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Development of CRISPR-Cas13a-based antimicrobials capable of sequence-specific killing of target bacteria

Kotaro Kiga¹, Xin-Ee Tan¹, Rodrigo Ibarra-Chávez², Shinya Watanabe¹, Yoshifumi Aiba¹, Yusuke Sato’o¹, Feng-Yu Li¹, Teppei Sasahara¹, Bintao Cui¹, Moriyuki Kawauchi¹, Tanit Boonsiri¹, Kanate Thitiananpakorn¹, Yusuke Taki¹, Aa Haeruman Azam¹, Masato Suzuki³, José R. Penadés² & Longzhu Cui¹

The emergence of antimicrobial-resistant bacteria is an increasingly serious threat to global health, necessitating the development of innovative antimicrobials. Here we report the development of a series of CRISPR-Cas13a-based antibacterial nucleocapsids, termed CapsidCas13a(s), capable of sequence-specific killing of carbapenem-resistant Escherichia coli and methicillin-resistant Staphylococcus aureus by recognizing corresponding antimicrobial resistance genes. CapsidCas13a constructs are generated by packaging programmed CRISPR-Cas13a into a bacteriophage capsid to target antimicrobial resistance genes. Contrary to Cas9-based antimicrobials that lack bacterial killing capacity when the target genes are located on a plasmid, the CapsidCas13a(s) exhibit strong bacterial killing activities upon recognizing target genes regardless of their location. Moreover, we also demonstrate that the CapsidCas13a(s) can be applied to detect bacterial genes through gene-specific depletion of bacteria without employing nucleic acid manipulation and optical visualization devices. Our data underscore the potential of CapsidCas13a(s) as both therapeutic agents against antimicrobial-resistant bacteria and nonchemical agents for detection of bacterial genes.

¹Division of Bacteriology, Department of Infection and Immunity, School of Medicine, Jichi Medical University, Tochigi, Japan. ²Institute of Infection, Immunity & Inflammation, University of Glasgow, Glasgow G12 8TA, UK. ³Antimicrobial Resistance Research Center, National Institute of Infectious Diseases, Tokyo, Japan. ✉email: longzhu@jichi.ac.jp

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The emergence and spread of antimicrobial resistance among pathogenic bacteria has been a growing global public health concern for several decades. According to the recent CDC’s report on antimicrobial resistance, >2.8 million antimicrobial-resistant infections occur in the U.S. each year, and >35,000 people die as a result. It is also predicted that antimicrobial-resistant infections occur in the U.S. each year, and only a limited number of antibiotics were in either phase II or III clinical trials. Therefore, there is an urgent need for new strategies to develop alternative therapeutic approaches to prevent infections of AMR bacteria.

To this end, various nucleic acid-based antibacterials, peptides, bacteriophage therapies, antibodies, bacteriocins, and anti-virulence compounds have been recently developed. Among these, CRISPR (clustered regularly interspaced short palindromic repeats)–Cas9 and Cas9-encoding phages provide a means to combat such threats by selectively killing AMR bacteria. The CRISPR-Cas3 and CRISPR-Cas9 genome-editing constructs, which were designed to target AMR genes, were delivered into bacteria by packaging them into phages to achieve AMR gene-specific bacterial killing and prevent the spread of AMR genes. However, because DNA cleavage of plasmid DNA does not result in bacterial death, at least not in the absence of other confounding variables, such as a toxin–antitoxin system, this strategy is ineffective in targeting bacteria with plasmid-borne AMR genes.

CRISPR-Cas13a is a CRISPR-Cas type VI class 2 system and is characterized by RNA-guided single-stranded RNA (ssRNA) cleavage activity. A 2016 study by Abudayyeh et al. demonstrated that CRISPR-Cas13a has promiscuous ssRNA cleavage activities and restricts host bacteria growth due to the degradation of the bacterial RNAs. This has turned out to be a defense system against phages. When phages infect bacteria, CRISPR-Cas13a recognizes transcript of phage genome, which leads to nonspecific degradation of bacterial transcripts and arrests bacterial cell growth to prevent the spread of infection. Recently, we identified four different subtypes of CRISPR-Cas13a systems from 11 strains of six Leptotrichia species. Among them CRISPR-Cas13a form Leptotrichia shahii (LshCas13a) had a significant inhibition effect on bacterial growth, consistent with the observation by Abudayyeh et al. We here report success in developing sequence-specific antimicrobials by packaging the LshCas13a into bacteriophage capsids, which can be used as both therapeutic agents against AMR bacterial infections and non-chemical agents to detect bacterial genes for diagnosis.

