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研究課題名（和文）Development of a novel antimicrobial capable of selectively killing Shiga toxin-producing Escherichia coli

研究課題名（英文）Development of a novel antimicrobial capable of selectively killing Shiga toxin-producing Rscherichia coli

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研究成果の概要（和文）：本申請では、腸管出血性大腸菌(EHEC)感染症の新しい治療法の開発を目的として、志賀毒素産生遺伝子(stx)を認識するCRISPR-Cas13aを大腸菌ファージ80に搭載した抗菌カプシドを構築した。部分的な stx 遺伝子を持つ大腸菌に対して殺菌活性があることを明らかにした。次に、臨床分離大腸菌706株を用いてファージ誘発試験を行った。その結果、広くEHEC大腸菌に感染できる最適ファージを得ることが出来なった。現在、stx 遺伝子を標的とした抗菌カプシドがEHEC大腸菌を死滅させることから、今後のEHEC感染症への新しい治療法として有効である可能性が期待される。

研究成果の学術的意義や社会的意義
現在、抗菌薬によりEHEC大腸菌が殺菌されると細菌が持つ毒素が菌体外に放出されることが問題となっている。本申請は、CRISPR-Cas システムと薬剤耐性菌を選択的に殺菌できる遺伝子組み換えファージを組み合わせた新しい抗菌剤の開発を行なった。本申請の成果は、治療困難なEHEC 感染症の新しい治療法に資する基盤技術を提供することができる。特に、志賀毒素産生遺伝子(stx)を認識するCRISPR-Cas13aを搭載した抗菌カプシドにより、志賀毒素の産生を抑制するのみならず、同時にEHEC大腸菌を死滅させることができる。そのため、新しいEHEC 感染症の治療法となる可能性がある。

研究成果の概要（英文）：This research aimed to develop a new therapeutic agent to treat EHEC infections which can be accomplished by packaging stx-targeting CRISPR-Cas13a into the capsid of an E. coli phage. The stx-targeting Cas13a has been loaded into the capsid of a narrow-range phage80, generating Cas13a-stx. The bactericidal activity of Cas13a-stx was then confirmed. Furthermore, a candidate phage isolated from sewage water which has a satisfactory infectivity against EHEC strains (3/5) was isolated. It carried large genome size and difficult genome-manipulation. 706 clinical E. coli strains were chemically induced to further isolate broad-host-range phages. A candidate broad-host-range phage (killing activity of 33.67%) with small genome size (about 30 kbp) was isolated but it cannot infect EHEC strains. Although this work does not progress as proposed, CRISPR-Cas13a-loaded capsids were shown to kill E. coli expressing target sequence, proving their potential as an alternative therapeutic against EHEC.

研究分野：感染症学・微生物学

キーワード：EHEC　AB capsid　CRISPR-Cas13a

１．研究開始当初の背景

Recently, the supportive therapy without administering antibiotic therapy is suggested for Enterohemorrhagic *Escherichia coli* (EHEC) infection. Thus, more than 2.8 million people around the world suffer in abdominal pain and hemolytic uremic syndrome (HUS) from EHEC infection every year[1]. These EHEC bacteria can produce a potent toxin, called Shiga toxin (Stx) from Shiga toxin gene (stx). About 5-10% of EHEC patients develop to hemolytic uremic syndrome (HUS) with HUS mortality between 3-5% but most patients show chronic kidney disease (50%) and neurological complication (25%). Thus, development of new therapeutic agents for EHEC infection have been an ongoing challenge. Previous studies in our group successfully generated CRISPR-Cas13a-based antimicrobial by loading CRISPR-Cas13a into the head (capsid) of a bacteriophage, which is bacterial-infecting virus[2] . This CRISPR-Cas13a-loaded phage capsid, named antimicrobial capsid (AB-capsid), kills bacteria by cleaving targeted RNA encoded specific drug-resistant bacterial genes and other non-specific RNAs.

２．研究の目的

This study proposed to develop a new therapeutic agent for EHEC infections for which there is still no effective antibacterial therapy. Figure 1 displays comparison between new strategy from this study and antibiotic treatment. Treatment of EHEC infection with antibiotic significantly stimulates production and releases of Stx toxin after bacterial death, causing HUS complication[3] (Fig 1B). The phage capsid loaded with CRISPR-Cas13a is the novel strategy for treatment of EHEC infections without drugs administrations (Fig 1A). [Capsid] is the part of shell that surround the genome of [Phage], a bacterial virus. When this phage attaches to the target bacterium, it will inject the phage genome to hijack the bacterial components for producing new phages and explode bacterial cells by using endolysins. [CRISPR-Cas13a] is an RNA-targeted CRISPR-system which contain CRISPR RNA (crRNA) for recognition of target RNA and Cas13a endonuclease for single-stranded RNA (ssRNA) cleavage[4]. Interestingly, the formation of crRNA:Cas13a complex exhibits the effect of collateral cleavage (non-specific nearby RNA cleavage), leading to bacterial death. The ability of Cas13a against drug-resistant bacteria to decompose bacterial endogenous RNA resulting in bacterial death was clarified[2]. Thus, the ability of CRISPR-Cas13a-loaded capsid is applied in the new strategy for treatment of EHEC infection with bactericidal activity together with suppression of Shiga toxin production.

