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CRISPR-CAS SYSTEMS IN PATHOGENIC BACTERIA

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ABSTRACT

Developing preventive measures, diagnosing, and treating diseases caused by bacteria remains a problem worldwide. Hence, understanding the mechanisms contributing to the virulence and adaptive immune systems of pathogenic bacteria is extremely important. Moreover, the latest events in the world show that we still need prompt safe diagnosis of various infectious diseases during severe outbreaks. Throughout evolution, pathogenic bacteria have developed many protective mechanisms against bacteria-targeting viruses. One such mechanism is the CRISPR-Cas system – a part of the bacterial adaptive immune system. Nowadays, the CRISPR-Cas system is considered the best molecular tool for gene editing in molecular biology applications. Moreover, CRISPR-Cas systems play a role in the virulence of pathogenic bacteria. The variety of these systems is amazing, as is their use in various fields of biology and medicine. In this review, we summarize different types of CRISPR-Cas systems in pathogenic bacteria and discuss the role of CRISPR-Cas in bacterial virulence and its application for biotechnology.

Key words: CRISPR-Cas, adaptive immune system, pathogenic bacteria, gene editing, virulence, pathogen detection.

INTRODUCTION

CRISPR - a Clustered Regularly Interspaced Short Palindromic Repeats – a system of adaptive immunity of bacteria and archaea, aimed to destroy any foreign DNA or RNA that penetrates into the cell [1]. It was first discovered in *Escherichia coli* in 1987 [2]. This system consists of two main parts: 1) the CRISPR- cassette, which contains palindromic repeats 24-49 nucleotides size alternating with spacers, and 2) *cas* genes, encoding nucleic acid processing enzymes, including nuclease or helicase proteins [3]. Repeats can form recognizable loop-structures, while spacers are identical to fragments of bacterial phages and protect against virus invasions [4.]. It was a great breakthrough when discovered that CRISPR spacers are also sometimes identical to fragments of mobile genetic elements (MGE). Studies on CRISPR-driven inhibition of plasmid conjugation and transformation in *Staphylococcus epidermidis* proved the preventive ability against not only phages but plasmids, transposons, etc. [5].

Evolutionarily prokaryotes developed a variety of defense mechanisms for survival after phages attack such as restriction-modification system, an abortive infection, toxin-antitoxin systems, which are well studied and described [6]. There are several molecular methods of genome editing based on these mechanisms: zinc finger

nucleases (ZFN) and TALE-associated nucleases (TALEN), CRISP-Cas [7]. But after important discovery that Cas9 nucleases induce blunt double stranded breaks in DNA [8, 9], CRISPR-Cas9 technology became leading in manipulations with genomes. And now it stands at the forefront due to its simplicity and widespread use at many molecular fields.

CRISPR-Cas systems have been found in 50% of complete bacterial and 90% of archaeal genomes, respectively [10, 11]. According to NCBI data, 3914 works related to CRISPRs were published in 2018 (fig.1).

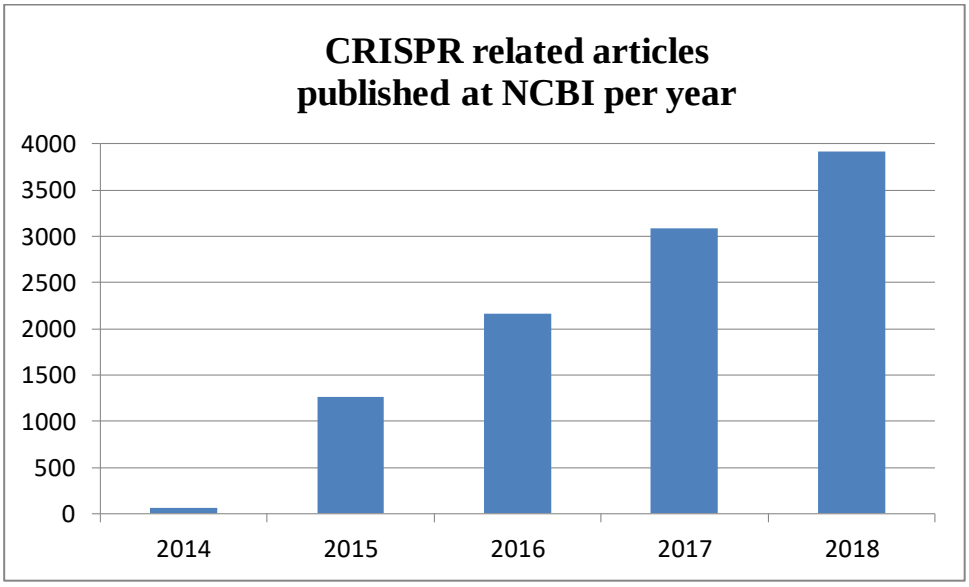

Fig. 1. CRISPR related articles published at NCBI per year

Authors of the latest 2019 work announced that they created quick, cheap and sensitive CRISPR–Cas-based perfect diagnostics platform for pathogen detection during the outbreak with a potential for cancer, and genetic diseases [12].