**Results**

**Bactericidal activity of Cas13a.** First, we verified the growth inhibition ability of CRISPR-Cas13a (LshCas13a) in comparison with CRISPR-Cas9 by using Escherichia coli carrying the carbapenem resistance gene blaatIMP-1 on a chromosome or plasmid. To this end, we constructed two plasmids (pKLC21 and pKLC54) harboring CRISPR-Cas13a or CRISPR-Cas9 with spacers targeting blaatIMP-1 (spacer sequence was optimized, see later), and introduced them into the E. coli, respectively, to test their growth inhibition effect against the host cells carrying blaatIMP-1 (Fig. 1a). As expected, the LshCas13a with targeting blaatIMP-1 decreased the number of bacteria by two to three orders of magnitude against the bacteria carrying blaatIMP-1 both on chromosome (from 2.6 × 10^{10} to 2.0 × 10^{8} CFU/ml) and plasmid (from 2.3 × 10^{10} to 8.7 × 10^{6} CFU/ml). However, the CRISPR-Cas9 decreased the number of bacteria by three orders of magnitude against the bacteria carrying the blaatIMP-1 only on the chromosome (from 6.3 × 10^{10} to 3.6 × 10^{7} CFU/ml), but not on the plasmid (from 2.8 × 10^{10} to 2.2 × 10^{10} CFU/ml), when compared with their respective nontargeting controls (Fig. 1b, c). These results agreed with the fact that while CRISPR-Cas9 caused cell death by double-strand DNA breaks, CRISPR-Cas13a induced cell dormancy by collateral nonspecific cleavage of RNA (ref. 14). However, it is not clear whether the above decrements in cell number by Cas13a is due to cell death.

In order to determine whether Cas13a causes cell death or not, we have constructed a system in which CRISPR-Cas13a targeting mRNA of red fluorescent protein (RFP) transcribed from plasmid inhibits the growth of bacteria harboring RFP, following the method by Abudayyeh et al. We carried out the experiment in broth culture medium using LshCas13a by introducing an anhydrotetracycline (aTc)-inducible RFP plasmid (pKLC56-rfp), as well as an RFP-targeting LshCas13a (Cas13a_rfp) into E. coli (Fig. 1d), where the same spacer sequence used by Abudayyeh et al. was used (target rfp (ref. 14)). We observed that similar cell growth restriction by LshCas13a upon induction of the target rfp transcription compared to nontarget control and non-induction control (Fig. 1e, left and middle panels). Interestingly, when the same experiment was carried out using blaatIMP-1 as target gene, the bacteria growth curve stopped rising upon induction of blaatIMP-1 transcription (Fig. 1e, right panel), indicating cell growth might be completely stopped due to the cell death or dormancy. To make this point clear, viable cells carrying blaatIMP-1, targeting spacer were counted during the culture with and without aTc induction of blaatIMP-1 transcription. As seen in Fig. 1f, the number of viable cells were decreased by about three orders of magnitude from 5.5 × 10^{6} to 8.8 × 10^{3} during the 8 h upon induction of blaatIMP-1 transcription. We interpreted these results as demonstration of CRISPR-Cas13a-mediated sequence-specific killing of host cells. However, reasons of the viable cells that are in dormancy or resistant to CRISPR-Cas13a are not clear at the moment, but we did not find any mutation in target sequence after sequencing 32 randomly selected colonies from the above two experiments (Fig. 1b). We also observed that the induction of blaatIMP-1 transcription inhibited the host cell growth (Fig. 1e, right panel), which could be attributed to fitness cost of blaatIMP-1 overexpression. It is well known that the accumulation of carbapenemase compromises the bacterial growth.

