# CRISPR/Cas Systems as Antimicrobial Agents: Current Status and Prospects (Postprint)

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**Date:** 2018-07-15T00:00:00+00:00

## Abstract

The long-term misuse of antibiotics has led to dysbiosis of the human microbiota and the emergence of bacterial drug resistance, necessitating the search for novel, targeted antibacterial approaches to treat infections caused by resistant bacteria. In recent years, in-depth research on the CRISPR/Cas system has provided new insights for designing therapeutics that specifically target resistance genes and directionally eliminate resistant bacteria. Herein, this article introduces the CRISPR/Cas system as a novel antibacterial modality that achieves specific clearance of resistance genes or pathogens through targeted cleavage of resistance plasmids or bacterial genomes, and evaluates the selection of different nuclease types for CRISPR-based antimicrobials as well as the delivery vehicles for CRISPR delivery systems.

## Full Text

## The Progress of CRISPR/Cas System Used as Antimicrobials

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**Abstract:** Antibiotics are used to arrest essential bacterial signaling and/or metabolic pathways, causing bacterial cell death. Overuse and misuse of antibiotics have led to dysbacteriosis and drug resistance. The application of CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR associated) systems provides a new method to kill drug-resistant microbes specifically by designing programmable sequence-specific antimicrobials. Currently, the most efficient CRISPR/Cas systems are type I and type II as a novel antimicrobial tool for selective removal of bacterial pathogens. Bacteriophage has

been developed as a delivery vehicle, but membrane vesicles have more potential for transporting the CRISPR/Cas systems into the targeted resistant pathogen.

**Keywords:** Bacterial drug-resistance; Selective removal; CRISPR/Cas; Delivery vehicles

**Funding:** This work was supported by the National Natural Science Foundation of China (31460699), the Hainan Natural Science Foundation Innovation Research Team Project (2017CXTD005), and the Hainan Natural Science Foundation General Project (317071).

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The long-term abuse of antibiotics has led to intestinal flora imbalance and bacterial drug resistance in humans, necessitating the development of novel, targeted antimicrobial approaches to treat infections caused by resistant bacteria. In recent years, intensive research on CRISPR/Cas systems has provided new strategies for designing drugs that specifically target resistance genes and directionally eliminate resistant bacteria. This article introduces CRISPR/Cas systems as a novel antimicrobial method that achieves specific clearance of resistance genes or pathogens by targeting and cutting resistance plasmids or bacterial genomes, and evaluates the selection of different nucleases for CRISPR antimicrobials as well as the delivery vehicles for CRISPR delivery systems.

Due to antibiotic misuse, an increasing number of resistant bacteria are continuously isolated from hospitals and laboratories, making bacterial resistance a serious global health concern. Traditional antibiotics typically kill bacteria by blocking metabolic pathways or signaling pathways, but they often fail to distinguish between pathogenic and beneficial microorganisms, leading to dysbacteriosis and drug resistance. According to a 2014 report released by the UK government, if no new antimicrobial methods are found, infections by resistant bacteria are projected to cause 10 million deaths annually worldwide by 2050 [1]. Therefore, there is an urgent need to develop novel antimicrobial methods to treat resistant bacterial infections while specifically clearing pathogens.

In recent years, the emergence of CRISPR/Cas—an artificial endonuclease technology—has provided a completely new solution to the problem of bacterial resistance. The CRISPR/Cas system is an adaptive immune system present in most bacteria and archaea that resists invasion by foreign genetic material such as phages or exogenous plasmids, consisting of CRISPR repeat sequences and tandem cas genes [2]. Based on their specific cas genes, CRISPR/Cas systems can be divided into three types: Type I, Type II, and Type III. All three types contain common genes cas1 and cas2, and each type has its own specific genes: cas3, cas9, and cas10, respectively. Among them, the Type II CRISPR/Cas9 system has gradually become the third-generation genome editing technology

following zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN), thanks to its low cost, convenient operation, and high efficiency, and has been widely applied in genome editing.

The gene editing principle of CRISPR/Cas systems is based on DNA cleavage and directional repair. In eukaryotic cells, cleaved DNA is efficiently repaired through ubiquitous mechanisms such as homologous-directed repair (HDR) or non-homologous end joining (NHEJ) [3]. However, bacteria lack the NHEJ mechanism [4], and therefore cannot repair the double-strand DNA breaks produced by CRISPR/Cas nucleases, leading to cell death. Thus, by leveraging the ability of CRISPR/Cas systems to target and cleave DNA to cause bacterial lethality, they can be developed into a highly efficient and novel antimicrobial method for specific bacterial clearance.