Remarkable discovery was that CRISPR systems in pathogenic bacteria not only acquired immunity system but also play role in bacterial virulence [13]. In *Francisella novicida*, knockout mutants of *cas9* were not able to cause lethal infections in a mouse model [14]. And addition of Cas9 to an isolate of *C.jejuni* lacking a CRISPR-Cas system led to a significant increase of virulence of this isolate, showing that Cas9 is very important for virulence [15].

Thus, in this review, we discuss current diversity of CRISPR-Cas systems in pathogenic bacteria and its role in virulence, summarize today's emerging applications of CRISPR-Cas in biotechnology.

CRISPR-Cas biology and classification

At the molecular level CRISPR-Cas systems function through three stages: adaption, crRNA maturation and interference (fig. 2). There are wonderful reviews dedicated to these processes with a detailed description of each stage [16, 17, 18].

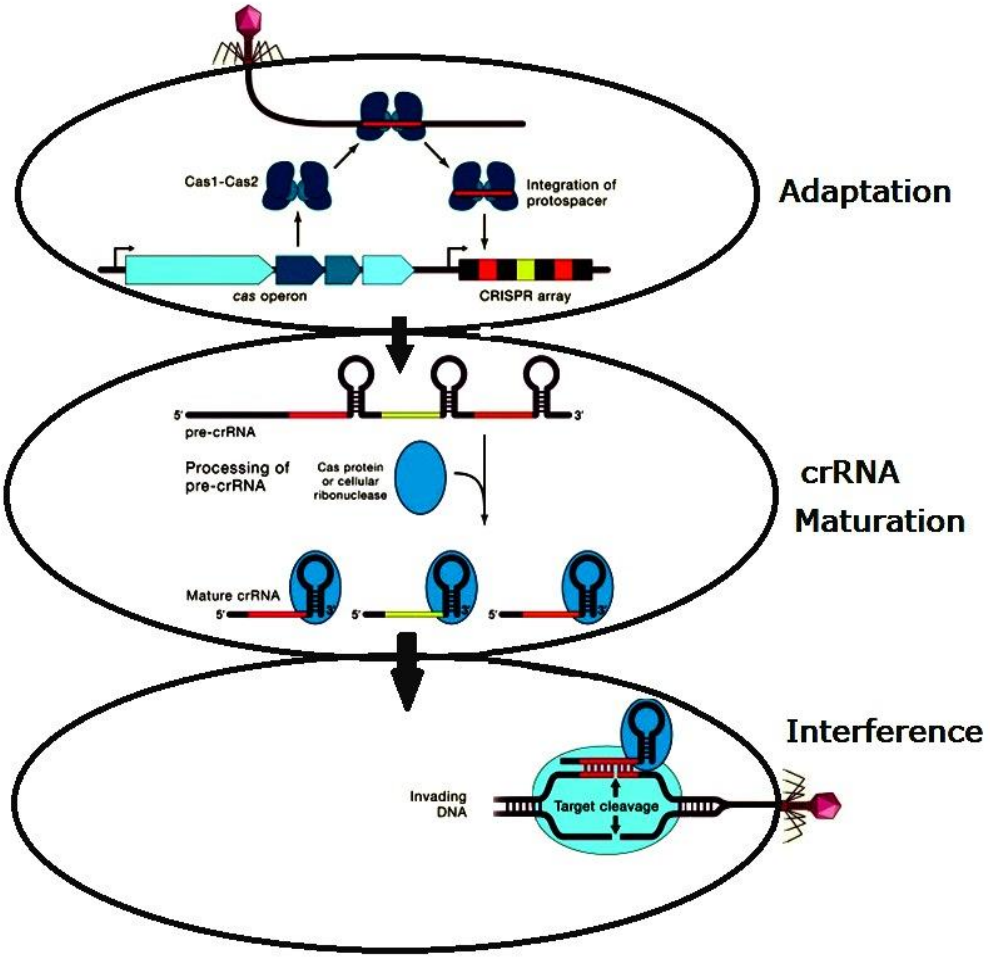

Fig. **2.** Three stages of CRISPR-Cas machinery

During adaptation, foreign nucleic acids are cut from "digested" bacteriophage or MGE and inserted to the CRISPR array providing memorization of infection. During crRNA maturation - memory is activated when the CRISPR array is transcribed for producing a pre-crRNA - precursor crRNA (CRISPR-assosiated RNA), and then a yield mature crRNAs. During next re-infection interference starts up by crRNAs and complementary sequences in the foreign nucleic acid called protospacers are split. Thus, CRISPR-Cas systems protect prokaryotes from secondary infection. It is important that the binding and degradation of the DNA of the protospacer occurs only in the presence of a specific nucleotide segment (protospacer adjacent motif, PAM), which may have different sequence and size in different species of bacteria [11]. The presence of the PAM site serves as a kind of identification signal for foreign DNA and allows avoid cleavage own bacterial spacer regions of the CRISPR locus during interference.

One organism can contain different types of CRISPR-Cas systems, and spacers can distinguish in different cells of one population. According to Makarova's classification, diversity of cas genes and interference complexes allowed to classify the CRISPR-Cas systems for today into two classes, which are then divided into six types and several subtypes with selected cas gene [11, 19]. Most effectors destroy DNA, only one – RNA [20], and very rare – both molecules.

Systems of class 1 CRISPR-Cas

Systems of class 1 CRISPR-Cas (types I, III and IV) use multicomponent protein complexes during interference to destroy target DNA or RNA. The CRISPR-Cas type I interference is called CRISPR-associated complex for antiviral defense (Cascade, depends on type I subtypes, from I-A to I-F and I-U) [21]. It consist of PAM recognition on foreign targets by Cas8 subunits, Cas6- or Cas5-mediated crRNA binding to target DNA, a Cas7 formation of backbone, R-loop stabilization by two small Cas11 subunits, and target cleavage by Cas3 [22] (fig. 3).