**Packaging of CRISPR-Cas13a into bacteriophage capsid.** Having verified the bactericidal activity of the CRISPR-Cas13a_blaatIMP-1 construct against blaatIMP-1-positive bacteria, we came up with the idea that AMR bacteria-specific CapsidCas13a constructs could be synthesized by loading the CRISPR-Cas13a system into a phage capsid. The CRISPR-Cas13a_blaatIMP-1 was loaded into E. coli phage M13 capsid, to generate EC-CapsidCas13a_blaatIMP-1 (Fig. 2a), which demonstrated sequence-specific killing activity against bacteria carrying the blaatIMP-1 gene in an EC-CapsidCas13a_blaatIMP-1 concentration-dependent manner (Fig. 2b). Subsequently, we confirmed the same sequence-specific killing activities with a series of CapsidCas13a constructs programmed to target different genotypes of carbapenem-resistant genes (blaIMP-1, blaOXA-48, blaVIM-2, blaOXA-51, and blacKPC-2) and colistin-resistant genes (mcr-1 and mcr-2), all of which are currently a problem in the clinical setting (Fig. 2c, Supplementary Fig. 1). Conversely, nontargeting CapsidCas13a (with nontargeting spacer) killed no bacteria under any circumstance, indicating that this construct has robust specificity for gene-directed antimicrobial therapy against AMR bacterial infections.
Fig. 1 Sequence-specific bactericidal activity of CRISPR-Cas13a. a A schematic diagram of transformation of CRISPR-Cas13a and CRISPR-Cas9 with targeting bla<sub>IMP-1</sub> into bla<sub>IMP-1</sub>-expressing E. coli STBL3. b E. coli STBL3 expressing bla<sub>IMP-1</sub> from a plasmid (plasmid-borne bla<sub>IMP-1</sub>) and chromosome (chromosome-borne bla<sub>IMP-1</sub>) were prepared, and transformed with CRISPR-Cas13a or CRISPR-Cas9, both with spacer targeting bla<sub>IMP-1</sub> or no spacer (nontargeting). The resulting transformants were plated on an LB plate containing kanamycin (Km) to test sequence-specific bacterial killing by CRISPR-Cas13a and CRISPR-Cas9. Km was used to maintain the plasmids. c The number of bacteria on the plate obtained in the experiment of b was counted. The statistical significance was determined by two-sided Student’s t-test. Each bar represents the mean with standard deviation (n = 3). d A schematic diagram of the experiment to test CRISPR-Cas13a-dependent cell growth inhibition. Anhydrotetracycline (aTc)-inducible bla<sub>IMP-1</sub> or rfp-expression plasmid (pKLC56) was co-transformed with pKLC21 plasmid expressing CRISPR-Cas13a with spacer targeting bla<sub>IMP-1</sub> or rfp into E. coli STBL3. The resulting transformants were then cultured and the aTc induction in the presence of the antibiotics Km and Cm (Km and Cm for maintaining plasmids) was carried out at the indicated time points. Thereafter, the OD values were measured every hour. e Growth curves were plotted. Each line of the growth curves represents the mean with standard deviation. f The number of viable cells was counted to calculate the ratio of cell death caused by bla<sub>IMP-1</sub>-targeting CRISPR-Cas13a. The bacterial culture prepared in d was diluted to 1/100, then aTc was added to induce bla<sub>IMP-1</sub>. Km and Cm were also added to maintain the CRISPR-Cas13a and the aTc-inducible bla<sub>IMP-1</sub> plasmids. Thereafter, the bacterial cultures were sampled at the indicated time points, and the number of surviving bacteria was counted on fresh LB plates. Each bar represents the mean with standard deviation of four biological replicates. p-values were determined by two-sided Student’s t-test. Source data are available in the Source Data file.
In order to compare the characteristics of CapsidCas13a with previously reported Cas9-based antimicrobial agents, we generated a Cas9-based EC-CapsidCas9$_{_{\text{bla}\text{IMP-1}}}$ construct with the same protocol, but in this instance CRISPR-Cas9$_{_{\text{bla}\text{IMP-1}}}$ was replaced with CRISPR-Cas13a$_{_{\text{bla}\text{NDM-1}}}$ and its bactericidal manner was compared with that of EC-CapsidCas13a$_{_{\text{bla}\text{NDM-1}}}$.
Fig. 3 Potential of CapsidCas13a as a tool for modifying bacterial flora.
Programmed CapsidCas13a altered the composition of bacterial population. A mixed cell population was prepared by mixing E. coli NEB5α F′/F (control) with equal numbers of NEB5α F′/F expressing blaIMP-1, and mcr-2, respectively. The cell mixtures were then independently treated with blaIMP-1-targeting, mcr-2-targeting, and nontargeting CapsidCas13a. Note that each AMR gene-targeting CapsidCas13a reduced the corresponding target cell population. The percentage represents the mean of three biological replicates. Source data are available in the Source Data file.

Fig. 4 Potential of CapsidCas13a as a therapeutic against AMR bacteria infections. Examination of the therapeutic effect of EC-CapsidCas13a-blaIMP-1 using a Galleria mellonella infection model. Administration of EC-CapsidCas13a-blaIMP-1 (MOI 100) into G. mellonella larvae infected with R10-61 (carbapenem-resistant clinical isolates of E. coli carrying blaIMP-1) significantly improved host survival compared to controls, EC-CapsidCas13a-nontargeting (p = 0.044), and phosphate-buffered saline (PBS; p = 0.0016). The p-value between two groups infected with R10-61, and treated with EC-CapsidCas13a-nontargeting and PBS was 0.30. The p-values are calculated by log-rank test. The results are presented as the aggregate values of three independent experiments performed using ten larvae per group. Source data are available in the Source Data file.
The \textit{bla}\textsubscript{IMP-1},563 spacer sequence GACTTTGGCCAAGGGTCC TATATTGCGT, which had the highest depletion rate of 99.7, was chosen as the best spacer sequence for use in subsequent experiments (Supplementary Fig. 2c, Supplementary Table 4). Then, the carrier M13 phage was replaced with the lysogenic phage \textit{Phi}80 for use with the phage-inducible chromosomal island (PICI) packaging system (Supplementary Fig. 3)\textsuperscript{15,26}, which is more flexible in genome manipulation. Finally, the kanamycin (Km) resistance gene (KanR) was inserted as a selection marker to generate the constructs of PICI-based EC-CapsidCas13a::KanR\textit{bla}\textsubscript{IMP-1} and EC-CapsidCas13a::KanR nontargeting as a nontargeting spacer control (Fig. 5a).

