRISPR-Cas systems in 2017 suggested potential

anti-symbiotic functions for these systems, in agreement with findings demonstrating reduced levels of infection in CRISPR-Cas positive strains. As CRISPR-Cas systems are active against invading nucleic acids, H. pylori could use this mechanism to combat viruses, plasmids, or foreign DNA to maintain the integrity of the genome [86]. Indeed, functional analysis of CRISPR-Cas systems in other bacteria has revealed roles in plasmid loss, limitation on the uptake of exogenous DNA, and maintenance of genome fidelity after phage infection. However, some of these functions, such as plasmid loss, are unlikely to have a major impact in H. pylori as it does acquire and maintain plasmids [87].

Novel functions for the H. pylori CRISPR-Cas systems are explored, which may have evolved in parallel with the emergence of this pathogen. Putative CRISPR-Cas systems of type I-F and I-C were discovered in H. pylori strains involved in peptic ulcers and gastric cancer. Phylogenetic analysis indicated that H. pylori type I-F CRISPR-Cas systems were most closely related to those in Campylobacter fetus and the type I-C systems to those in Fusobacterium nucleatum. Similar to most other type I CRISPR-Cas systems, H. pylori Cas3 Csn2 effectors are followed by a helicase-hnh nuclease, and an auxiliary cse1 protein [88]. Surrounding cas genes include cas4 and the many CRISPR repeat-associated CRISPR-RNA processing genes needed for interference with invading nucleic acids. In contrast, H. pylori type I-C systems possess distinct Cas6e and Cse3e HNH family proteins, and contain only leader-proximal cas genes [89].

Type I-C systems are shorter than their type I-F counterparts, as they only contain crRNA biogenesis and interference genes, suggesting that CRISPR-Cas systems may have adapted to the H. pylori niche by acquiring diverse auxiliary genes. Loss of product from these CRISPR-Cas systems leads to a relaxed restriction of plasmid acquisition, suggesting that the CRISPR-Cas systems play an important role in protecting genome integrity in this bacterium [90].

9. Interactions Between CRISPR-Cas and Other Genetic Elements

A CRISPR-like sequence in Helicobacter pylori CRISPR-Cas systems has been detected in various bacteria, including Listeria monocytogenes. The concept of the CRISPR-Cas system as a prokaryotic defense mechanism against invasive genetic elements has been supported by a variety of studies. Whenever an exogenous genetic element is attacked, short (20-40 bp) sequences derived from the genetic element become integrated into a region of the bacterial genome called an inverse transcribed palindrome (ITP) [91]. Each time a foreign genetic element attacks a cell, the new relevant 20-40 bp in length sequence becomes integrated into the ITP. In the absence of any acquired sequence, this DNA sequence is then transcribed and then amplified in the form of small RNA (crRNA). This crRNA molecule then forms a complex with a variety of cas gene products encoded by the cas region and it becomes capable of degrading the exogenous genetic material derived from bacteriophages or plasmids similarly to a restriction enzyme [92]. Clustered regularly interspaced short palindromic repeats (CRISPR) sequences are detected in around 40% of bacteria and many archaea. CRISPR, together with the CRISPR-associated genes (cas) are a prokaryotic defense system against invasive bacteriophages or genetic elements. This system has been characterized to mediate RNA based immunity in bacteria, where a series of cas proteins function to cleave and degrade foreign nucleic acids of bacteriophages or plasmids [93]. CRISPR loci in the bacterial genome contain multiple direct repeat (DR) sequences. The size of DR varies but is mostly composed of 29-37 nt long sequences. In between each DR, a variable spacer sequence exists that is frequently targeted to invasive phage or plasmid DNA. DR sequences are commonly conserved whereas spacer sequences are diverse, and derived from the targets of the invasive bacteriophage or plasmid. These CRISPR sequences have been used successfully to develop a genetic typing method [94]. Nontypeable strains are likely to occur due to genetic alterations in either CRISPR or toxin genes. Prior knowledge of each allelic type can greatly help the manipulation of strains for experimental study, vaccine development and novel antibiotic discovery [95].

9.1. Plasmids and Transposons

Helicobacter pylori is a Gram-negative bacterium that colonizes the gastric mucosa of half of the human population, contributing to a range of diseases, including chronic gastritis and gastric cancer. Its pathogenicity is linked to the presence of virulence factors. However, genetic diversity and genome plasticity, including the presence of integrated mobile genetic elements, contribute to its high population diversity and result in variation of virulence factors [100].