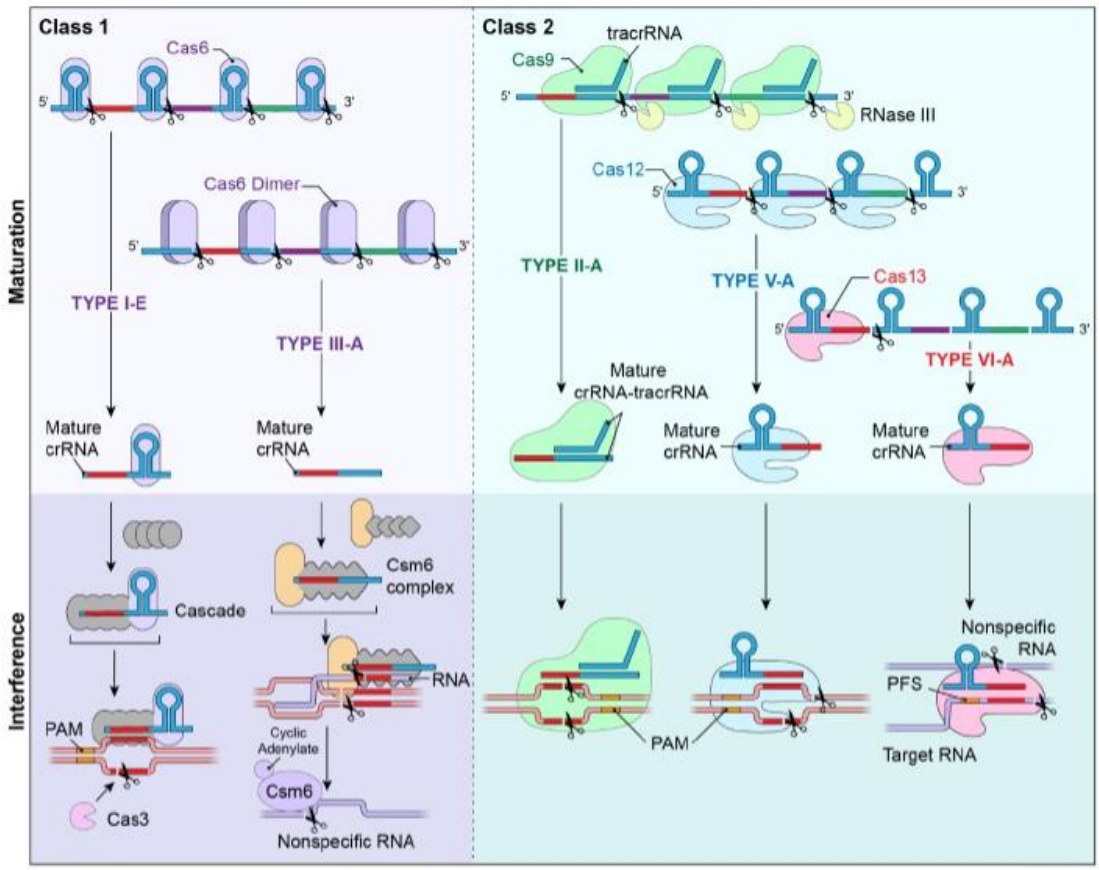

Fig. 3. Maturation and Interference in CRISPR-Cas Class 1 and Class 2 systems [21].

The type III (subtypes III-A and III-B) interference complex is similar to Cascade. Recognition of a PAM or RNA-PAM (present on transcribed RNA) is required by some but not all type III systems. However cleavage of foreign DNA depends upon binding of the interference complex to RNA transcribed from foreign DNA. When the interference complex is bound, Cas7 cleaves the ssRNA transcript at regular intervals and Cas10 cleaves target DNA [23]. The mechanism of the interference of other type I subtypes, subtypes III-C and III-D, and type IV is not yet fully studied.

Systems of class 2 CRISPR-Cas

Systems of class 2 CRISPR-Cas (types II, V and VI) interference is only effected by one effector protein instead of protein complex [24]. Type II systems (subtypes II-A, II-B, and II-C) are based on Cas9 protein and the requirement for tracrRNA along with crRNA serving to guide the Cas9 nuclease [25]. Cas9 recognizes PAM sequences on target DNA and crRNA-tracrRNA complex pairs with complementary DNA resulting in Cas9 double stranded cleavage.

Type V CRISPR-Cas system (subtypes V-A, V-B, and V-C) is based on 12a, 12b, and 12c proteins, respectively. tracrRNA is required for 12b activity but not 12a activity, and requirements of 12c need further characterization. After PAM recognition by the crRNA-Cas12 complex, target dsDNA is cleaved [26].

Interestingly, that type II CRISPR-Cas systems appears only in bacteria, not in archaea. More, type II CRISPR-Cas systems are presented in bacteria that use vertebrates as a host, including a wide variety of pathogens [27]. For genetic engineering most suitable system is CRISPR-Cas of class 2 type II– it is the most simple. Its effector protein called Cas9 in our days is most widely used molecular tool in different fields, especially in genome editing [28]. An example of Cas9 protein - nusclease SpCas9 from Streptococcus pyogenes. SpCas9 is class II type of CRISPR/Cas-system. SpCas9 does not need any additional co-factors for binding and cutting the DNA. In natural conditions two RNA are needed for SpCas9 activation - CRISPR-assosiated RNA (crRNA) from genome locus which stores phage's nucleotide fragments, and trans-activated-RNA (tracrRNA), which guides activity of Cas9-RNA complex [29]. But for genetic engineering it was enough to place the *cas9* gene and the CRISPR cassette on the vectors. And by changing the number and type of spacers, several different regions of the genome that need to be changed can be modified at once. Interesting thing was found, that both RNA can be synthetically integrate into one chimeric molecule of sgRNA (single guide RNA of 100-250 bp). It contains 19–20 nucleotides which are complementary to target DNA, functional elements tracrRNA и crRNA, and NGG-like three nucleotides at 3'-end of sgRNA. After binding sgRNA with complementary sequence of target DNA SpCas9 cleaved both DNA in 3 nucleotides distance from PAM and form a double-stranded DNA blunt breaks. Optimization of the system for eukaryotic cells is also needed - correct the codon composition and add the nuclear "address" so that it would follow to the chromosomes. The CRISPR- Cas9 system is easily undergo modifications, from changing the secondary structure of sgRNA to mutations of specific amino acids in SpCas9, or the addition of functional domains from other proteins [30].