Next, we tested the detection efficiency of the constructs for \textit{bla}\textsubscript{IMP-1} by spotting 2\(\mu\)L of tenfold serial dilutions of EC-CapsidCas13a::KanR\textit{bla}\textsubscript{IMP-1} and EC-CapsidCas13a::KanR nontargeting onto fresh top agar lawns of the test strains in Luria-Bertani (LB) agar with or without supplementation of Km (Fig. 5b). To improve the efficiency, we opted to determine the bacterial killing effect against Km-resistant cells on Km plates (Fig. 5c), rather than assessing the bacterial killing effect against original cells on Km-free plates (Fig. 5d). When the EC-CapsidCas13a::KanR\textit{bla}\textsubscript{IMP-1} carrying the Km resistance gene was applied on the soft agar bacterial lawn grown on bottom agar containing Km, the cells infected by this capsid acquired Km sensitivity by about three orders of magnitude against the bacteria carrying target gene.

Uncropped images of the gels are available in the Supplementary Information.

Fig. 5 Potential use of CapsidCas13a for bacterial gene detection. a A schematic illustration of generation of PICI-based EC-CapsidCas13a targeting \textit{bla}\textsubscript{IMP-1}. Mitomycin C induction promotes the packaging of PICImid carrying a CRISPR-Cas13a system and kanamycin (Km) resistance gene into the capsid of helper phage \textit{Phi}80. b-d Bacterial activity test of PICI-based EC-CapsidCas13a::KanR\textit{bla}\textsubscript{IMP-1} (in tenfold serial dilutions) against \textit{E. coli} MC1061 with or without the expression of target gene was carried out on LB agar plates (d); and the test results were judged by observing bacterial growth on LB bottom agar plates supplemented with Km (c), or observation of cell lysis on drug-free LB bottom agar plates (d): noting that the former assay had an enhanced sensitivity by about three orders of magnitude against the bacteria carrying target gene. e-j The PICI-based EC-CapsidCas13a(s) were applicable to detect various carbapenem resistance genes (\textit{bla}\textsubscript{IMP-1}, \textit{bla}\textsubscript{OXA-48}, and \textit{bla}\textsubscript{VIM-2}) regardless of their location on either the plasmid or chromosome (e, f), whereas EC-CapsidCas9 could detect genes located on the chromosome but not on the plasmid (g); and the PICI-based EC-CapsidCas13a(s) also effectively detected toxin-encoded genes (h), differentiated different genes located on the same plasmid (i), and were also applicable to clinical isolates (j, left panel) as being verified by PCR (j, right panel). k SA-CapsidCas13a::TetR\_mecA generated by packaging mecA-targeting CRISPR-Cas13a into capsid of \textit{S. aureus} phage 80\alpha exhibited mecA-specific bactericidal activity against MRSA, but not \textit{S. aureus} strains deficient in mecA. All assays were replicated three times. Uncropped images of the gels are available in the Supplementary Information.
resistance and, hence, could grow in the presence of Km. Nevertheless, with the bacterial cells carrying the target AMR gene \(b\text{la}_{\text{IMP}-1}\), there was no observable growth due to the bactericidal effect of the CRISPR-Cas13a construct (Fig. 5c). This method was shown to be almost three orders of magnitude more sensitive than direct observation of the bacterial growth inhibition on Km-free plates (Fig. 5d). We further confirmed the efficiency of this system, with the use of the M13 capsid-based EC-CapsidM13Cas13a::KanR\(_{b\text{la}_{\text{IMP}-1}}\) construct (Supplementary Fig. 4a–c). A subsequent experiment showed that the target AMR genes of interest located on either the plasmid or chromosome could be precisely detected (Fig. 5e, f, Supplementary Fig. 4d, e), as expected, whereas CRISPR-Cas9 construct could detect only the genes located on a chromosome but not on a plasmid (Fig. 5g). The detection ability was further confirmed with the CapsidCas13a constructs targeting other AMR genes \(b\text{la}_{\text{OXA-48}}\) and \(b\text{la}_{\text{NDM-1}}\) (Fig. 5e, f), toxin genes \(s\text{tx1}\) and \(s\text{tx2}\) (Fig. 5h), and two genes (\(b\text{la}_{\text{IMP}-1}\) and \(m\text{cr}-2\)) located on the same plasmid (Fig. 5i), indicating that this method is applicable for the detection of any bacterial genes regardless of their location on bacterial chromosome or plasmid. Although the sensitivity was slightly lower, it was even possible to detect target genes by directly spotting the CapsidCas13a(s) onto the bacteria swabbed on an agar plate instead of using the soft agar overlay method (Supplementary Fig. 5).