In addition to numerous putative plasmids with predicted functions such as toxin-antitoxin systems, restriction-modification systems, bacteriocins, virulence factors and genes involved in DNA repair, transposable elements of varying lengths were found in H. pylori genomes. These include short and long insertion sequences, and a second class of replicative transposons resembling those of the H. pylori transposon family, including transposon structures associated with plasmids or bacteriophage elements [101]. Other transposon classes were also predicted by bioinformatic searches. The presence of inserted transposons or remnants, as well as a number of Tn7-like transpososomes involved in cell adhesion and in the establishment of the pathogenic potential of the strains was also indicated at the sequence level [102].

This work expands the knowledge of mobile genetic elements in H. pylori and provides the basis for further studies on the role of plasmids and transposons in this human pathogen. ColE1-like plasmids provide a tool for one-way recombination in H. pylori, but not for a whole-genome approach. Instead, they lead to mixed-infection studies, which should narrow down the virulence genes of H. pylori. Analysis of H. pylori bacteriophage and their prophages provide insight into the addiction of H. pylori to integrate and transduce mobile genetic elements, and may also play a role in spread of antibiotic resistance genes among bacteria. Transposons of H. pylori may be involved in genome plasticity and confer diverse pathogenic potential [103].

9.2. Horizontal Gene Transfer

Effects on horizontal gene transfer were analyzed by dual fluorescent labeling. The MG1655-derived strains were transformed with pKMx vector-derived plasmids that carry gfp and mCherry genes under the lrp and hns promoters, respectively. Strains harboring gfp or mCherry plasmids were grown separately in M9 minimal media with 0.2% glucose. Cells were co-cultured on semi-solid M9 media with 0.3% agarose, and the cells were incubated for 4 h. Finally, the co-cultured cells were analyzed by fluorescence microscopy to investigate the transfer of fluorescence plasmids from transformed strains to the non-transformed one [104]. For analysis of co-cultured S. Typhimurium and E. coli K-12 cells, E. coli MG1655 was transformed by pKMx vectors that carry gfp and mCherry genes under the lrp- or hns-promoters. pKG and pKG-vakv mKate vector were transformed into S. Typhimurium. E. coli K-12 MG1655 and S. Typhimurium were co-cultured on a M9 agar plate with streptomycin incubated overnight at 37°C. The bacteria were harvested and resuspended in phosphate buffer, and a 2-3 μl suspension of the mixed population was placed on an agar slide and examined using a fluorescence microscope [105]. The following dyes were used: 488 nm for GFP, 515 nm for mCherry, and 543 nm for mKate. The images were captured by camera. The proportion of cell fluorescence for each species in the co-cultured cells was measured to represent interspecies transfer by a program written by custom image analysis software using the ImageJ plugin for the MatLab image processing toolbox. After capturing fluorescence images, color coding was applied. The data files of the resulting images were converted into numerical matrices of RGB values (8-bit grayscale for each color channel). Subsequently, the ratio of each color was computed for each pixel using a 230 × 230 pixel area and visualized for each cell by smoothing the value matrix (Gaussian filter size of 5) [106].

10. Experimental Approaches to Study CRISPR-Cas in H. pylori

Research on CRISPR-Cas has been actively studied in H. pylori since the latter was first identified as a human pathogen in the late 1980s. This model is widely used in culture-independent studies involving next-generation sequencing technology as magnitudes of data have yet to be

