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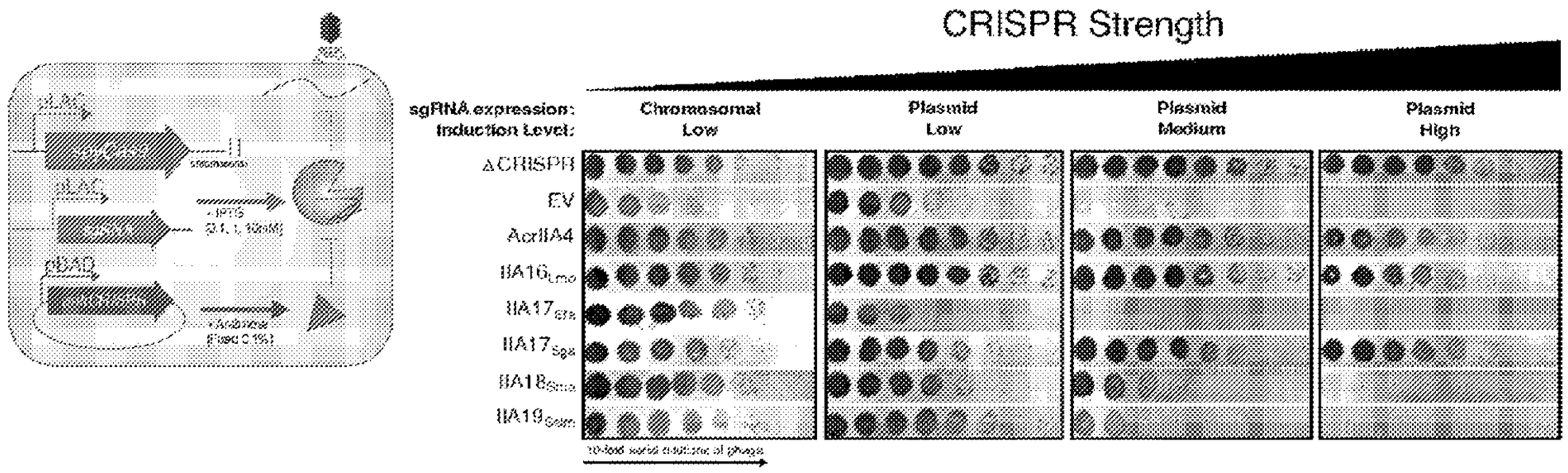
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(54) Title: BROAD SPECTRUM INHIBITORS OF CRISPR-CAS9

FIG. 1B



(57) **Abstract:** The present disclosure provides Cas9-inhibiting polypeptides and polynucleotides, and methods of using the same to inhibit Cas9 in cells.

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BROAD SPECTRUM INHIBITORS OF CRISPR-CAS9

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims priority to U.S. Provisional Pat. Appl. No. 62/932,383, filed on November 7, 2019, which application is incorporated herein by reference in its entirety.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] This invention was made with government support under grants OD021344 and R01 GM127489 awarded by the National Institutes of Health, and grant HR0011-17-2-0043 awarded by the Defense Advanced Research Projects Agency. The government has certain rights in the invention.

BACKGROUND

[0003] Bacteria are constantly exposed to invasive mobile genetic elements (MGEs) that can either benefit or harm the host. Many MGEs encode antibiotic resistance pathogenicity factors that can enhance microbe virulence (Palmer et al., 2010; Waldor and Mekalanos, 1996), although most are regarded as parasitic entities (Koonin, 2016). To combat MGE invasions, bacteria possess defense mechanisms, including restriction modification and CRISPR-Cas adaptive immunity (Labrie et al., 2010), which can limit the exchange of destructive genetic material (Price et al., 2016; Edgar and Qimron, 2010; Zhang et al., 2013). CRISPR-Cas systems are widespread, found in roughly half of bacteria and over 80% of archaea (Makarova et al., 2015), and can protect host genomes against phage infection and plasmid conjugation (Garneau et al., 2010). Nevertheless, the occurrence of horizontal gene transfer (HGT) persists across species, as is evident by DNA sequence estimates suggesting that 5-6% of genes in bacterial genomes are derived from HGT (Clark and Pazdernik, 2013).

