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Review Article

Future of Engineered Phage Therapy for *Clostridium difficile* Infections

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Abstract

Bacteriophages have the potential to eliminate both antibiotic-resistant and sensitive bacteria; as a result, they have become a major focus of such research. In contrast to antibiotics, which assault the entire bacterial population without discrimination, bacteriophages have a limited set of characteristics that allow them to target infectious microbes while avoiding friendly species (commensal microbiota). Nevertheless, large groups of naturally occurring bacteriophages that are well-differentiated and selective for the most clinically recognized pathogenic bacterial strains are required. Utilizing genetic engineering techniques that modify the target phage genome to synthesize phages with known characteristics in a brief period of time and at a low acquisition, characterization, and treatment cost. *Clostridioides difficile* is the leading cause of nosocomial acquired diarrhea, causing approximately 500,000 cases of *Clostridium difficile* infection (CDI) and nearly 29,000 deaths annually in the United States. Vancomycin is the most often used antibiotic to treat CDIs, and it is believed that it contributes to the disturbance of the gut microbiota, resulting in diminished colonization resistance against CDI and increased recurrence rates. This article provides a concise summary of existing CRISPR-Cas systems that can be utilized to create a lytic phage as a potential treatment for CDIs. While further study is needed, phage therapy appears to be a promising and perhaps more sustainable approach of preventing severe CDIs.

Keywords: Bacteriophage, CRISPR-Cas, *Clostridioides difficile*, *Clostridium difficile* infection.

مستقبل العلاج بالعاثيات المهندسة لعدوى المطثية العسيرة

الخلاصة

العاثيات البكتيرية لديها القدرة على القضاء على كل من البكتيريا المقاومة للمضادات الحيوية والحساسة. ونتيجة لذلك، أصبحت محورا رئيسيا لمثل هذه البحوث. على عكس المضادات الحيوية، التي تهاجم جميع البكتيريا دون تمييز، فإن البكتيريا لها مجموعة محدودة من الخصائص التي تسمح لها باستهداف الميكروبات المعدية مع تجنب الأنواع الصديقة (الجراثيم المتعايشة). ومع ذلك، هناك حاجة إلى مجموعات كبيرة من العاثيات البكتيرية التي تحدث بشكل طبيعي والتي تكون متمايزة بشكل جيد وانتقائية للسلالات البكتيرية المسببة للأمراض المعترف بها سريريا. استخدام تقنيات الهندسة الوراثية التي تعدل جينوم العاثيات المستهدفة لتخليق العاثيات ذات الخصائص المطلوبة في فترة زمنية وجيزة وبتكلفة منخفضة لتعديل الجينوم والتوصيف والمعالجة. المطثية العسيرة هي السبب الرئيسي للإسهال المكتسب من المستشفيات، مما تسبب في ما يقرب من 500000 حالة من عدوى المطثية العسيرة وما يقرب من 29000 حالة وفاة سنويا في الولايات المتحدة. يعتقد أن الفانكومايسين الذي يستخدم في علاج CDIs يساهم في تعطيل البكتيريا الصديقة في الأمعاء، مما يؤدي إلى انخفاض مقاومة االجسم ضد CDI وزيادة معدلات التكرار بسبب الاضطراب المستمر لميكروبات الأمعاء. تقدم هذه المقالة ملخصا موجزا لأنظمة CRISPR-Cas الحالية التي يمكن استخدامها لإنشاء عاثية محللة للجراثيم كعلاج محتمل ضد CDIs. على الرغم بأن هناك حاجة إلى مزيد من الدراسة، يبدو أن العلاج بالعاثيات يعد نهجًا واعدًا وربما أكثر استدامة لتجنب عدوى المطثية العسيرة الشديدة.

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INTRODUCTION

At the beginning of the 1900s, Frederick Twort and Felix d'Herelle made the separate discovery of bacteriophages (phages). Found in natural waters at a concentration of 2.5 × 108/ml, phages are the organisms with the greatest prevalence on this planet [1]. Ever since they became known, phages have been the basis for the formulation of natural strategies for bacterial infection treatment because they target bacteria [2–4]. However, despite ongoing investigations in a few countries in Eastern Europe, interest in the therapeutic potential of phages diminished when antibiotics were discovered, as the latter have a wider scope of efficacy and are less expensive [5]. Renewed attention has started to be paid to phages for antimicrobial applications as the rate of infections caused by antibiotic-resistant bacteria rises and fewer traditional antibiotics are discovered [6]. In general, the environment is a source of a variety of phages, but a small group of natural phages can be used in applications for the treatment of bacterial infections for a variety of reasons, including safety first and foremost as well as the complex relationship between bacteria and phages themselves. Bacteria may also resist natural phage infection; therefore, identifying a bacterial phage with a bactericidal effect and isolating it from its native environment has become a never-ending race [7]. The therapeutic potential of phages has been clearly demonstrated by the recent case of an individual from San Diego who acquired an infection with a strain of multi-drug-resistant Acinetobacter baumannii while in Egypt; a phage cocktail lysing this bacterium was intravenously injected, helping the individual to awaken from a nearly two-month coma after just two days of treatment and make a full recovery [8]. Furthermore, in the future, treatment options with pathogen specificity will come to play a significant role in drug development, given that antibiotic-mediated eradication of commensal bacteria can have adverse effects such as gut dysbiosis [9]. As a result, the approaches for modifying phage genomes vary in complexity and are dependent on the developmental strategies of the phage of interest.