CRISPR-Cas in pathogenic bacteria

Generally, most strains of the same species usually contain identical CRISPR-Cas types. But the diversity of CRISPR-Cas systems is so high, that there are exceptions like the type I-F system in *E.coli* strains and the type I-C system in some *Streptococcus sanguis* strains. One of the most unusual examples is in the *Campylobacter* genus: the *Campylobacter jejuni* contains a type II-C system, while other species (*Campylobacter concisus*, *Campylobacter curvus*, *Campylobacter fetus*, *Campylobacter hominis* and *Campylobacter rectus*) are based on a type I-B system [3].

Sometimes bacteria can contain more than one or combination of two CRISPR-Cas types (or even three types in the *Streptococcus* species). *Helicobacter* species (*Haemophilus*) and two genera belonging to *Pasteurellaceae* family (*Pasteurella*) have a type II-C system and a virulence-associated protein D (VapD) that shows homology to Cas2. In M*ycobacterium spp*. type III-A system is observed; in some strains only the VapD protein is detected. In the *Clostridium* species type I-B, type II-B, type II-C, and type III-B CRISPR-Cas systems have been detected. In the sequenced genomes of *Bacillus* species, mostly type I-B and type I-C CRISPR-Cas systems have been found, whereas in *Bacillus cereus*, only a large CRISPR array has been detected, reminiscent of a degenerate CRISPR-Cas system (fig. 4).