## 1. Selection of CRISPR/Cas Nucleases

Different types of CRISPR/Cas systems act as bacterial immune systems by cleaving DNA or RNA to resist foreign genetic material [5,6]. Among these, both Type I Cas3 and Type II Cas9 exhibit powerful nuclease characteristics for pathogen clearance. The Type II CRISPR/Cas9 system is currently the most commonly used gene editing tool in genome editing, with a basic structure comprising three sequence regions: the tracrRNA (trans-activating crRNA) region, the Cas region, and the CRISPR region [2,3]. When the system functions, the CRISPR region is transcribed to form crRNA, which then forms a dual complex with a tracrRNA segment to become sgRNA (single guide RNA). Since the sgRNA contains a 20 nt spacer sequence that can base-pair with the target DNA sequence, it guides the Cas9 nuclease to perform double-strand cleavage of the target sequence [7] (Figure 1 [Figure 1: see original paper]). However, due to this DNA double-strand cleavage principle of Cas9 nuclease, DNA repair mechanisms exist not only in eukaryotes but have also been found in some prokaryotes. For instance, Cui and Bikard reported homology-based repair of genomic DNA through additional copies in Escherichia coli [8].

In Type I systems, Cas3 possesses both ssDNA exonuclease and 3' -5' helicase activities [9,10], cutting and degrading DNA in the 3' -5' direction, thereby destroying most of the DNA upstream of the target sequence [11]. For example, the CasI-E system present in the E. coli genome requires 4-7 proteins: Cas1-3 and CasA-E (also known as Cse1, Cse2, Cas7, Cas5, and Cas6e) (Figure 2 [Figure 2: see original paper][12]). Its mechanism of action involves CasA-E binding with crRNA to form the Cascade complex, where Cas7 subunits constitute the backbone of the complex, the 3' end of crRNA binds to Cas6e subunits, and the 5' end binding includes a Cas7 subunit or Cas5 or Cse1. Subsequently, the Cascade complex recognizes the target DNA sequence through Cas3 mediation and cleaves the target DNA sequence at the 7 nt sequence at the 5' end of the PAM site, ultimately leading to DNA degradation.

Gomaa et al. [13] demonstrated the utility of the E. coli I-E system as a pro-

grammable antimicrobial, showing activity during both endogenous and heterologous expression and enabling simultaneous clearance of multiple bacterial species sharing common genomic sequences. Additionally, Yosef et al. [14] employed a similar approach to clear antibiotic resistance plasmids from E. coli, restoring antibiotic sensitivity and thereby killing the bacteria. Compared with the commonly used Type II CRISPR/Cas9 system, Type I systems have a lower off-target rate, and their ability to continuously degrade upstream DNA fragments of the target sequence makes DNA repair nearly impossible.

## 2. Delivery Systems

### 2.1 Application of Bacteriophages as CRISPR/Cas Delivery Vehicles

Currently, the most important challenge facing CRISPR technology for efficient and specific pathogen clearance is how to effectively deliver the CRISPR/Cas system to different bacteria, which requires finding an efficient delivery vehicle. The most commonly used delivery vehicles are bacteriophages [16,22], which bind to corresponding molecules on the bacterial surface through tail fiber proteins. After tail fiber binding to the receptor, two functions are achieved: anchoring the phage and inducing conformational changes in tail fiber and tail sheath proteins, allowing the tail tube to contact the bacterial surface. The tail tube possesses lysozyme activity, degrading the bacterial cell wall and inserting into the bacterium, while the contracted tail sheath injects nucleic acids into the bacterial cell [14]. For example, Citorik et al. [16] utilized phagemid transduction to deliver the CRISPR/Cas9 system, which contained sequences targeting antibiotic resistance genes on plasmids in enterohemorrhagic E. coli, effectively removing these plasmids from bacteria and restoring antibiotic sensitivity. Chen et al. [22] used phagemid-mediated CRISPR/Cas9 for sequence-specific clearance of Staphylococcus aureus containing virulence genes, effectively removing plasmids carrying resistance genes and resensitizing bacteria to antibiotics.