Key Message: Bacteriophages provide a focused strategy to tackle antibiotic-resistant bacteria while protecting beneficial microbiota species. Current antibiotic therapy may disturb the gut microbiota and cause considerable morbidity and death from CDIs. Using CRISPR-Cas systems to create lytic phages as a targeted treatment for CDIs is explored in this article.

Future Therapy for *Clostridium difficile* Infection by Engineered Phage

Phages targeting both Gram-positive and Gram-negative bacteria can now be easily developed and assessed, and implicitly, their uses have broadened considerably, owing to the latest innovations in synthetic biology techniques, especially genome engineering [10-14]. In particular, Clostridioides difficile infection (CDI) could be managed via treatment based on phages due to the narrow-spectrum action of the latter [15], which could minimize or altogether prevent gut microbiota changes that enable CDI to re-emerge following treatment [16,17]. Although antibiotics with a wide action spectrum have made treatments successful, relapse occurs in around 30% of cases and is most likely caused by ongoing gut microbiota disruption [14]. The use of phages to treat CDI is a good idea because preclinical studies have shown that phages specifically target *C. difficile* in complex bioreactor models and work well in in vivo models [18]. So far, the *C. difficile* phages that have been studied are thought to be temperate, but the sequencing of prophage genomes and the sequencing of the *C. difficile* genome have shown that a lot of them are lysogenic [19]. As reported by Bondy-Denomy and his colleagues [20], it is possible to create phage resistance to the lysogenic host by repressor-mediated immunity or superinfection exclusion by integrating a prophage into the bacterial genome. Obligatory lytic phages are yet to be identified for *C. difficile*. Therefore, this paper seeks to genetically engineer an existing *C. difficile* phage displaying elevated lytic activity, potentially yielding a virulent phage for this organism. The path to the creation of engineered phages has been paved by technological innovations like high-throughput sequencing, genome editing, and synthetic biology. Such innovations can make phages more efficient by fostering the development of valuable features of modular designer-phages as versatile biologics that efficiently control multidrug-resistant bacteria, as well as supplying new approaches to pathogen detection, drug development, Degradation of biofilms, an increased host range, the eradication of lysogeny, the inclusion of genes to arm phages with secondary antimicrobial payloads, and other developments [11,21-24].

CRISPR-Cas Systems as a Modern Technology for *C. difficile* Phage Genetic Engineering

The study of model organisms in any biological category has been transformed by the genetic tools that have become available since prokaryotic immune systems called Clustered, Regularly Interspaced, Short Palindromic Repeats (CRISPR) and cas (CRISPR-Cas: CRISPR-associated) were discovered more than ten years ago. Some such tools have been developed for phages, the viruses that exclusively infect bacteria and are the focus of CRISPR-Cas immunity. However, the functions of most phage genes are unknown, even though no other organism is more prevalent than phages [25,26]. Estimates suggest that around 45% of bacteria and 87% of archaea possess at least one CRISPR-Cas system, as revealed by the CRISPR web server, which is an online service

