様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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研究課題名（和文）Development of a novel MRSA genotyping method that does not require nucleic acid manipulation and optical device

研究課題名（英文）Development of a novel MRSA genotyping method that does not require nucleic acid manipulation and optical device

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研究成果の概要（和文）：本申請は、特異的な標的遺伝子認識によるCRISPR-Cas13aシステムを用いた簡便かつ安価なMRSA遺伝子型別法の構築を目的とした。MRSAの遺伝子型を鑑別するために、タイピング遺伝子の保存領域を決定し、crRNAオリゴヌクレオチドを設計した。次に、このcrRNAをファージのパッケージングサイトとCas13aを持つファージミドを作製した。さらに、広宿主域ファージに搭載することで、MRSAの耐性遺伝子を標的とした遺伝子型別のライブラリーを作製した。最後に、標的とする耐性遺伝子を発現した黄色ブドウ球菌に感染させることで、構築した抗菌カプシドの配列特異的な殺菌活性を評価した。

研究成果の学術的意義や社会的意義
本申請の成果は、ファージとCRISPR-Cas13aシステムを組み合わせた新しいMRSA遺伝子型別法の構築である。本システムは、スペーサー配列を設計するだけで、MRSAの遺伝子型別であるPFGE、SCCmec、MLST、coaおよびspa型別を安価かつ簡便に決定できる新しいMRSA検査法を提供できる。また、遺伝子の増幅を必要とせず、標的遺伝子を高感度に検出できる。さらに、高精度に標的遺伝子を認識できることで再現性にも優れている。特に、crRNA配列とファージの宿主相互作用による特異性により、明確な鑑別と解釈が可能であり、試験結果に差異が生じないことも大きな利点である。

研究成果の概要（英文）：This study aimed to establish a simple and cheap MRSA genotyping system by exploiting the collateral RNA cleavage ability of CRISPR-Cas13a ensuing sequence-specific recognition of target gene. For this purpose, different genetic discriminants commonly used for MRSA genotyping have been selected. Their conserved regions were determined and used as templates for the design of potential crRNA oligonucleotides. Next, crRNAs were loaded onto a plasmid carrying phage packaging site and CRISPR-Cas13a (phagemid). These phagemids were then packaged into our candidate broad-host-range phage, generating a library of ‘typing tools’ targeting different genetic determinants of MRSA. Finally, these CRISPR-Cas13a-based ‘typing tools’ were used to infect S. aureus RN4220 expressing the corresponding target genes. The ability of these capsids to kill the target bacteria sequence-specifically were confirmed. Our study showed that AB-capsids have the potential to be developed as MRSA genotyping tools.

研究分野：感染症学・微生物学

キーワード：MRSA genotyping　CRISPR-Cas　Phagemid

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様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Increased emergence of antibiotic-resistant bacteria (ARB) has become one of the biggest threats to global health. The relative prevalence of ARB varies among regions with the majority of ARB infections in Japan attributable to methicillin-resistant *Staphylococcus aureus* (MRSA) **[Uematsu, H. *et al., AJIC* 2016]**. Due to the rise in antimicrobial resistance and dried-up antibiotic pipelines, alternatives to antibiotics have thus become a priority for the treatment of ARB infections. Of equal importance, however, is the prevention of spread of the communicable diseases. Infection control practices have long been recognized as an important way to prevent transmission of ARB. Containment of MRSA was shown to play a part in eradication of these pathogens, as evidenced by the low, or even negligible, rates of MRSA infections in Scandinavian countries and the Netherlands owing to their 'search-and-destroy' policy.

Implementation and assessment of control and prevention measures is supported by epidemiological typing as discrimination among bacterial isolates enhances outbreak detection/confirmation and contributes towards identification of the source of transmission. To date, many microbial genotyping methods have been developed. Pulsed-field gel electrophoresis (PFGE) is considered the 'gold standard' for molecular typing of MRSA strains. However, this method is tedious, time-consuming, expensive, and not widely available. Other typing methods seize variations found in the sequences of coagulase (*coa*) and staphylococcal protein A (*spa*) genes. In these cases, however, the analyses are considered to be costly. Hence, there is a need to establish a simple and cheap, but yet robust system that can be applied for MRSA bio-typing; whereby it will act as a molecular typing tool to improve understanding of the epidemiology of MRSA infections, thereby contributing to the implementation of control strategies and subsequent eradication of MRSA infections.