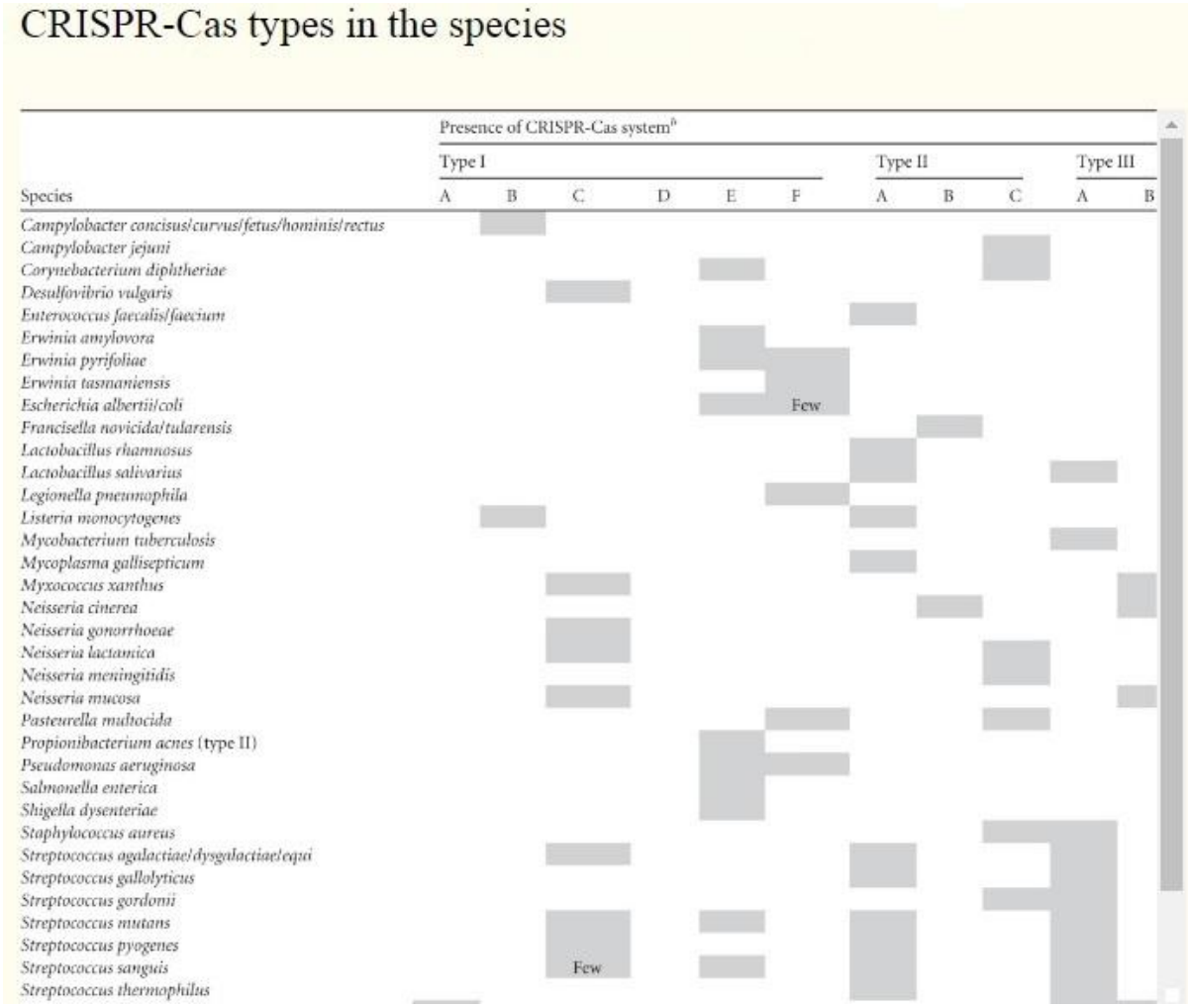

Fig.4. CRISPR-Cas types in different pathogenic bacteria [13].

In 2015 at triennial international meeting in Lausanne of the European Society for the study of *Chlamydia*, *Coxiella*, *Anaplasma* and *Rickettsia* (ESCCAR) for the first time have been revealed the presence of CRISPR in *Chlamydiae* [31]. In *Chlamydia*-related organism *Protochlamydia naegleriophila*, strain *KNic*, a clustered regularly interspaced short palindromic repeats found in the chromosome. It consist of small CRISPR from eight repeats 28 bp long repeats separated by 33 bp-long spacers and associated *cas-cse* genes of the subtype I-E. The upstream operon of CRISPR-associated genes from the *E. coli* subtype I-E consists of the core genes *cas1-2*, the type I gene *cas3* and subtype-specific genes *cse1-2, cas5, cas6e, and cas7*. The novelty of this work for CRISPR systems is that although confirmed CRISPR locus was predicted by CRISPR finder [32] in the recently released genomes of *Neochlamydia sp*. [33], but no *cas* genes could be identified before this work. And since it was shown that the CRISPR locus is also present in another *Pr. naegleriophila* strain of *Chlamydia sp. - Diamant*, it was assumed that the CRISPR system was inherited from common ancestor [34].

CRISPR-Cas systems in biotechnology and role in virulence

The application of crisps is amazingly wide. CRISPR-Cas9 systems are currently used in biotechnology for the following purposes: to improve the properties of animals and plants (CRISPR systems for rice, wheat, corn and many other crops are already have been tested [35]); to protect industrially important bacterial strains against bacteriophages and unwanted plasmids by incorporating CRISPR-Cas systems with

desired properties; for gene therapy and fighting with chronic diseases like HIV [36]; to identify bacterial antibiotic resistance genes through combination CRISPR-Cas9 with optical DNA mapping [37]; to detect viruses (for example, Zika and Denge vuris directly from clinical isolates [38, 39]) and bacteria (combination CRISPR-Cas with FISH method to detect Methicillin-resistant *Staphylococcus aureus* [40]).