[0004] Bacteriophages have responded to CRISPR-Cas with anti-CRISPR (Acr) proteins (Bondy-Denomy et al., 2013), which can inhibit CRISPR-Cas complex formation/stability

(Harrington et al., 2019; Zhu et al., 2019), target DNA binding, or cleavage (Bondy-Denomy et al., 2015; Dong et al., 2019; Knott et al., 2019). To date, 46 distinct families against various CRISPR-Cas subtypes have been discovered, of which type II-A Cas9 inhibitors alone constitute 11 (Rauch et al., 2017; Hynes et al., 2017, 2018; Uribe et al., 2019; Forsberg et al., 2019). Numerous strategies have been employed for Acr discovery, including bioinformatic (Pawluk et al., 2016; Rauch et al., 2017), experimental (Bondy-Denomy et al.; 2013, Hynes et al., 2017), and metagenomic screening (Uribe et al., 2019; Forsberg et al., 2019). Many of these approaches have discovered Acrs on phages and prophages. It is not clear, however, how other MGEs avoid CRISPR targeting. In the opportunistic pathogen *Enterococcus faecalis*, for example, where integrated conjugative elements (ICEs) encode antibiotic resistance, their presence is associated with non-functional CRISPR-Cas systems (Palmer and Gilmore, 2010; Hullahalli et al., 2018). It is unclear whether Acrs play a role in the horizontal spread and vertical maintenance of non-phage MGEs by compromising the host immune defense systems.

[0005] The present disclosure provides previously unknown CRISPR-Cas9 inhibitors from plasmids and other conjugative elements in *Firmicutes* bacteria. The present inhibitors are encoded by mobile genetic elements in bacteria and possess a wide range of inhibition capacity, making them suitable for use as broad regulators of different Cas9 nucleases.

BRIEF SUMMARY OF THE INVENTION

[0006] In one aspect, the present disclosure provides a method of inhibiting a Cas9 polypeptide in a cell, the method comprising, introducing a Cas9-inhibiting polypeptide into a cell, wherein: the Cas9-inhibiting polypeptide is heterologous to the cell, and the Cas9-inhibiting polypeptide is substantially (e.g., at least about 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%) identical to any one or more of SEQ ID NOS: 1-8; thereby inhibiting the Cas9 polypeptide in a cell.

[0007] In some embodiments, the method comprises contacting the Cas9-inhibiting polypeptide with a Cas9 polypeptide in the cell. In some embodiments, the Cas9-inhibiting polypeptide comprises one of SEQ ID NOS: 1-8. In some embodiments, the Cas9-inhibiting polypeptide comprises SEQ ID NO: 1, 2, 4 or 7. In some embodiments, the cell comprises an expression cassette comprising a promoter operably linked to a polynucleotide encoding the Cas9 polypeptide. In some embodiments, the cell comprises the Cas9 polypeptide before the introducing. In some such embodiments, the promoter is inducible and the method comprises

contacting the cell with an agent or condition that induces expression of the Cas9 polypeptide in the cell prior to the introducing of the Cas9-inhibiting polypeptide. In some embodiments, the cell comprises the Cas9 polypeptide after the introducing of the Cas9-inhibiting polypeptide. In some such embodiments, the promoter is inducible and the method comprises contacting the cell with an agent or condition that induces expression of the Cas9 polypeptide in the cell after the introducing of the Cas9-inhibiting polypeptide.

[0008] In some embodiments of the method, the introducing of the Cas9-inhibiting polypeptide comprises expressing the Cas9-inhibiting polypeptide in the cell from an expression cassette that is present in the cell and is heterologous to the cell, wherein the expression cassette comprises a promoter operably linked to a polynucleotide encoding the Cas9-inhibiting polypeptide. In some embodiments, the promoter is an inducible promoter and the introducing of the Cas9-inhibiting polypeptide comprises contacting the cell with an agent that induces expression of the Cas9-inhibiting polypeptide. In some embodiments, the introducing of the Cas9-inhibiting polypeptide comprises introducing an RNA encoding the Cas9-inhibiting polypeptide into the cell and expressing the Cas9-inhibiting polypeptide in the cell from the RNA. In some embodiments, the introducing of the Cas9-inhibiting polypeptide comprises inserting the Cas9-inhibiting polypeptide into the cell or contacting the cell with the Cas9-inhibiting polypeptide.

[0009] In some embodiments of the method, the cell is a eukaryotic cell. In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is a human cell. In some embodiments, the cell is a blood cell or an induced pluripotent stem cell. In some embodiments, the method occurs *ex vivo*. In some such embodiments, the cells are introduced into a mammal after the introducing of the Cas9-inhibiting polypeptide, and optionally after the contacting of the Cas9 polypeptide. In some embodiments, the cells are autologous to the mammal.