We also tried to apply the CapsidCas13a constructs to detect carbapenem-resistant clinical isolates of \(E.\text{coli}\) carrying \(b\text{la}_{\text{IMP}-1}\) or \(b\text{la}_{\text{NDM-1}}\). As these strains were not susceptible to Km, the KanR of EC-CapsidCas13a::KanR was replaced with the hygromycin (Hygro) resistance gene, HygroR, to generate the constructs EC-CapsidCas13a::HygroR\(_{b\text{la}_{\text{IMP}-1}}\) and EC-CapsidCas13a::HygroR\(_{b\text{la}_{\text{NDM-1}}}\). The test results with these two CapsidCas13a(s) showed that the \(E.\text{coli}\) clinical isolates carrying \(b\text{la}_{\text{IMP}-1}\) and \(b\text{la}_{\text{NDM-1}}\) were precisely detected, which was consistent with the results of polymerase chain reaction (PCR) analysis (Fig. 5), Supplementary Fig. 5d), suggesting that the CapsidCas13a(s) can be applied to detect bacterial genes. With regard to the application potential of CRISPR-Cas13a, our data demonstrated above has opened up a new field in which it can be developed not only as an antibacterial therapeutic agent, but also as a new bacterial identification system where no nucleic acid manipulation is necessary once the CapsidCas13a(s) are established. There was an elegant report on the CRISPR-Cas13-based nucleic acid detection method, called as SHERLOCK system, which was developed by combination of nucleic acid amplification technique and CRISPR-Cas13. This method can detect DNA or RNA in vitro with attomolar level sensitivity. In contrast, the proposed detection system using the CapsidCas13a constructs can be performed without amplification of DNA or RNA, electrophoresis equipment or optical devices, as only bacterial culture plates are required. In addition, 10\(^{10}\) transducing forming units (TFU) of CapsidCas13a constructs can be harvested per liter of host bacterial culture, and only 2–3 mL of 10\(^{3}\) constructs per mL of solution is required for a single spot test to accurately determine the presence or absence of target genes. Although these features highlight the elegant potential of CapsidCas13a(s) for bacterial gene detection, there are still limitations, at least: (1) it is necessary to construct corresponding CapsidCas13a for each bacterial species and gene, (2) turnaround time for test results can be long since interpretation of the results is dependent on bacterial growth, (3) it cannot be used when the bacteria cannot be cultured or the target gene is not transcribed.

\textit{Staphylococcus aureus CapsidCas13a}. Lastly, in addition to demonstrating the bactericidal activity of CRISPR-Cas13a against Gram-negative bacteria, we also attempted to confirm the col-lateral activity of CRISPR-Cas13a against Gram-positive bacteria using \textit{Staphylococcus aureus}. First, a set of \(E.\text{coli}\)–\(S.\text{aureus}\) shuttle vectors, namely \(p\text{KLC4}(s)\), were generated carrying the CRISPR-Cas13a construct with or without a spacer sequence targeting the \(S.\text{aureus}\)\(_{r\text{psE}}\) genes. Transformation of the vector into \(S.\text{aureus}\) strain RN4220 showed that the bactericidal activity of CRISPR-Cas13a with an appropriate spacer sequence was similar to that in \(E.\text{coli}\) (Supplementary Fig. 6a). Then, a SA-CapsidCas13a\(_{m\text{ecA}}\) construct was produced to target methicillin-resistant gene \(m\text{ecA}\) of methicillin-resistant \(S.\text{aureus}\) (MRSA), one of the most prevalent AMR pathogens worldwide. We optimized the spacer sequences (Supplementary Fig. 6b–e) and the CRISPR-Cas13a\(_{m\text{ecA}}\)-carrying vector (\(p\text{KLC-SP}\_m\text{ecA}\)) construction were carried out in the same way as above for CRISPR-Cas13a\(_{b\text{la}_{\text{IMP}-1}}\). The packing of CRISPR-Cas13a\(_{m\text{ecA}}\) into \(S.\text{aureus}\) phage 80a capsid was performed in accordance with the method established by Ubeda et al. using the \(S.\text{aureus}\) pathogenicity island (SaPI) system. This packing system simultaneously imparted tetracycline (Tet) resistance to the resulting SA-CapsidCas13a::TetR\_mecA construct, since the SaPI carried the Tet resistance gene, which made it possible to be tested for both bactericidal ability against MRSA and capability of MRSA detection by targeting \(m\text{ecA}\). As expected, when the methicillin-susceptible \(S.\text{aureus}\) strain RN4220 and MRSA USA300 or \(m\text{ecA}\) knockout USA300 (USA300\_\(\Delta m\text{ecA}\)) were infected with SA-CapsidCas13a\(_{m\text{ecA}}\), the growth of only the USA300 strain carrying \(m\text{ecA}\) was significantly inhibited (Fig. 5k, Supplementary Fig. 8), clearly demonstrating the sequence-specific bacterial killing ability of CRISPR-Cas13a against the Gram-positive bacteria \(S.\text{aureus}\).