When using bacteriophages as delivery vehicles, there are two common methods for integrating CRISPR/Cas systems: integrating the system into a phagemid or into the phage genome (Figure 3 [Figure 3: see original paper][17]). A phagemid is a filamentous phage-derived vector containing both plasmid replication origins and antibiotic resistance selection markers. It encodes almost no additional phage components that affect cell physiology, containing only the intergenic region (IG) and capsid protein coding sequences of filamentous phages [18]. Integration into the genome is more cumbersome and time-consuming than integration into a phagemid, but compared with phagemids, the phage genome can avoid degradation and modification of foreign DNA within bacteria.

The greatest obstacle to using bacteriophages as CRISPR/Cas delivery vehicles is their lack of broad host spectrum. Commonly used phages can only adsorb to one or a few types of bacteria, and this narrow adsorption range poses a significant challenge for using phages as universal CRISPR/Cas delivery vehicles [19]. To address this issue, researchers are exploring solutions. One approach

is the "cocktail" therapy, which combines multiple phages to expand bacterial infection coverage [24,25]. Theoretically, the more phage types the cocktail therapy contains, the broader the host range coverage, enabling effective killing of multiple pathogens. Żaczek et al. [25] treated 20 patients with Streptococcus infections using phage cocktail therapy, significantly improving therapeutic efficacy. Another method involves broadening the host spectrum by modifying or exchanging the fiber proteins responsible for binding to the bacterial cell surface. Ando et al. [20] developed a phage that altered DNA delivery range by exchanging tail fiber genes, enabling it to accommodate tail fiber proteins from related phages and thereby deliver to different intestinal bacteria. Yosef et al. [21] developed hybrid particles displaying various phage tail/tail fiber proteins for extending the host range of T7 phage DNA transduction. These modular particles were programmed to package and transduce DNA into hosts that restrict T7 phage reproduction, while also developing an innovative universal platform that enhances DNA transfer to new hosts through efficient transduction via artificial tails.

In vivo studies have demonstrated that using bacteriophages as CRISPR/Cas delivery vehicles can play a role in targeted clearance of clinically relevant pathogens [15,16,22]. However, phage-based delivery systems still have some deficiencies in actual clinical treatment, such as low delivery efficiency in vivo, narrow host range, and potential for transferring virulence genes through generalized transduction [23]. To address these issues, Park et al. [15] overcame these disadvantages of phage-based delivery to some extent by integrating the CRISPR/Cas9 system into temperate phage genomes, improving delivery efficiency to target cells. They also expanded host specificity by supplementing phage tail fiber proteins, removed virulence factor genes from host strains to prevent contamination of harmful bacterial products in phage lysates, and avoided diffusion of virulence genes through generalized transduction. Therefore, using phage-carried CRISPR/Cas systems as antimicrobials still holds considerable potential for clinical application.

### 2.2 Utilizing Extracellular Vesicles to Deliver CRISPR/Cas Systems

Extracellular vesicles produced by living cells have different names depending on the cell type that secretes them, generally referred to as membrane vesicles (MVs) or extracellular vesicles (EVs). Vesicles secreted by Gram-negative bacteria are called outer membrane vesicles (OMVs); those secreted by Gram-positive bacteria are called membrane vesicles (MVs); and those secreted by eukaryotic cells are called exosomes. Although initially considered to be randomly produced by cell lysis, it is now established that MVs are secreted by living cells [26,27] as spherical vesicles wrapped by lipid bilayers with molecular diameters between 10-500 nm. They can carry various cellular components such as lipopolysaccharide (LPS), toxins, cholesterol, sphingomyelin, and nucleic acids [28].

Although the mechanisms by which cells bind to and uptake MVs remain un-

clear and appear to vary depending on the type of recipient cell [33], researchers have paid extensive attention to utilizing the biological characteristics of cell secretion and uptake of MVs as a novel vaccine delivery vector. Through contact between MVs and cell surface ligands, effector molecules can be delivered to target cells to complete intercellular material exchange [32,33] (Figure 4 [Figure 4: see original paper]). For example, in 2017 Kim et al. [34] used MVs secreted by cancer cells as a CRISPR/Cas9 system carrier to achieve targeted clearance of cancer cells in vivo. Our laboratory [43] utilized outer membrane vesicles secreted by E. coli as a delivery vehicle for CRISPR/Cas9 targeting Streptococcus agalactiae, achieving low-efficiency but specific clearance of S. agalactiae through co-culture.