[0010] In some embodiments of the method, the cell is a prokaryotic cell. In some such embodiments, the introducing comprises introducing a polynucleotide encoding the Cas9-inhibiting polypeptide into the cell using bacteriophage, and expressing the Cas9-inhibiting polypeptide in the cell from the polynucleotide. In some embodiments of any of the herein-described methods, the Cas9 polypeptide is SpyCas9, Efa1Cas9, or Efa3Cas9.

[0011] In another aspect, the present disclosure provides a cell comprising a Cas9-inhibiting polypeptide, wherein the Cas9-inhibiting polypeptide is heterologous to the cell

and the Cas9-inhibiting polypeptide is substantially (e.g., at least about 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%) identical to any one or more of SEQ ID NOS: 1-8.

[0012] In some embodiments, the cell is a eukaryotic cell. In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is a human cell. In some embodiments, the cell is a prokaryotic cell.

[0013] In another aspect, the present disclosure provides a polynucleotide comprising a nucleic acid encoding a Cas9-inhibiting polypeptide, wherein the Cas9-inhibiting polypeptide is substantially (e.g., at least about 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%) identical to any one or more of SEQ ID NOS: 1-8.

[0014] In some embodiments, the Cas9-inhibiting polypeptide inhibits one or more Cas9 polypeptides selected from the group consisting of SpyCas9, Efa1Cas9, and Efa3Cas9. In some embodiments, the polynucleotide is RNA. In some embodiments, the polynucleotide is DNA.

[0015] In another aspect, the present disclosure provides an expression cassette comprising any of the herein-described polynucleotides encoding a Cas9-inhibiting polypeptide, operably linked to a promoter. In some embodiments, the promoter is heterologous to the polynucleotide encoding the Cas9-inhibiting polypeptide. In some embodiments, the promoter is inducible.

[0016] In another aspect, the present disclosure provides a vector comprising any of the herein-described expression cassettes. In some embodiments, the vector is a viral vector.

[0017] In another aspect, the present disclosure provides a bacteriophage comprising any of the herein-described expression cassettes.

[0018] In another aspect, the present disclosure provides an isolated Cas9-inhibiting polypeptide, wherein the Cas9-inhibiting polypeptide is substantially (e.g., at least about 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%) identical to any one or more of SEQ ID NOS:1-8. In some embodiments, the Cas9-inhibiting polypeptide inhibits one or more Cas9 polypeptides selected from the group consisting of SpyCas9, Efa1Cas9, and Efa3Cas9.

[0019] In another aspect, the present disclosure provides a pharmaceutical composition comprising any of the herein-described Cas9-inhibiting polypeptides or polynucleotides encoding a Cas9-inhibiting polypeptide.

[0020] In another aspect, the present disclosure provides a delivery vehicle comprising any of the herein-described Cas9-inhibiting polypeptides or polynucleotides encoding a Cas9-inhibiting polypeptide. In some embodiments, the delivery vehicle is a liposome or nanoparticle.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] **FIGS. 1A-1C**. Identification of four new Type II-A inhibitors, AcrIIA16-19. **FIG. 1A**: Schematic representation of Type II-A acr genes, with vertical arrows indicating relationships between acr loci and percent protein sequence identity. Numbers in genes correspond to AcrIIA number. Grey genes are proteins of unknown function that tested negative for AcrIIA activity. **FIG. 1B**: Schematic of phage plaque assays to assess CRISPR-SpyCas9 inhibition. 10-fold serial dilutions of targeted phage (black circles) are spotted on a lawn of *P. aeruginosa* (grey background) expressing the Type II-A CRISPR-Cas system and indicated acr genes. CRISPR strength is determined by expression of sgRNA from the chromosome (low), or from a multicopy plasmid at increasing induction levels [0.1, 1, 10 mM IPTG]. ΔCRISPR lacks a phage-targeting sgRNA. EV, empty vector. **FIG. 1C**: Schematic of CRISPRi to assess AcrIIA inhibition of dCas9 binding to target DNA. Chromosomally-integrated dCas9 (yellow asterisks) in *P. aeruginosa* programmed to bind the phzM gene promoter with sgRNA expressed from a multicopy plasmid at low or medium IPTG induction levels, in the presence of indicated AcrIIA proteins. CRISPRi inhibition was assessed by quantification of pyocyanin levels in response to dCas9 phzM gene repression, relative to ΔCRISPR. Representative pictures of at least three biological replicates at medium CRISPR strength are shown (bottom).