Discussion

In this study, we employed the promiscuous RNA cleavage ability of CRISPR-Cas13a via recognition of target RNA by CRISPR-RNA (crRNA), which resulted in host cell death, to generate a new type of sequence-specific bacterial antimicrobials. To deliver the CRISPR-Cas13a to target bacteria, we packaged the CRISPR-Cas13a into carrier phage capsid using the PICI packaging system for \(E.\text{coli}\), and SaPI packaging system for \(S.\text{aureus}\). Since synthesized CapsidCas13a does not carry phage genome, it thus belongs to the category of a nucleic acid drug or gene drug, not an organism, thereby easily being put into practical use as a therapeutic drug. Although there are still many questions to be answered concerning practical application—such as host range of the phage capsids, catalytic mode of Cas13a (refs. 14,15), the efficiency of phage capsid packaging, and ethical issues regarding genetic recombinants, etc.—our strategy demonstrated that the CapsidCas13a antimicrobials are promising to be developed for at least three application categories: (1) as promising antibacterial therapeutic agents targeting any bacterial gene, including AMR genes, or selectively killing targeted toxin-producing bacteria, (2) as a simple and inexpensive bacterial gene detection system for bacterial identification and efficient molecular epidemiological investigations without the need for the amplification of nucleic acids or optic devices, (3) as tools to manipulate the bacterial flora by targeting and eliminating a specific bacterial population without disrupting other irrelevant bacterial populations. In conclusion, the proposed CRISPR-Cas13a-based antimicrobials are expected to have a great impact in the field of antimicrobial resistance for infection control, as well as bacterial flora control.

\textbf{Methods}

\textbf{Ethics declarations}. Ethics approval for the use of invertebrates was given by Jichi Medical University ethics committee.
Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Supplementary Table 1. Bacterial strains were grown at 37°C in LB medium with 100 μg/mlampicillin. Undiluted overnight cultures were used to inoculate LB medium with 100 μg/mlampicillin to grow to mid-log phase, before the following final concentrations: 100 μg/mlampicillin for ampicillin (Amp), 30 μg/mlKm, 34 μg/mlchloramphenicol (Cm), 4 μg/mlcolistin, and 200 μg/mlHyg.

CRISPR-Cas13a and CRISPR-Cas9 gene targeting vectors. The CRISPR-Cas13a expression vector (pC003), which carries LshCas22 loci on pACYC184, was kindly provided by Dr. Feng Zhang (Addgene plasmid # 79152; http://n2t.net/addgene: 79152; RRID: Addgene_79152)34. In order to generate an efficient vector series carrying a CRISPR-Cas13a system targeting various genes, we conducted genomic manipulations. First, a DNA fragment of cas13a-cas13-I2 locus was amplified from L. shahii strain JC1M6776 (ref. 14), using a primer set of LsCas13a clo-f and LsCas13a clo-R to obtain CRISPR-Cas13a_nontargeting (control). Finally, we individually generated two PCR products between the 5′ and 3′ region of bap on pMAY-bapUp/Down with In-Fusion HD Cloning Kit to generate pKLC-SP_mecA and pKLC-SP null control.