experimentally validated. Without an easy, approachable system for H. pylori, researchers may miss a great opportunity for advancing our understanding of the other CRISPR-mediated processes. For in vivo experiments, at least a strain that expresses a plasmid-born reporter god can be engineered [107]. As the density of H. pylori explodes in the human stomach, this method of employing reporters could greatly enhance understanding of the system. Using H. pylori as a model to study this CRISPR defence system would be especially advantageous. Suggested experimental approaches include the construction of a vector that allows foreign DNA to be inserted into the cas9 gene along with the guide RNA and the establishment of a transgenic strain that allows this plasmid to be maintained [108]. Modular systems now exist that can program Cas9 with complex gRNAs or multiplexed sgRNAs. There exists a natural genetic resource that involves searching the number of traces. Juxtaposition of bioinformatic pipelines with bacterial genetics and molecular biology should allow functional analysis of many previously uncharacterized and orphan Cas proteins. Understanding defense pathways is likely to be of great importance, although H. pylori must employ additional uncharacterized proteins to combat other types of foreign nucleic acids [109]. The use of genome transformation along with other existing external resources could shed light on this long respected but poorly understood group of viral enemies. Finally, a CRISPR–Cas immune system with a long evolutionary history must greatly influence species diversity in bacteria. Studies analogous to those on mammalian adaptive immunity that assess the outcome of immunity at the population level could yield fascinating new insights into how CRISPR immunity shapes the genetic and functional diversification of H. pylori in human populations [110].

10.1. Genetic Manipulation Techniques

To understand the various factors underpinning genomic manipulation in Helicobacter pylori (H. pylori), it is essential to know about the bacterium, its physiology, and attributes that differentiate it from other bacteria. H. pylori is a gram negative, spiral-shaped, and unicellular microaerophilic bacterium. Genomic information is at the heart of all things biological. To acquire, analyze, and manipulate genome information, it is vital to have the correct techniques in place [111]. Genetic manipulation techniques include cloning, screening transgenic individuals, transferring natural or synthetic genes into the genome, incorporating tailored mutations in targeted genes, or depleting specific proteins, preventing their transcription and translation. These are key components of the overall process, such as designing vectors or selecting screening methods [112].

Genome modification is accomplished using protein tools such as meganucleases, zinc finger nucleases, transcription activator-like effector nucleases, and the more recent clustered regularly interspaced short palindromic repeats associated with CRISPR-associated proteins. These tools can be engineered to target specific genes in a variety of organisms. However, in bacteria, induced genome changes can be subject to adaptive immunity mediated by restriction-modification systems, CRISPR-Cas systems, and DNA targeting small RNAs. Engineering CRISPR-Cas systems to repurpose them as genome targeting tools is a foundational recent advance in genetic engineering [113]. Although a large number of CRISPR systems encode the genes needed for RNA based targeted genome modification, the functional details of these systems are poorly understood. Understanding the bases of CRISPR functioning is essential for effectively using these tools in biotechnology and for developing effective CRISPR interference and immune evasion strategies against possible natural applications of ribonucleoprotein complexes [114].

10.2. Bioinformatics Analysis

The CRISPR searching query was performed using the BLASTN algorithm against the H. pylori genome and spatially mapped to visualize homology and synteny using the BLAST ring image generator. To elucidate the complex evolutionary history of H. pylori CRISPR-A1 sequences, phylogenetic comparison was conducted along with interspecies analysis using representative CRISPR-Cas I-A sequences from other bacterial species [115]. Crassphage was selected to draw the phylogenetic tree, as it is a proposed model of CRISPR-Cas GE spread among the phage.

Representative CRISPR, DR and cas sequences of each strain were aligned via MUSCLE and the ML phylogenetic trees were constructed via IQ-TREE with 1,000 ultrafast bootstrap replicates. Pairwise dN/dS ratios of protein coding genes and BLAST results were also calculated to estimate GE-GE adaptation coevolution and played the role of genomic stories. To visualize the GRNs, the GRN with bacterium-induced time points were displayed via Cytoscape and generated structural modeling of interacting core genes and proteins using Chimera and I-TASSER [116]. Selected GWAS candidate genes were classified and illustrated via Cytoscape. Phylogenomic trees were constructed using the IQ-TREE employing LG+G4, for which genomic integrity was controlled by utilizing Sconservate. The above analysis of another GE with a CRISPR-Cas I-C system can be performed as well. A CRISPR database was also built to elucidate evolutionary history. The presence of bacterial virulence was performed [117]. H. pylori strain assemblies were downloaded and the virulence gene detection tool was run across databases. Amendment of acid stress was simulated on H. pylori to investigate the post-immune response with regards to CRISPR-cas systems, growth rate measurements, antimicrobial susceptibility detection, stress enzyme activity measurements, Caco-2 monolayer passage assay' cytotoxicity measurements and transcriptome sequencing. Taking passage assay as an example, 24 hours post-infection the 100 μg/mL penicillin was supplemented for another 24 hours, after which the remaining viable bacteria in the medium was retrospectively counted using the bacteria survival assay via serially diluting and dropping on plates [118].