[0022] **FIGS 2A-2C**. Prevalence of acrIIA genes in integrative mobile genetic elements and their effect on CRISPR-targeting during conjugation. **FIG 2A**: Left: Host distribution of acrIIA16-19 based on phylogenetic analysis, see **FIG. 5A**. Right: Mobile genetic element distribution of acrIIA16-19 based on genomic neighbors characteristic of phage or plasmid genes. Unknown/Host denote genomic regions that could not be identified as either phage or plasmid-like elements. **FIG. 2B**: Schematic of conjugation in *E. faecalis* encoding a Type II-A CRISPR system that targets the protospacer-bearing plasmid in the presence of indicated acrIIA genes episomally expressed in recipient cells. Conjugation frequency is quantified as transconjugants per donor relative to a non-targeted plasmid. **FIG. 2C**: Schematic of plasmid

conjugation in *E. faecalis* from a donor to recipient. The conjugating plasmid carries the indicated acrIIA gene and is targeted by the host's Type II-A CRISPR-Cas system.

[0023] **FIGS. 3A-3D**. *In vitro* binding and inhibition activities of AcrIIA16-19 against SpyCas9. **FIG. 3A**: Time courses of SpyCas9 cleavage reactions targeting a double-stranded linear DNA template in the presence of purified Acr proteins. (L) 1 kb dsDNA ladder, (–) DNA template alone. **FIG. 3B**: Immunoprecipitation (IP) of Myc-tagged SpyCas9-sgRNA. Left: Immunoblot probed with α-Myc (top), α-GST (middle), and α-*E. coli* RNA polymerase β as a loading control (bottom). Image is cropped to show only the bands corresponding to full-length SpyCas9, see **FIG. 7B** for uncropped version. Right: SDS-PAGE analysis and Coomassie staining. **FIG. 3C**: Time courses of target DNA cleavage reactions using SpyCas9 co-immunoprecipitated with AcrIIA-proteins from **FIG. 3B**. Top band present in EV, AcrIIA14, 15 and 16 lanes are co-purifying nucleic acid contaminants. (L) 1kb dsDNA ladder, (–) DNA template alone. **FIG. 3D**: Immunoprecipitation (IP) of GST-Acr proteins in the presence of Myc-tagged SpyCas9 either sgRNA-bound (left) or Apo- without sgRNA (right). Immunoblot for Myc-Cas9 (top) or GST-Acr (bottom).

[0024] **FIGS. 4A-4B**. Schematic of acr loci and lethal self-genome cleavage assay. **FIG. 4A**: Full schematic of acr loci with relevant neighboring genes displayed. **FIG. 4B**: Schematic of SpyCas9 in *P. aeruginosa* programmed to cause lethal self-genome cleavage to assess bacterial survival in the presence of AcrIIA proteins. CRISPR strength is determined by titrating levels of IPTG, which induces expression of sgRNA targeting the chromosomal phzM gene from a multicopy plasmid.

[0025] **FIGS. 5A-5D**. Anti-CRISPR distribution in integrative mobile genetic elements across bacterial taxa. Phylogenetic analysis of acrIIA16-19 homologs (**FIG. 5A** to **5D**, respectively) reconstructed from a midpoint rooted minimum-evolution of full-length protein sequences identified following an iterative PSI-BLASTp search. Branches are labeled with species name and colored according to species class (see legend). Species for which AcrIIA homologs have been tested in this study are shown in bold.

[0026] **FIGS. 6A-6D**. AcrIIA enhance conjugation-mediated horizontal gene transfer in *E. faecalis*; related to **FIG. 2**. **FIG. 6A**: Schematic of the native CRISPR-Cas system in *E. faecalis* strains OG1RF for CRISPR1 and T11RF for CRISPR3 utilized for all conjugation experiments. Black diamonds denote spacers in the CRISPR array and red indicates spacer that match the protospacer in the targeted plasmids. **FIGS. 6B, 6C**: Mating outcomes during

plasmid conjugation of a targeted plasmid from donor to recipient cells where indicated acrIIA genes are (**FIG. 6B**) pre-expressed in recipient cells, or (**FIG. 6C**) encoded on conjugating plasmid. Data displayed as 10-fold colony serial dilution spots of donor, recipient or transconjugant cells on selective antibiotic plates. **FIG. 6D**: Schematic of *E. faecalis* conjugation of protospacer and acrIIA-bearing plasmid transferring into CRISPR-defective recipients. For CRISPR1, the bona fide AcrIIA4 is utilized to suppress CRISPR-targeting, and a ΔCas9 strain from previously reported work is used for CRISPR3 (Price et al., 2016). Red * denotes plasmids that have lost conjugation ability.