Construction of target gene expression vectors. We constructed two plasmid systems for expression of target genes of the CRISPR-Cas13a systems: a pSp272 tACS regulatory vector in which the cloning site for target gene expression was under the control of the tACS regulatory element. The PCR amplification was carried out with primer pairs of LsCas22 mecA5-F and LsCas22 mecA5-R to obtain CRISPR-Cas13a_mecA, and LsCas22 mecA5-F and LsCas22 mecA5-R to obtain CRISPR-Cas13a_mecA (crispr-Cas13a_non-targeting). The PCR amplification was carried out with primer pairs of LsCas22 mecA5-F and LsCas22 mecA5-R to obtain CRISPR-Cas13a_mecA, and LsCas22 mecA5-F and LsCas22 mecA5-R to obtain CRISPR-Cas13a_mecA (control). Finally, we individually generated two PCR products between the 5′ and 3′ region of bap on pMAY-bapUp/Down with In-Fusion HD Cloning Kit to generate pKLC-SP_mecA and pKLC-SP null control.
amplified from pBAD33 using a primer pair of pBAD33 PCR InFusion-F and pBAD33 PCR InFusion-R. The ligation was performed with In-Fusion HD Cloning Kit and transformed into E. coli strain C600 to select cells free of plasmid. CRISPR-Cas13a systems were generated by PCR. Finally, resulting clones were chemically induced by mitomycin C to generate SA-CapsidCas13a_mecA and SA-CapsidCas13a_non-targeting (control) by using the method described elsewhere. The E. coli strain with chromosomally integrated genes. The generation of E. coli strain expressing foreign gene on its own chromosome was achieved by using ARA-inducible Red recombination system. First, we transformed E. coli strains NEB-alpha F′ (New England Biolabs, US) and MC1061 with plasmid pKD46 that carries ARA-inducible Red recombination system. Next, the desired genes, e.g., _bla_IMP-1, were knocked-in into the above strains following the methods described by Tomoya Baba et al.35 The _bla_IMP-1 vector), and selected on the LB agar plate containing Amp, colistin, and drug free, respectively, were counted, and cell number of surviving cells was monitored.

**Bacterial cell growth inhibitory test in culture medium.** The pKLC21_bla_IMP-1_rfp, the pKLC21_bla_IMP-1_non-targeting were transformed into E. coli STBL3(pKLC63) (as a control), STBL3(pKLC63_bla_IMP-1) (Ara-inducible _bla_IMP-1 expression plasmid), and STBL3(pKLC63_RFP) (Ara-inducible RFP expression plasmid). Each transformant was then plated onto LB plates containing Km and Cm, and incubated at 37 °C for 10 h. After that, bacteria on the plates were collected and serially diluted with 0.8 % NaCl, and a colony count for the number of surviving cells was carried out.

**Cell viability test after introducing CRISPR-Cas13a.** The pKLC21_bla_IMP-1_rfp, the pKLC21_bla_IMP-1_non-targeting were transformed into E. coli STBL3(pKLC63) (as a control), STBL3(pKLC63_bla_IMP-1) (Ara-inducible _bla_IMP-1 expression plasmid), and STBL3(pKLC63_RFP) (Ara-inducible RFP expression plasmid). Each transformant was then plated onto LB plates containing Km and Cm, and incubated at 37 °C for 10 h. After that, bacteria on the plates were collected and serially diluted with 0.8 % NaCl, and a colony count for the number of surviving cells was carried out.

**Sequence-specific bacterial killing by EC-CapsidCas13a.** The logarithmic phase cultures of three E. coli strains with overexpression of _bla_IMP-1, _mecA_2, and _mcr-2 in plasmid and carrying control plasmid NEBS-alpha _P'_i (pKLC62_bla_IMP-1) and NEBS-alpha _P'_i (pKLC62_bla_IMP-1), and NEBS-alpha _P'_i (pKLC62_bla_IMP-1) were transferred to LB medium and incubated at 37 °C for 1.5 h with vigorous shaking (500 rpm). Then, 1 µg/ml of _A_Δc was added to induce the expression of _bla_IMP-1, and rfp, and incubated at 37 °C for 1 h with vigorous shaking. After that, antibiotics (Km and Amp) were added into the culture, and incubated at 30 °C for 23 h with vigorous shaking. OD600 of each well was monitored every hour.

**Generation of PICI-based EC-CapsidCas13a and EC-CapsidCas9.** The E. coli JP12507 (ref. 25), derived by lysogenizing phage E. coli f1 origin of M13 (i.e., pKLC21_bla_IMP-1, Vector), and the resulting phagemid was termed pKLC25. Next, CRISPR-Cas13a-loaded E. coli strains NEB-alpha _P'_i (New England Biolabs, US) and MC1061 with plasmid pKD46 that carries ARA-inducible Red recombination system. First, we transformed E. coli strains NEB-alpha _P'_i (New England Biolabs, US) and MC1061 with plasmid pKD46 that carries ARA-inducible Red recombination system. Next, the desired genes, e.g., _bla_IMP-1, were knocked-in into the above strains following the methods described by Tomoya Baba et al.35 The _bla_IMP-1 vector), and selected on the LB agar plate containing Amp, colistin, and drug free, respectively, were counted, and cell number of surviving cells was monitored.