11. Clinical Implications of CRISPR-Cas Research

Further understanding the roles of CRISPR-Cas systems in H. pylori may provide insights to advance public health and treatment of related diseases. In addition to the established association between CRISPR-Cas systems and genome stability, naturally-acquired H. pylori infection has been established as one of the strongest risk factors for development of several human diseases including gastric cancer. The observed direct human-to-human transmission of H. pylori provides a perfect opportunity to study the dynamics of CRISPR-Cas systems and their effect on the pathogenesis of H. pylori [119]. Given the complexity of associations between H. pylori alteration, immune response, inflammation and gastric diseases, future studies in this area will require multidisciplinary approaches, from bioinformatics to molecular biology, microbiology and immunology. In addition, utilizing relevant animal models, such as gnotobiotic SPF mice, will also be helpful in collecting further evidence on these conclusions. While this study has established some direct associations between CRISPR-Cas systems, H. pylori genome evolution and disease outcome, H. pylori is a highly heterogeneous organism with a relatively large genome size, up to 1600 predicted protein encoding genes, and ~130 variable effectors [120]. Given this complexity, more extensive sampling of relevant CRISPR-Cas strains from different origins, and more thorough genotypic and phenotypic analyses on a case-by-case basis will be required to study their contributions in affecting genome evolution outcomes. Further dissection of the underlying mechanisms, such as prediction of DNA repair pathways, genome-wide association mapping and integration of transcriptomic data, will also be required, and help to recruit new research teams from a variety of disciplines [121]. H. pylori are already a common target for therapeutic vaccinology of gastric diseases. Further understanding of H. pylori genome alteration processes, and how they affect layers of the host response immune and pathological networks, will enhance the current understanding of this organism, and may lead to more effective immunotherapies and treatments of related diseases. Meanwhile, the rapid yet highly stochastic genome-wide CRISPR and COG mutation acquisitions and associated gene knockouts and rearrangements provide a novel model to study the mechanisms of innate genome alteration, and co-evolution of genomes and populations. These findings may have broad implications that extend beyond H. pylori [122].

11.1. Potential Therapeutic Applications

Helicobacter pylori (H. pylori), a Gram-negative spiral-shaped bacterium, is an important human pathogen. As the etiology of chronic gastritis, peptic cells rupture, and gastric cancer, it has been

classified as a class I human carcinogen by the World Health Organization. The pathogenesis of gastric disease is heterogeneous and influenced by virulence factor acquisition and loss. The most important factors associated with gastric cancer development are virulence factors encoded by the cytotoxin-associated gene A (cagA) and vacuolating cytotoxin A (vacA) [123]. However, the prevalence of H. pylori in gastric disease and control population is much higher than the incidence of gastric cancer. Genetic instability of H. pylori is theorized to favor survival in the diverse environments encountered within the host. Some evidence suggests a role for CRISPR-Cas as a defense mechanism against bacteriophage or plasmids. The identification of a CRISPR-like, putative defense system in Streptococcus pyogenes has had major implications in molecular biology and gene targeting [9]. In this study, bioinformatic analysis was used to evaluate possible CRISPR-Cas systems in public H. pylori genomes [124]. The analysis shows that H. pylori acquired CRISPR-Cas systems from different sources with differing capacities to defend against genetic invaders. To provide initial insight into the role of CRISPR-Cas in H. pylori, a transparent, helical enteric bacterium, the H. pylori Cag pathogenicity island-2 (HPCAP-2) transmission vectors incorporating the PHR, CRISPR-Cas, Dcr complexes and diverse endonucleases were examined in D. acidovorans. It is the first report that associations of CRISPR and Rab-type GTPases, suggested active CRISPR-Cas systems [125]. The functions of CRISPRs in H. pylori showed dynamic evolution and diverse origins. In particular, CRISPRs of HP_01624-HP_01626 and HP_12171-HP_12179 are noteworthy because their Cas3 functions have been retained over longer phylogenetic distances. The association of using fragment homology with cognate CRISPR and PHR systems in H. pylori with unusual sequences considered as putative safeguard mechanisms is proposed [126].