２．研究の目的

This study aimed to establish a simple, customizable and inexpensive MRSA typing system by adopting CRISPR-Cas13a system and its collateral effect of killing bacterial cells once the system is activated **(Figure 1)**. CRISPR-Cas13a is a bacterial class II type VI CRISPR-Cas system which is demonstrated to display RNA endonuclease activity upon recognition of target RNAs by crRNA. After targeting a specific RNA sequence, activation of CRISPR-Cas13a proteins ensue 'collateral' cleavage of nearby non-targeted RNAs, conferring programmed cells death or induced dormancy **[Abudayyeh, O.O. *et. al., Science* 2016]**. Note, for example, that the widely used Cas9 system only cleaves double-stranded DNA without causing a collateral effect on untargeted genes. Therefore, this system cannot kill ARB if the gene(s) of interest is located on plasmids or in other mobile genetic elements. The ability to destroy any mRNA following activation thus makes CRISPR-Cas13a system unique, which happens to be the major defining feature involved in molecular typing of MRSA

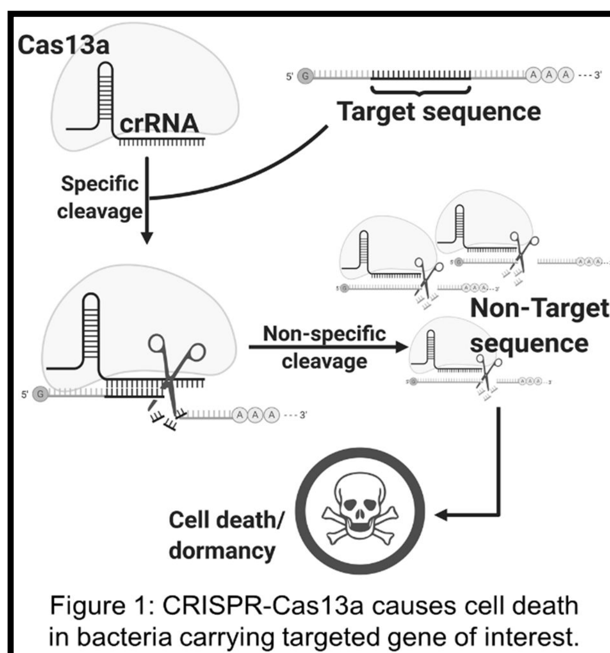

Figure 1: CRISPR-Cas13a causes cell death in bacteria carrying targeted gene of interest.

Herein, **I proposed to establish a simple, customizable and inexpensive MRSA typing system by adopting CRISPR-Cas13a-based chimeric phage that our laboratory has recently developed**. <u>This system requires neither nucleic acid amplification nor optic devices, and yet can recognize bacterial gene sequence-specifically and has high discriminating power</u>

equivalent to the currently available MRSA typing methods (SCC*mec*, MLST, POT, *spa*, *agr*, etc.). Furthermore, it can easily be customized to be applicable to other bacterial species, hence is believed to be able to bring revolutionary progress in bacterial epidemiological study and infection control practices.

３．研究の方法

Principally, a series of **(1) potential crRNA sequences** located on the gene(s) selected for MRSA typing will be identified. crRNA oligonucleotide(s) with optimal sequence variation detection ability will be **(2) custom-synthesized, annealed and cloned onto phagemids, together with CRISPR-Cas13a system**. These constructed phagemids will finally be **(3) packaged into the capsid of a candidate helper phage**. One important quality of any genotyping methods is to have high discriminating power between different bacterial strains of the same species. Since phagemids adopted the phage machinery for packaging and transfer, it is necessary to identify and characterize phages with broad-host-range, which will enable us to deliver the constructed phagemids carrying CRISPR-Cas13a to many different *S. aureus* strains. Hence, we will be **(4) screening for broad-host-range phages** from environmental/clinical samples using different propagating hosts. Finally, the engineered antibacterial capsids (AB-capsids) will be purified and used as a typing agent. The ability of AB-capsids to distinguish between *S. aureus* strains **(5) (MRSA typing) will then be evaluated**. Bacterial strains carrying the targeted sequence will be killed due to the lethal activity of CRISPRCas13a, forming a clear lytic zone at the spotted area.

４．研究成果

Our laboratory has demonstrated that CRISPR-Cas13a can be applied for specific killing of ARB. We have previously co-transformed *E. coli* with *mecA* gene and CRISPR-Cas13a system targeting *mecA*. The activation of CRISPR-Cas13a system upon recognition of *mecA* gene retarded *E. coli* growth, as shown by OD measurement of cell growth as well as observation of turbid appearance of broth cultures. We have also succeeded in generating functional chimeric phage(s) targeting *mecA* gene which can result in MRSA-specific killing. In this context, gene-targeting CRISPR-Cas13a system were loaded onto bacteriophage capsid using SaPI packaging system. SaPI will parasitize the life cycle of helper phage, inhibit phage production, and redirect phage packaging of SaPI element. The generated chimeric phages will then be used to infect target bacteria and the programmed CRISPR-Cas13a system will be delivered into host cells through normal viral transduction. We lysogenized phage 80α into *S. aureus* RN4220 to produce all necessary phage protein products and with the deletion of DNA packaging site *terS*, SaPI element carrying CRISPR-Cas13a was packaged into the capsid instead of the natural phage genome. Finally, this generated phi80α-SaPICas13a was used to infect USA300 (*mecA*-positive *S. aureus*), RN4220 (*mecA*-negative *S. aureus*) and USA300Δ*mecA* (*mecA*-negative *S. aureus*). The results showed that phi80α-SaPICas13a could specifically recognize and kill target bacteria USA300 but not the control strains RN4220 and USA300Δ*mecA*.