[0027] **FIGS. 7A-7C**. AcrIIA16-19 biochemical analysis, related to **FIG. 3**. **FIG. 7A**: Coomassie-stained polyacrylamide gel showing AcrIIA proteins purified from *E. coli*. AcrIIA proteins are eluted from Heparin or Ni-NTA columns as indicated and fractionated by SEC. **FIG. 7B**: Uncropped version of **FIG. 3B**, displaying all fragments of SpyCas9 present and both Myc and GST pulldowns. **FIG. 7C**: Immunoblot of Myc and GST pulldowns from *P. aeruginosa* expressing GST-tagged AcrIIA proteins and Myc-tagged Apo-SpyCas9.

DETAILED DESCRIPTION

1. Introduction

[0028] The present disclosure provides new polypeptide inhibitors of Cas9 nuclease ("Cas9-inhibiting polypeptides"), and methods of using the Cas9-inhibiting polypeptides, that have been identified from plasmids and other conjugative elements in *Firmicutes* bacteria. These Cas9-inhibiting polypeptides are designated AcrIIA16, AcrIIA17, AcrIIA18, and AcrIIA19. AcrIIA16 corresponds, e.g., to SEQ ID NOS: 1 and 2 (showing AcrIIA16 from *Listeria monocytogenes* and *Enterococcus faecalis*, respectively); AcrIIA17 corresponds to, e.g., SEQ ID NOS: 3 and 4 (showing AcrIIA17 from *Enterococcus faecalis* and *Streptococcus gallolyticus*, respectively); AcrIIA18 corresponds to, e.g., SEQ ID NOS: 5 and 6 (showing AcrIIA18 from *Streptococcus macedonicus* and *Streptococcus gallolyticus*, respectively); and AcrIIA19 corresponds to, e.g., SEQ ID NO: 7 and 8 (showing AcrIIA19 from *Staphylococcus simulans* and *Staphylococcus pseudintermedius*, respectively).

[0029] The Cas9-inhibiting polypeptides described herein possess a wide range of inhibition capacity, inhibiting, for example, one or more of SpyCas9 (i.e., Cas9 from *Streptococcus pyogenes*), CRISPR1 from *Enterococcus* (Efa1Cas9), and CRISPR3 from *Enterococcus* (Efa3Cas9), and as such can be used to regulate multiple different Cas9

proteins, including those often used for gene editing. For example, the proteins can be used as broad-sectrum inhibitors, providing a single option for providing a Cas9 "off-switch" *in vivo*.

[0030] The present polypeptides can be used in numerous ways to inhibit unwanted Cas9 activity. For example, the proteins can be used to limit excess Cas9 nuclease activity and thereby enhance the specificity of Cas9. They can be used to protect organisms against Cas9-mediated genome manipulations in the wild, such as gene drives. The proteins can also be used to reduce virulence of infectious pathogens that possess functional CRISPR-Cas9 systems. The proteins are also useful for engineering into phage therapeutics to enhance their potency. These and other uses and features of the proteins are described in more detail elsewhere herein.

2. Definitions

[0031] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0032] The terms "a," "an," or "the" as used herein not only include aspects with one member, but also include aspects with more than one member. For instance, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the protein" includes reference to one or more proteins known to those skilled in the art, and so forth.

[0033] The terms "about" and "approximately" as used herein shall generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Typically, exemplary degrees of error are within 20 percent (%), preferably within 10%, and more preferably within 5% of a given value or range of values. Any reference to "about X" specifically indicates at least the values X, 0.8X, 0.81X, 0.82X, 0.83X, 0.84X, 0.85X, 0.86X, 0.87X, 0.88X, 0.89X, 0.9X, 0.91X, 0.92X, 0.93X, 0.94X, 0.95X, 0.96X, 0.97X, 0.98X, 0.99X, 1.01X, 1.02X, 1.03X, 1.04X, 1.05X, 1.06X, 1.07X, 1.08X, 1.09X, 1.1X, 1.11X, 1.12X, 1.13X, 1.14X, 1.15X, 1.16X, 1.17X, 1.18X, 1.19X, and 1.2X. Thus, "about X" is intended to teach and provide written description support for a claim limitation of, *e.g.*, "0.98X."