11.2. Challenges in Clinical Translation

Clinical interventions targeting H. pylori have been sharply limited by a variety of issues related to the organism's distinctive physiology and genetic variability that enables rapid development of antibiotic resistance. H. pylori does not grow on cell-free defined media and thus unable to reach suitable cell densities for ex vivo manipulation. A similar limitation applies for several other gastric bacteria such as Campylobacter jejuni and some strains of Enterohemphelicobacter. H. pylori is highly diverse with different strains showing large variation in genotypes, virulence, and antibiotic resistance profiles [127]. Furthermore, once established, this pathogen generates a chronic infection in which daughter cells are maintained stably over time. In addition, efforts in H. pylori vaccine generation have faced obstacles since this organism employs strategies to evade colonic antibodies and gained persistence without inducing gastric inflammation. Despite significant progress in understanding genetic interactions in H. pylori revealed by genomics and transposon mutagenesis, H. pylori pathogenesis remains poorly understood [128].

A promising new system is the use of phage particles and phagemids as vectors to deliver auto-targeting CRISPR-Cas components inside a pathogenic cell. This idea exploits Cas9 of Type II CRISPR-Cas systems, which together with crRNA and tracrRNA, binds and cleaves almost any dsDNA in a sequence-specific way. Many pathogens such as Helicobacter pylori or Clostridium difficile, which are associated with peptic ulcer or colitis, possess endogenous active CRISPR-Cas systems and could be repurposed for self-targeting. In these pathogenic bacteria, anti-Cas9 should not need to be applied as serotype-matched opposing RNA is usually stable and costly to provide and can be defeated by phage evolution [129]. The attraction of such a strategy is that much of its operation proceeds on the precise template of the pathogenic DNA, targeting almost any input sequence. The system also shares advantages common to other phage-derived agents such as nano-medicines or phage-antibody conjugates. Such a CRISPR system should be applicable to flexible dual diversity, multiplex confrontation of both viral and plasmid invaders, target vector delivery, broad host pan-virulance, and the eventual recombination and reprogramming of target genomes with fewer failed dosages [130].

12. Future Directions in CRISPR-Cas Research

CRISPR-Cas systems were first discovered as a transformative tool for targeted genome editing, and understanding the biological role of these systems continues to garner wide interest. It has become increasingly evident that CRISPR-Cas systems are involved in a multitude of unexpected processes across various fields, including pathogenesis, phylogenomics, physiology, and even human evolutionary studies [131]. Meanwhile, a spatially and temporally separate reconsideration of CRISPR-Cas function in a variety of traditional biological processes is presenting new avenues for investigation, as well as new questions. For example, the findings that CRISPR-Cas systems in H. pylori might target essential genes and contribute to post-transcriptional regulation is completely at odds with current expectations [132].

Some parts of Cas proteins are being repurposed in novel biosensing schemes, revealing the potential significant evolutionary and application divergence of CRISPR systems. CRISPR-Cas systems possess an inherent capacity to perform CRISPRi, which may be exploited as a regulatory tool. Representative CRISPR RNA-guided nucleic acids are either small guide RNAs and transcription units of emerging regulatory architectures that enhance the power of this approach [133]. The expected outcomes of new CRISPR-Cas inquiries can differ significantly from those anticipated. For example, while a treasure chest of new information on an old biological questions can be extracted using CRISPR-Cas to study genome regulation, was it expected that Cas proteins would be exploited as a robust technology for identifying ligand-protein interactions in living cells? Unforeseen consequences of CRISPR-Cas research on basic biology may yet surface [134].

12.1. Emerging Technologies

A variety of technologies have been developed to study CRISPR-Cas systems and use them for biotechnological purposes. Since the initial description of CRISPR-Cas systems in model organisms, attention has been drawn to their genome-editing potential, and the versatility of these systems has been studied in laboratory strains of Escherichia coli, Staphylococcus aureus, and other bacterial species. However, CRISPR-Cas systems are widespread in bacteria and archaea. While the genetic tractability of many of the latter has been a challenge, endogenous CRISPR-Cas systems have been discovered in Helicobacter pylori, and the possibilities of using the latter as a technology platform have been reviewed [135]. In the case of H. pylori, which is a gram-negative pathogen infecting half of the world's population, studies of CRISPR-Cas systems haven't only familiarized with H. pylori CRISPR-Cas systems but also added to knowledge about CRISPR-Cas systems in general. Applying this technology in a pathogen with profound effects on human health has raised a myriad of challenging questions about a system that has not been much studied up till now [136].