Currently, research on using MVs to carry CRISPR/Cas systems for targeted pathogen clearance is limited, possibly due to the low natural secretion of MVs by bacteria [32]. Additionally, compared with Gram-negative bacteria, Gram-positive bacteria have thicker cell walls that further increase the difficulty of vesicle fusion, resulting in lower vesicle transfer efficiency [27]. However, studies have confirmed that bacterial horizontal gene transfer (HGT) is closely related to MVs (Table 1 ), with HGT occurring through MVs secreted by bacteria as carriers. Therefore, theoretically, bacterial-secreted MVs can deliver the CRISPR/Cas system into pathogenic bacteria through non-specific fusion with pathogens, thereby achieving targeted clearance. To verify this hypothesis, our laboratory constructed a CRISPR/Cas9 shuttle plasmid targeting the CAMP factor-encoding gene (cfb), a virulence factor of S. agalactiae. Using the original shuttle plasmid without targeting activity as a control and E. coli X6097 as the donor cell, we tested whether the plasmid could be transferred to eliminate S. agalactiae through co-culture and colony counting analysis. The results showed that no colonies formed on plates with specific selection for S. agalactiae and plasmid resistance, while a few colonies appeared on control plates. PCR analysis of these colonies confirmed the presence of the shuttle plasmid. Furthermore, MVs prepared from the co-culture supernatant and PCR analysis confirmed that the shuttle plasmid was indeed present in MVs, thus demonstrating that E. coli can inefficiently transfer plasmids into S. agalactiae and specifically clear the pathogen, with MVs playing a crucial role in this plasmid transfer process. This study provides factual evidence for using MVs as delivery vehicles for CRISPR/Cas systems to specifically clear pathogens [43].

## 3. Future Development and Prospects

CRISPR/Cas antimicrobials have become a highly promising novel antimicrobial weapon to replace traditional antibiotics due to their advantages in sequence-specific pathogen clearance. However, it should be noted that CRISPR/Cas systems may also cause damage to beneficial microorganisms in the body, as high expression of Cas nucleases in vivo can easily cause cleavage of non-target DNA sequences [7]. Here, we propose a concept: by transferring only the sgRNA sequence containing bacteria-specific target sites,

the endogenous CRISPR/Cas system of bacteria can be utilized to cleave their own sequences, thereby killing the bacteria. This approach can avoid non-target cleavage by Cas nucleases in beneficial microorganisms or even human cells, simplifies the transferred plasmid, and reduces operational difficulty. However, this method is limited to bacteria with functional CRISPR/Cas systems and is sensitive to the expression and genetic stability of CRISPR/Cas loci.

Meanwhile, the effectiveness and safety of delivery systems based on phages and membrane vesicles are still insufficient. Although other delivery vehicles have been developed, such as polymer-based nanoparticles developed by Kang et al. [41] for delivering Cas9-sgRNA ribonucleoprotein complexes into bacterial cells, their efficiency is also very low and ultimately cannot achieve pathogen control. Therefore, the journey from CRISPR/Cas antimicrobial therapy in the laboratory to animal models and then to human application remains long. Nevertheless, the concept of using CRISPR/Cas systems as antimicrobials is novel and highly promising. For example, CRISPR/Cas plasmids containing multiple pathogen-specific target sites [42] could be designed and transferred into probiotics such as lactic acid bacteria. After human consumption, these probiotics could secrete MVs carrying CRISPR/Cas plasmids that non-specifically fuse with intestinal flora to clear pathogens, thereby regulating the gut microbiome. Although the delivery efficiency using MVs as carriers is low [29,43], probiotics that persist in the intestine could provide long-term prevention and control of pathogens, reducing the probability of human infection. We believe that in the future, efficient and convenient delivery systems will be developed, and the off-target effects of CRISPR/Cas systems will be satisfactorily resolved, thereby promoting this system to become a novel antimicrobial weapon that replaces traditional antibiotics.

## References

[1] O'Neill J. Antimicrobial resistance: tackling a crisis for the health and wealth of nations[J]. The Review on Antimicrobial Resistance, 2014, 20.

[2] Sander J D, Joung J K. CRISPR-Cas systems for editing, regulating and targeting genomes[J]. Nature biotechnology, 2014, 32(4).