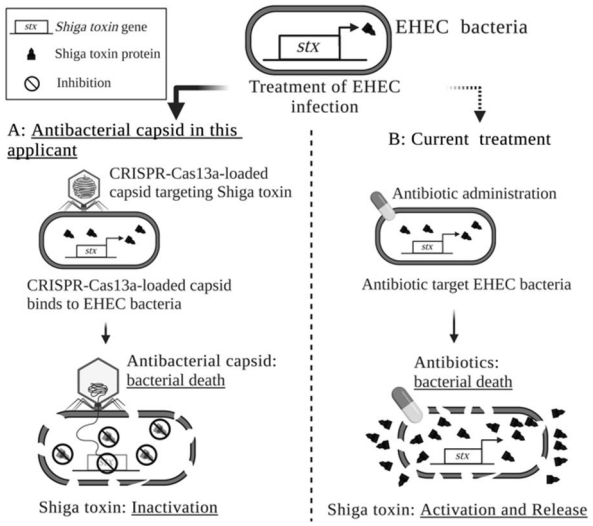

Figure 1: EHEC-bacterial death after suppression of Shiga toxin production due to new strategy with CRISPR-Cas13a-loaded capsid

３．研究の方法

3.1 Bacteriophage isolation

Phage induction and isolation were performed on a total of 706 clinical *E. coli* strains, which were obtained from Japanese hospitals and other countries. Briefly, bacteria were grown at 37^{o}C in LB medium supplement with $CaCl_2$ and $MgSO_4$- until reaching an optical density at 620 nm (OD_{620}) of 0.5. A final concentration of 2 μg/mL mitomycin C was then added to induce temperate phage and continued overnight incubation at 30^{o}C. After centrifugation at 8,000 x *g* for 10 minutes at 4^{o}C, the supernatants were filtered by passage through 0.45 micron filter and stored at 4^{o}C for further analysis.

Sewage samples were collected from Jichi Medical University, Japan and filtered the solution with bottle top vacuum filter (0.22 micron filter). The phage Y was isolated from the filter solution Briefly, the phage mixture in the filter solution was precipitated overnight with 20%PEG8000/2.5M NaCl. These concentrated phages were then centrifuged at 12,000xg for 60 minutes and resuspended in SM buffer. The equal volume of this phage mixtures was mixed with *E. coli* O157:H7 without *stx* gene and incubated at 37^{o}C for 20 minutes. This mixture was added to semi-solid agar (0.3%) and transferred onto a plate with LB agar. Each

single plaque was randomly picked and removed bacteria contaminant by passage through 0.45 micron filter. In addition, the phage Z was isolated from the solid on the paper filter. This solid part was diluted with LB broth to five dilution factors. 100 µL of each dilution was spread on the *E. coli* selective medium, X-MG. After overnight incubation, green colonies on the plate were randomly picked and induced prophage by mitomycin C as mentioned above. All phage solutions were stored at 4°C.

3.2 Host range determination of isolated phages

Standard spot test assays were used to determine the host ranges of the collected phages against 689 *E. coli* strains. 250 µL of bacterial culture (OD_{620} of 0.3) was mixed with 4 mL of LB soft agar (0.5%) and poured onto a LB plate to create an overlay agar. Then 2 µL of isolated phage suspension was spotted onto overlaid top agar. The plates were incubated at 37°C overnight to evaluate bacterial growth inhibition as indicated by clearing zones where the lysate was spotted. The bactericidal ability of each lysate was graded as plaque morphology as followed:

- Level 3 refers to complete lysis or clear zone
- Level 2 refers to lysis with resistant colonies
- Level 1 refers to hazy lysis
- Level 0 refers to no clear zone

3.3 Genome characterization

Genomic DNAs from potential phage candidates were extracted using phenol-chloroform method and subsequently purified using DNeasy Blood and Tissue Kit following the manufacturer's protocol. The purified DNAs was determined whole-genome sequencing using next-generation sequencing technology. To analyze the sequencing data, several bioinformatics tools were employed such as PHASTER (prophage identification) and RAST Server (gene annotation).