for the identification of CRISPR systems in sequenced prokaryotes [27]. Given that they are naturally prevalent in various hosts, CRISPR-Cas systems have potential for use as genetic tools to engineer the viruses infecting them. Therefore, reliable genetic tools for researching such systems are urgently required. According to the latest studies, it is possible to employ CRISPR-Cas systems, particularly type I, II, and III systems, to genetically engineer phages infecting a range of hosts [14,28,29]. Classified as adaptive immune systems, CRISPR-Cas systems are capable of identifying and eradicating foreign nucleic acids based on small CRISPR RNAs (crRNAs) and Cas nucleases [30–32]. The Cas proteins and CRISPR locus are the major constituents of CRISPR-Cas systems. The former represents the catalytic core of the system and are in charge of cleaving DNA, while the latter constitutes the genetic memory guiding catalytic activity against foreign DNA [33]. In turn, CRISPR loci comprise a number of non-contiguous direct repeats divided by short stretches of variable DNA sequences known as spacers and originating from extrachromosomal components [34,35]. Adaptation, crRNA biogenesis, and interference are the three steps of CRISPR-Cas immunity. The adaptation step involves the capture and incorporation of 30–40 nucleotide spacers into CRISPR loci among comparable long, partly palindromic DNA repeats. The crRNA biogenesis step involves transcription of the repeat-spacer array into an extensive precursor crRNA, which undergoes additional processing for the release of fully developed crRNAs, each of which focuses on just one target. The interference step involves the formation of an effector complex through the integration of crRNAs with at least one Cas protein; this effector complex is capable of detecting and breaking down nucleic acids called "protospacers" complementary to the crRNA. The steps of crRNA biogenesis and interference make up the defense phase of CRISPR-Cas immunity. This pathway is standard for every known CRISPR-Cas system, and yet these systems are remarkably diverse in terms of phylogenetics and mechanisms. According to the present categorization framework, CRISPR-Cas systems are distinguished into two major categories, six distinct types (I–VI), and numerous subtypes depending on cas gene composition and mechanism discrepancies [32,36]. Thus, multi-subunit effector complexes are encoded by class 1 systems (types I, III, and IV), while foreign nucleic acids are eradicated by class 2 systems (types II, V, and VI) based on one subunit. Furthermore, as indicated by recent studies, phage genome engineering could be achieved on the basis of CRISPR-Cas systems of types I, II, and III [12,25,37,38]. Hatoum-Aslan [39] pointed out, however, that type III systems can't be used to change late genes in lysogenic phages because they only work when the targeted locus is being actively transcribed. A proportion of almost 60% of systems detected so far have been classified as CRISPR-Cas3, which is therefore the system of greatest prevalence in prokaryotes [32]. Most *C. difficile* isolates that have been sequenced have type I-B CRISPR-Cas systems, which make it easier to block DNA from outside the cell [40,41]. Selle and his research group reported that bacterial load was markedly decreased both in vitro and in vivo through stimulation of lytic activity by modifying *C. difficile* temperate phages. It caused lysis and bacterial genome damage via the type I-B CRISPR-Cas system, producing an additive antimicrobial effect [14]. The alteration of viruses targeting Gram-positive bacteria is usually highly challenging. However, a novel platform technology has been used to achieve fast, precise, and selection-free engineering of synthetic, customized phages targeting Gram-positive bacteria. To that end, Listeria monocytogenes temperate phage genomes were altered in a targeted fashion to assemble synthetic phage genomes in vitro from smaller DNA fragments based on the creation of virulent phages with Rebooting Synthetic Genomes. This strategy has been described as ‘virulent conversion, and the phages developed in this way have been proven to have high destructive power [13].

Conclusion

The advent of synthetic biology, along with the enormous variety of phages, has resulted in potent applications in medicine, diagnostics, and materials research. The development of new genetic engineering methods has resulted in more accurate and quicker phage genome modification for both fundamental science and engineering. Phages have previously been employed to develop novel anti-infective agents, diagnostics, drug delivery systems, and vaccinations, as well as nanoscale electronics, imaging, and tissue scaffolds. Nevertheless, despite the progress detailed earlier, phage research is still in its early stages. The wide range of phage types and architectures seen in nature has yet to be completely exploited [42,43]. In reality, the majority of existing phages have yet to be propagated in the laboratory. In order to create specialized phages faster and to more effectively transform academic work that has been done as a proof-of-concept into practical application, new technologies are still required. As shown above, there is still a shortage of highly effective, quick methods that are applicable to a variety of phages. The capacity to genetically alter their bacterial hosts or to effectively transfer exogenous DNA into these hosts is a requirement for many engineering phage techniques, although this is still difficult for many bacterial species. Therefore, new instruments for DNA or genetic modification are required. In an ideal world, various genetic modifications could be efficiently and precisely introduced into phage genomes. Ultimately, the majority of the research included in the present article has produced genetically altered phages that may be useful for developing novel materials, identifying and treating infectious diseases caused by bacteria, and treating nonbacterial disorders. Although there are possible advantages, various parts of the world may have

varied levels of approval for using genetically altered phages in practical applications. Such worries may be alleviated by methods for inactivating phages so they cannot multiply outside of the lab, such as by removing crucial protein genes from the phage genome and providing them in trans in production hosts. In the event of human application, it will be crucial to choose regions with a clear medical need and to provide specific proof of safety. In conclusion, phage engineering using CRISPR-Cas systems to control the microbiome composition in illness as well as health is a field of study that is receiving a lot of attention and has a lot of potential, but it has not yet been completely utilized.

Conflicts of interest

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