[3] Xu, K. Establishment, optimization and application of a eukaryotic CRISPR/Cas9 system derived from Streptococcus thermophilus [D]. Northwest A&F University, 2015.

[4] Staals R H J, Jackson S A, Biswas A, et al. Interference-driven spacer acquisition is dominant over naive and primed adaptation in a native CRISPR–Cas system[J]. Nature communications, 2016, 7: 12853.

[5] Koonin E V, Makarova K S, Zhang F. Diversity, classification and evolution of CRISPR-Cas systems[J]. Current opinion in microbiology, 2017, 37: 67-78.

[6] Makarova K S, Wolf Y I, Alkhnbashi O S, et al. An updated evolutionary classification of CRISPR–Cas systems[J]. Nature Reviews Microbiology, 2015,

13(11): 722.

[7] Jiang Y, Qian F, Yang J, Liu Y, Dong F, Xu C, Sun B, Chen B, Xu X, Li Y, et al.: CRISPR-Cpf1 assisted genome editing of Corynebacterium glutamicum. Nat Commun 2017, 8:15179.

[8] Cui L, Bikard D. Consequences of Cas9 cleavage in the chromosome of Escherichia coli[J]. Nucleic acids research, 2016, 44(9).

[9] Beloglazova N, Petit P, Flick R, et al. Structure and activity of the Cas3 HD nuclease MJ0384, an effector enzyme of the CRISPR interference[J]. The EMBO journal, 2011, 30(22): 4616-4627.

[10] Sinkunas T, Gasiunas G, Fremaux C, et al. Cas3 is a single-stranded DNA nuclease and ATP-dependent helicase in the CRISPR/Cas immune system[J]. The EMBO journal, 2011, 30(7): 1335-1342.

[11] Caliando B J, Voigt C A. Targeted DNA degradation using a CRISPR device stably carried in the host genome[J]. Nature communications, 2015, 6: 6989.

[12] Westra E R, Swarts D C, Staals R H J, et al. The CRISPRs, they are a-changin' : how prokaryotes generate adaptive immunity[J]. Annual review of genetics, 2012, 46: 311-339.

[13] Gomaa A A, Klumpe H E, Luo M L, et al. Programmable removal of bacterial strains by use of genome-targeting CRISPR-Cas systems[J]. MBio, 2014, 5(1): e00928-13.

[14] Yosef I, Manor M, Kiro R, et al. Temperate and lytic bacteriophages programmed to sensitize and kill antibiotic-resistant bacteria[J]. Proceedings of the national academy of sciences, 2015, 112(23): 7267-7272.

[15] Park J Y, Moon B Y, Park J W, et al. Genetic engineering of a temperate phage-based delivery system for CRISPR/Cas9 antimicrobials against Staphylococcus aureus[J]. Scientific reports, 2017, 7: 44929.

[16] Citorik RJ, Mimee M, Lu TK: Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. Nat Biotechnol 2014, 32:1141–1145.

[17] Fagen J R, Collias D, Singh A K, et al. Advancing the design and delivery of CRISPR antimicrobials[J]. 2017, 4.

[18] Zhang, W.Q., Li, H.Q. Application and research progress of phagemid vectors in phage display[J]. Biotechnology, 2014, 24(01): 96-100.

[19] Denyes J M, Dunne M, Steiner S, et al. Modified bacteriophage S16 long tail fiber proteins for rapid and specific immobilization and detection of Salmonella cells[J]. Applied and environmental microbiology, 2017, 83(12): e00277-17.

[20] Ando H, Lemire S, Pires DP, Lu TK: Engineering modular viral scaffolds for targeted bacterial population editing. Cell Syst 2015, 1:187–196.

[21] Yosef I, Goren MG, Globus R, Molshanski-Mor S, Qimron U: Extending the host range of bacteriophage particles for DNA transduction. Mol Cell 2017, 66:721–728. e3.

[22] Bikard D, Euler CW, Jiang W, Nussenzweig PM, Goldberg GW, Duportet X, et al. Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. Nat Biotechnol. 2014; 32(11):1146–50.

[23] Chen J, Novick R P. Phage-mediated intergeneric transfer of toxin genes[J]. science, 2009, 323(5910): 139-141.

[24] Yen M, Cairns LS, Camilli A: A cocktail of three virulent bacteriophages prevents Vibrio cholerae infection in animal models. Nat Commun 2017, 8:14187.