3.4 Generation of AB capsid

A phage was lysogenized into a prophage-free laboratory strain *E. coli* 594, and then the packaging machinery was deleted to prevent self-assembly of the phage genome following previous report[5]. A phagemid or PICImid was transformed into *E. coli* 594-phage80*Δcos* at 42°C for 45 seconds. The transformed cells were recovered by S.O.C media and incubated at 37°C with constant agitation for 1 hour. The bacteria carrying phagemid were allowed to grow on Luria-Bertani (LB; Becton Dickinson, USA) agar containing 30 µg/mL kanamycin. All transformed cells carrying CRISPR-Cas13a and targeted gene sequence were confirmed by PCR and Sanger sequencing and then kept in a final concentration of 40% glycerol at -80°C. All bacteria harboring the correct sequence were revived through cultivation in LB at 37°C with constant agitation. Once the fresh bacterial culture reached an OD_{600} of 0.5, mitomycin C was added to a final concentration of 2 µg/mL and the culture will be incubated overnight at 30°C with shaking at 80 rpm. After incubation, the supernatant will be harvested by centrifugation at 8,000 x g for 10 minutes and passed through a 0.22 µm filter. The AB-capsid solutions were kept in 4°C.

3.5 Inhibition of bacterial growth

E. coli targets were cultured in appropriate medium until they reached an OD_{600} of 0.3. The equal volume of bacterial solution was mixed with antibacterial capsids and their dilutions. After incubation at 37°C for 20 minutes, the solutions were directly spotted onto the appropriate medium supplemented with antibiotic marker and incubated at 37°C for a specific time period (e.g., 14-18 hours). Bacterial killing will be observed by the absence of bacterial growth on the antibiotic plates.

3.6 Endotoxin removal

The methods for endotoxin removal were adapted from previous method[6]. Briefly, final concentration of 20 mM $MgCl_2$ were added into phage concentration and overnight incubation at 4°C. 0.4 volume of 1-butanol was then added and incubated at room temperature with constant agitation for 3 hour. The mixture solution was kept in the 4°C for 3 h. The aqueous phase after centrifugation was collected by using syringe and dialyzed four times at 15, 18, 21 and 24 hours with 25% (v/v) ethanol and three times at 15, 19 and 24 hours with 0.15 M NaCl. The endotoxin in phage purification was measured by ToxinSensor™ Chromogenic LAL endotoxin Assay.

4．研究成果（result）

Firstly, the AB-capsid loaded with phagemid was evaluated the sequence-specific killing activity. This phagemid composed of CRISPR-Cas13a programmed to target imipenem (IMP) gene and packaging *cos* site of phage was introduced into lysogenized *E. coli* carrying phage80 (helper phage) with deleted packaging *cos* site (*E. coli* 594-phage80Δ*cosN*). The transformant was subsequently chemically triggered with Mitomycin C in order to generate the AB-capsid (Cas13a-IMP). Lysogenized phage 80 in this case produced all of the essential phage protein products but the natural phage genome lacked the DNA packaging *cos* site, the phagemid was packaged into the capsid. Evaluation of phi80-pCas13a was then performed on *E. coli* strains with or without the IMP resistance gene. The result displayed that phi80-pCas13a could specifically recognize and kill the *E. coli* carrying IMP resistance gene but not in the control strain (Fig 2).

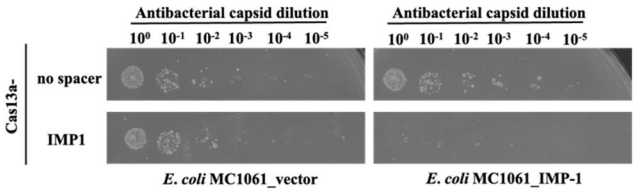

Figure 2: Inhibition of bacterial growth from antibacterial capsid targeting IMP-1 gene

Phage-inducible chromosomal island (PICIs) encode all the proteins to inhibit the phage's life cycle and package themselves into phage capsid. Therefore, we took advantage of PICI to load the CRISPR-Cas system into phage capsid to avoid the homologous recombination from packaging site between phagemid and phage genome. The packaging *cos* site in the phagemid was replaced with packaging system of PICI to generate PICImid. This PICImid, which consisted of CRISPR-Cas13a targeting *stx* gene, was then transformed into *E. coli* 594-phage80Δ*cosN* and induced by Mitomycin C to generate PICImid-loaded capsid (Cas13a-stx). *E. coli* carrying partial *stx* gene on the plasmid was used for evaluation of bactericidal activity of this generated AB-capsid and also showed specific killing activity only *stx*-positive *E. coli*, indicating that both systems (phagemid or PICImid) has the potential to be developed specifically targeting EHEC bacteria.