Many bacteria and archaea possess a defense system called clustered regularly interspaced short palindromic repeats associated proteins (CRISPR-Cas system) against invaders such as phages or plasmids. The CRISPR-Cas system consists of genomic CRISPR array and several cas genes. The numbers of spacer in CRISPR array differ among bacterial strains and can be used as a genetic marker for bacterial typing. Besides typing, there are many studies about application of CRISPR as a biotechnological tool; for example, as a molecular clock for estimation of time of evolution. Its attractive and promising features make it an ideal candidate for a versatile tool in genetic studies of h. The use of next generation sequencing enables high-throughput sequencing of CRISPR array, for typing and analysis of genomic evolution on large scale [137].

12.2. Unresolved Questions

CRISPR-Cas systems have been shown to be important for genome stability, as they counteract the most common type of cellular challenge: infection with foreign nucleic acids. However, for some bacteria, such as Helicobacter pylori, the very CRISPR-Cas systems that are thought to mitigate foreign DNA invasion can also precipitate genomic instability. Some of the most well-characterized examples of this include the contribution of BraE and Cas9 (which are part of type

II-A CRISPR-Cas systems) to DNA damaging double pay break formation via aberrant CRISPR targeting. Though such studies can serve a generalized model for several CRISPR-Cas systems across different species, the dual role algorithms were previously interrogated in a single bacterial species: H. pylori [138].

H. pylori is a Gram-negative bacterium colonizing the gastric mucosa of nearly half the human population worldwide. Infection with H. pylori is strongly associated with the development of various diseases, including peptic ulcer and gastric adenocarcinoma. During infection, the bacterium faces physical, chemical, and immune stress conditions that might endanger its genome integrity. CRISPR-Cas systems have been shown to provide prokaryotes with immunity against foreign nucleic acids, and HP-HMR (type I-F) and HP-IR (type II-C) CRISPR-Cas systems have been characterized in H. pylori. Both CRISPR-Cas systems are thought to mitigate horizontal gene transfer and result in genome stability, but they could also precipitate genomic instability [139,140].

The future fruit of microbiome research will shed light on symbiotic, commensal, and pathogenic relationships between bacteria and their hosts, including regulation of bacteria in the mammalian stomach and the role of H. pylori in health and disease, particularly gastric cancer [141]. Better understanding of the evolution, ecology, and genetic diversity of H. pylori could offer effective strategies for vaccine development/therapy against gastric diseases and design tools to control H. pylori transmission [142].

13. Conclusion

Helicobacter pylori is the most common bacterial pathogen affecting humans, with more than half of the human population harboring this bug in their gastric mucosa. In most cases, it remains asymptomatic, substantially increasing the risk of different gastroenteric diseases such as chronic gastritis, peptic ulcer disease, and inflammation-associated stomach cancers. Attempts at complete eradication using antibiotics showed only 40–80% success, resulting in an urgent need for novel approaches to treat the infections caused by this pathogen. One promising route to treat H. pylori infections consists of using CRISPR-Cas systems, composed of CRISPR repeat-spacers and a set of associated cas genes. These systems are present in around half of all bacteria and almost all archaea. They are universally divided into two main classes based on differences in their composition, architecture, and mechanism of action. In the first class, type I, type III, and type IV systems are characterized by a multi-protein interference complex containing large Cas3/3- and Csm- or Cmr-like proteins, plus small Cas7-like proteins. Cas6-like proteins are involved in pre-crRNA processing. In class two, the type II systems are composed of the nuclease-helicase Cas9 together with one or two other proteins and a standalone reverse transcriptase in the case of type V-C systems. Prototypical type II-C systems such as SpyCas9 cleave dsDNA, while other members of this class such as hC2c2 or Cas13d target ssRNA instead. Cas9-based systems have been used for diverse biotechnological applications such as genome editing, transcriptional regulation, biosensitivity, and epigenome editing. Virulence-associated genes, as well as their evolutionary relationship, may have been acquired by the bacteria via horizontal gene transfer. However, the systems of H. pylori and their association with any forged virulence-related factors remain to be determined. Moreover, the primary type of CRISPR-Cas system present in virulent strains of this bacterium and its correspondent protospacer genes have not been studied.

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