**Bacterial cell growth inhibitory test in culture medium.** The pKLC21_bla_IMP-1_rfp, the pKLC21_bla_IMP-1_non-targeting were transformed into E. coli STBL3(pKLC63) (as a control), STBL3(pKLC63_bla_IMP-1) (Ara-inducible _bla_IMP-1 expression plasmid), and STBL3(pKLC63_RFP) (Ara-inducible RFP expression plasmid). Each transformant was then plated onto LB plates containing Km and Cm, and incubated at 37 °C for 10 h. After that, bacteria on the plates were collected and serially diluted with 0.8 % NaCl, and a colony count for the number of surviving cells was carried out.

**Cell viability test after introducing CRISPR-Cas13a.** The pKLC21_bla_IMP-1_rfp, the pKLC21_bla_IMP-1_non-targeting were transformed into E. coli STBL3(pKLC63) (as a control), STBL3(pKLC63_bla_IMP-1) (Ara-inducible _bla_IMP-1 expression plasmid), and STBL3(pKLC63_RFP) (Ara-inducible RFP expression plasmid). Each transformant was then plated onto LB plates containing Km and Cm, and incubated at 37 °C for 10 h. After that, bacteria on the plates were collected and serially diluted with 0.8 % NaCl, and a colony count for the number of surviving cells was carried out.

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ratios were calculated. The sequence-specific killing activity of SA-CapsidCas13a-

Measurement of phage titers. The M13 phage/PI-CI-based EC-CapsidCas13a(s) were serially diluted with SM buffer ranging from 10^2 to 10^−7. In the meantime, overnight culture of E. coli strain NEB5-alpha FP4 or MC1061 diluted 1:100 with LB broth was incubated with agitation at 37 °C until an OD_600 of ~0.1 was obtained. Then, 10 µl of each dilution of M13 phage/PI-CI-based EC-CapsidCas13a(s) was added to 100 µl of bacterial suspension and the mixture was incubated at 37 °C for 30 min. Subsequently, all of the culture solution was plated on LB plates containing Cm or Km, and the plates were incubated overnight at 37 °C. The colonies grown on the Km plate but not on the Cm were counted to calculate the TFF/mL.

Detection of bacterial genes with CapsidCas13a(s). The E. coli strains to be determined were grown to an OD_600 of ~0.5. Then, 100 µl of the culture were mixed with 3 ml molten soft agar (LB solution with 0.5% agarose) prewarmed at 50 °C and poured onto an LB plate containing Km or Hygros. The plates were solidified at room temperature. Meanwhile, M13 phage/PI-CI-based EC-CapsidCas13a with known titers was adjusted to 10^5 TFF/mL and its tenfold serial dilutions were prepared. Finally, 2 µl of each dilution of the M13 phage/PI-CI-based EC-CapsidCas13a were spotted on the solidified soft agar and the plates were incubated at 37 °C. The result was interpreted as positive if bactericidal plaque formed on the plate.

pKLC21_blaIMP-1, library sequencing. The pKLC21_blaIMP-1 (CRISPR-Cas13a- blasmp-1 expression plasmid) library targeting the whole region of blaIMP-1 was constructed as aforementioned. Equal amounts of each of the 121 pKLC21_blaIMP-1 carrying spacers against different position of blaIMP-1 and pKLC21_nontargeting (as a control) were mixed and transformed into E. coli MC1061(pKLC26) and MC1061 (pKLC26_blaIMP-1). Each transformant was then plated onto 20 LB plates containing Cm and Km, and incubated at 37 °C for 16 h. Next, >10,000 colonies for each transformant were harvested by using LB medium, and plasmids were extracted using QIAGEN Plasmid Midi Kit (QIAGEN). Pair-end sequencing libraries were constructed from the plasmids using Nextera XT Library Prep Kit (Illumina). Sequencing was performed using Illumina MiSeq platform (2 × 310 bp) with MiSeq reagent kit version 3 (Illumina).

G. mellonella survival assay. The M-sized G. mellonella larvae purchased from Ikisa factory (Osaka, Japan) were used for the survival assay to assess the effect of PI-CI-based EC-CapsidCas13a on treatment of E. coli infections. Upon receipt, the larvae were acclimated to the laboratory environment by leaving them in a dark room for 24 h before starting the assay. Larvae with weak movement, dark color, unusual shape, and sizes that differed distinctly from other larvae were excluded from the experiment. A Hamilton syringe (701LT, Hamilton) and a KF color, unusual shape, and sizes that differed distinctly from other larvae were

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Author contributions

K.K. and L.C. designed the study, analyzed the data, and wrote the manuscript. X.-E.T. and R.-I.C. contributed to the acquisition, analysis and interpretation of data, and assisted the preparation of the manuscript. J.R.P. contributed to design of the study, interpretation of data, and assisted the preparation of the manuscript. All other authors contributed to data collection and interpretation and critically revised the manuscript. All authors approved the final version of the manuscript and agreed on all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to L.C.

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