Next, we moved on to isolate the candidate broad-host-range phage. To this end, in-house collections of *S. aureus* were induced with Mitomycin C and these phage solutions were then screened against various clinical *S. aureus* strains for host range determination. A broad-host-range staphylococcal phage exhibiting more that 90% killing activity against a total of 300 clinical *S. aureus* strains was isolated (**manuscript in preparation**). This broad-host-range phage is then utilized, replacing phage 80α, to generate AB-capsids.

Our laboratory previously exploited SaPI element as vehicle for the loading of CRISPR-

Cas13a system into phage capsids. However, cloning of CRISPR-Cas13a onto SaPI element involved homologous recombination which are both laborious and time-consuming. We therefore sought to simplified CRISPR-Cas13a loading by establishing a phagemid system. Here, plasmid vector carrying CRISPR-Cas13a system and phage packaging site (phagemid) was constructed and then transformed into RN4220 lysogenized with the candidate broad-host-range phage. Similar with that of phage 80α, the packaging site of this prophage was also eliminated. Treatment with Mitomycin C then induced the packaging of CRISPR-Cas13a-loaded phagemid into the capsid to generate pure AB-capsids. Following the establishment of this packaging system, a series of AB-capsids programmed to target other commonly used MRSA typing genes was generated by simply designing the desired crRNA sequences to guide the CRISPR-Cas13a system. We successfully demonstrated that the established antibacterial capsids could effectively distinguish variations among different MRSA typing genes and subsequently killed the target bacteria sequence specifically. As shown in Figure 2, targeting AB-capsids (carrying CRISPR-Cas13a with targeting spacer) specifically eliminated bacterial cells carrying the targeted gene, whereas non-targeting AB-capsids did not affect bacterial growth. This data presentation allows easy interpretation of the typing genes present in the tested MRSA strains. However, due to confidentiality constraint (patent application), data generated from this study will not be shown in detail.

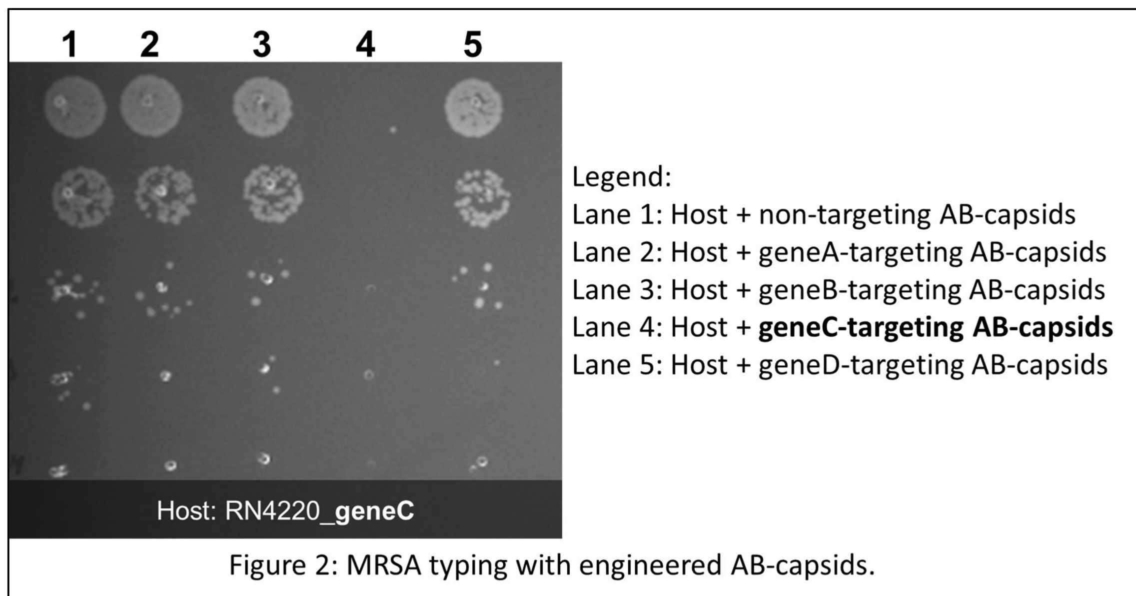

Figure 2: MRSA typing with engineered AB-capsids.

5. References

Uematsu H. et al. Effect of methicillin-resistant *Staphylococcus aureus* in Japan. *Am J Infect Control* **46** (1142; 2018).

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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