[25] Żaczek M, Łusiak-Szelachowska M, Jończyk-Matysiak E, et al. Antibody production in response to staphylococcal MS-1 phage cocktail in patients undergoing phage therapy[J]. Frontiers in microbiology, 2016, 7: 1681.

[26] Brown L, Wolf J M, Prados-Rosales R, et al. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi[J]. Nature Reviews Microbiology, 2015, 13(10): 620.

[27] Domingues S, Nielsen K M. Membrane vesicles and horizontal gene transfer in prokaryotes[J]. Current opinion in microbiology, 2017, 38: 16-21.

[28] Gerritzen M J H, Martens D E, Wijffels R H, et al. Bioengineering bacterial outer membrane vesicles as vaccine platform[J]. Biotechnology advances, 2017, 35(5): 565-574.

[29] Shu, C.Y., Wang, S.J., Gao, F.L., et al. E. coli outer membrane vesicles as a novel exogenous plasmid delivery vector[J/OL]. Chinese Journal of Biologicals: 1-5.

[30] Andaloussi S E L, Mäger I, Breakefield X O, et al. Extracellular vesicles: biology and emerging therapeutic opportunities[J]. Nature reviews Drug discovery, 2013, 12(5): 347.

[31] Li, S.D., Hou, X., Qi, H.Z., et al. Exosomes: naturally occurring endogenous nanocarriers for effective drug delivery strategies[J]. Progress in Chemistry, 2016, 28(Z2): 353-362.

[32] Schwechheimer C, Kuehn M J. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions[J]. Nature reviews microbiology, 2015, 13(10): 605.

[33] Fulsundar S, Harms K, Flaten G E, et al. Gene transfer potential of outer membrane vesicles of Acinetobacter baylyi and effects of stress on vesiculation[J]. Applied and environmental microbiology, 2014, 80(11): 3469-3483.

[34] Kim S M, Yang Y, Oh S J, et al. Cancer-derived exosomes as a delivery platform of CRISPR/Cas9 confer cancer cell tropism-dependent targeting[J]. Journal of Controlled Release, 2017, 266: 8-16.

[35] Dorward D W, Garon C F, Judd R C. Export and intercellular transfer of DNA via membrane blebs of Neisseria gonorrhoeae[J]. Journal of bacteriology, 1989, 171(5): 2499-2505.

[36] Yaron S, Kolling G L, Simon L, et al. Vesicle-Mediated Transfer of Virulence Genes from Escherichia coli O157: H7 to Other Enteric Bacteria[J]. Applied and environmental microbiology, 2000, 66(10): 4414-4420.

[37] Rumbo C, Fernández-Moreira E, Merino M, et al. Horizontal transfer of the OXA-24 carbapenemase gene via outer membrane vesicles: a new mechanism of dissemination of carbapenem resistance genes in Acinetobacter baumannii[J]. Antimicrobial agents and chemotherapy, 2011, 55(7): 3084-3090.

[38] Ho M H, Chen C H, Goodwin J S, et al. Functional advantages of Porphyromonas gingivalis vesicles[J]. PloS one, 2015, 10(4): e0123448.

[39] Blesa A, Berenguer J. Contribution of vesicle-protected extracellular DNA to horizontal gene transfer in Thermus spp[J]. Int Microbiol, 2015, 18(3): 177-187.

[40] Klieve A V, Yokoyama M T, Forster R J, et al. Naturally occurring DNA transfer system associated with membrane vesicles in cellulolytic Ruminococcus spp. of ruminal origin[J]. Applied and environmental microbiology, 2005, 71(8): 4248-4250.

[41] Kang YK, Kwon K, Ryu JS, et al. Nonviral genome editing based on a polymer-derivatized CRISPR nanocomplex for targeting bacterial pathogens and antibiotic resistance. Bioconjugate Chem 2017, 28:957–967.

[42] Guo L, Xu K, Liu Z, et al. Assembling the Streptococcus thermophilus clustered regularly interspaced short palindromic repeats (CRISPR) array for multiplex DNA targeting[J]. Analytical biochemistry, 2015, 478: 131-133.

[43] Hou, G.F., Zeng, J.W., Yang, X.C., et al. Study on the combination of bacterial membrane vesicles and CRISPR to eliminate Streptococcus agalactiae[J/OL]. Journal of Tropical Biology: 1-7.

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