One type of endotoxin known as lipopolysaccharide (LPS) is one of most effective stimulators of proinflammatory cytokine from immune response. Thus, the removal endotoxin is necessary for phage or AB-capsid solution. The butanol method for purification in this studies were tested with T7 phage due to ease of proliferation and strong bactericidal activity. The endotoxin after phage purification was then measured by ToxinSensor™ Chromogenic LAL endotoxin Assay. The results showed the significant reduction of endotoxin and still exhibit the bactericidal activity (Fig 3). This purification methods will further do in next step after generation of AB-capsid targeting EHEC strains.

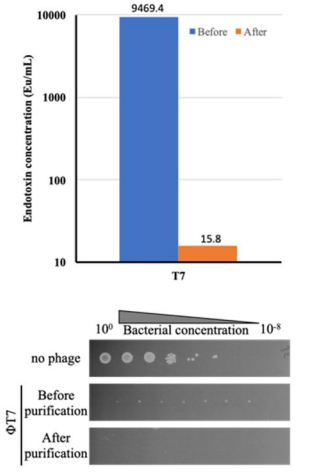

Figure 3: Endotoxin concentration and bactericidal activity before and after ΦT7 purification

The *stx* gene is a crucial role for guide RNA in CRIPRS-Cas system to selectively recognize and activate Cas endonuclease to cleave the targeting gene. Since the *stx* genes are susceptible to being lost during subculture process[7], the PCR primers were designed to verify the presence of *stx* genes among in-house collection of EHEC strains. There are two major groups of *stx* genes produced by *E. coli*, *stx1* and *stx2*. In contrast to the *stx1*-derived toxin, the shiga toxin produced from the *stx2* gene showed symptoms associated with HUS complications. In this study, 5 of 6 strains showed stx-positive *E. coli*. Both *stx1* and *stx2* gene were detected in 42.9% (3 of 7) of these strains, while 2 strains carried only *stx1* or *stx2* gene, respectively.

Previous phi80 (helper phage) harboring CRISPR-Cas13a system cannot infect any EHEC strains. Therefore, the successful delivery of programmed CRIPR-Cas13a to various types of *E. coli* strains requires the identification and characterization of broad-host-range helper phages. Mitomycin C induction of 706 *E. coli* lysogens, including EHEC strains, was the first step in finding for novel phages. The resulting 706 crude-induced lysates were then tested for their respective host ranges against 6 EHEC strains and 391 other *E. coli* strains, adherent invasive *E. coli* or multidrug-resistance strains. Among them, 63 out of 706 lysates showed distinctive bactericidal activity ranging from 0.15 – 63.43%. Further analysis revealed that only 1 of crude-induced lysate displayed the bactericidal activity in 1 of EHEC strain. However, this lysate and other 21 lysates were characterized in bacteriocins which are trypsin-sensitive antimicrobial peptides co-induced during phage isolation. The activity

of bacteriocins was confirmed by the loss of killing activity after trypsin exposure. Although phage, named phiX in 41 phage solutions collected in this study, was recorded the highest killing activity (33.67%) and showed the plaque characterization, phiX cannot kill any EHEC strains.

Sewage water is other source of hunting phage because it has a high amount of organic and non-organic material that is appropriate for the proliferation of bacterial hosts. 167 of phages were collected for testing bactericidal activity among EHEC bacteria and some types of *E. coli* strains. phiY showed the highest infection rate (76.14%), 1 of 6 EHEC strains and 108 of drug-resistance strains. Phage DNA sequence analyze exhibited a genome size 165 kbp, encoded 278 proteins and 35% GC content. This phiY was further modified amber mutation (premature termination of a polypeptide chain) at the packaging site, TerL by using the method of Cas9 in vitro digestion. The transformant cell carrying assembly genome between digested TerL genome and amber fragment did not show any plaques, indicating that it is difficult to modify the large-genome phage. Next, the other phage isolated from sewage water was clarified. This phiZ showed the potential of bactericidal activity among 3 of 6 EHEC strains and other types of *E. coli* strains, drug-resistant strain and adherent invasive *E. coli*. Phage DNA sequence analyze revealed a genome size of 241 kbp, encoded 264 proteins and 48% GC content. However, the genetic manipulation in the large phage (Jumbo phage; >200 kbp) by inserting other genes inside DNA needs to further study. The utilization of CRISPR-Cas13a-loaded capsids for the particular killing of *E. coli* expressing target sequence was confirmed, validating the possibility of this system as an alternative treatment against EHEC. Currently, we are searching for new broad-host-range phages, which can infect EHEC strains and other types of *E. coli*.

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５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関