A separate direction of CRISPR-Cas use is regulation of gene activity and DNA repair in bacteria [41]. Cas9 proteins (type II systems) regulate the virulence of pathogens *Legionella pneumophila, Francisella novicida, Campylobacter jejuni* and, possibly, *Neisseria meningitides*. It was a great discovery that CRISPR systems in pathogenic bacteria not only acquired immunity system but also play role in bacterial virulence [13]. In *Francisella novicida*, knockout mutants of *cas9* were not able to cause lethal infections in a mouse model [42]. And addition of Cas9 to an isolate of *C. jejuni* lacking a CRISPR-Cas system led to a significant increase of virulence of this isolate, showing that Cas9 is very important for virulence [15]. Studies of the virulence of *Enterococcus faecalis* isolates suggest that CRISPR-Cas systems in addition to other genomic differences may influence bacterial pathogenicity via two ways: defense by CRISPR-Cas may reduce the potential bacterial virulence when mobile elements could introduce foreign DNA carrying potential virulence factors (toxins or antibiotic resistance genes); and control of gene expression by CRISPR-Cas may enhance bacterial virulence.

CONCLUSION

Prokaryotes have CRISPR-Cas systems that provide heritable adaptive immunity. The emergence of the CRISPR-Cas9 system in the arsenal of genetic engineering had a tremendous impact on biology: gene therapy, chromosomal engineering and genomic screenings, detection pathogens and gene regulation. For pathogenic bacteria CRISPR-Cas systems not only acquired immunity system but it also involved into bacterial virulence mechanisms. As new discovers in structure, mechanisms and function of these systems appear, biological and medical applications for CRISPR technology will continue to extend.

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