[0034] The term "nucleic acid" or "polynucleotide" refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded

form. Unless specifically limited, the term encompasses nucleic acids containing known analogs of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); and Rossolini *et al.*, *Mol. Cell. Probes* **8**:91-98 (1994)).

[0035] "AcrIIA16" refers to a Cas9 inhibitor protein, e.g., a protein comprising the amino acid sequence shown as SEQ ID NO:1 or SEQ ID NO:2, or a protein comprising an amino acid sequence substantially identical to SEQ ID NO:1 or SEQ ID NO:2, e.g., a protein comprising 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more identity to SEQ ID NO: 1 or SEQ ID NO:2, or variants, derivatives, or fragments of any of these proteins. AcrIIA16 proteins can be from any source, and can bind to and/or inhibit Cas9 proteins, including, e.g., SpyCas9, Efa1Cas9, Efa3Cas9, and others, e.g., as assessed *in vitro*, in prokaryotic or eukaryotic cells, or *in vivo*. AcrIIA16 can refer to an AcrIIA16 protein from any organism, e.g., *Listeria monocytogenes* (IIA16-Lmo, e.g., SEQ ID NO: 1 or Accession no. WP_061665674.1) or *Enterococcus faecalis* (IIA16-Efa; e.g., SEQ ID NO: 2 or Accession no. WP_025188019.1).

[0036] "AcrIIA17" refers to a Cas9 inhibitor protein, e.g., a protein comprising the amino acid sequence shown as SEQ ID NO:3 or SEQ ID NO:4, or a protein comprising an amino acid sequence substantially identical to SEQ ID NO:3 or SEQ ID NO:4, e.g., a protein comprising 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more identity to SEQ ID NO: 3 or SEQ ID NO:4, or variants, derivatives, or fragments of any of these proteins. AcrIIA17 proteins can bind to and/or inhibit Cas9 proteins, including, e.g., SpyCas9, Efa1Cas9, Efa3Cas9, and others, e.g., as assessed *in vitro*, in prokaryotic or eukaryotic cells, or *in vivo*. AcrIIA17 can refer to an AcrIIA17 from any organism, e.g., *Enterococcus faecalis* (IIA17-Efa; e.g., SEQ ID NO: 3 or Accession no. WP_002401839.1) or *Streptococcus gallolyticus* (IIA17-Sga; e.g., SEQ ID NO: 4 or Accession no. WP_074626943.1).

[0037] AcrIIA18 refers to a Cas9 inhibitor protein, e.g., a protein comprising the amino acid sequence shown as SEQ ID NO:5 or SEQ ID NO:6, or a protein comprising an amino acid sequence substantially identical to SEQ ID NO:5 or SEQ ID NO:6, e.g., a protein comprising 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more identity to SEQ ID NO:5 or SEQ ID NO:6, or variants, derivatives, or fragments of any of these proteins. AcrIIA18 proteins can be from any source, and can bind to and/or inhibit Cas9 proteins, including, e.g., SpyCas9, Efa1Cas9, Efa3Cas9, and others, e.g., as assessed *in vitro*, in prokaryotic or eukaryotic cells, or *in vivo*. AcrIIA18 can refer to an AcrIIA18 from any organism, e.g. *Streptococcus macedonicus* (IIA18-Sma; e.g., SEQ ID NO: 5 or Accession no. WP_099390844.1) or *Streptococcus gallolyticus* (IIA18-Sga; e.g., SEQ ID NO: 6 or Accession no. WP_074627086.1).

[0038] AcrIIA19 refers to a Cas9 inhibitor protein, e.g., a protein comprising the amino acid sequence shown as SEQ ID NO:7 or SEQ ID NO:8, or a protein comprising an amino acid sequence substantially identical to SEQ ID NO:7 or SEQ ID NO:8, e.g., a protein comprising 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more identity to SEQ ID NO:7 or SEQ ID NO:8, or variants, derivatives, or fragments of any of these proteins. AcrIIA19 proteins can be from any source, and can bind to and inhibit Cas9 proteins, including, e.g., SpyCas9, Efa1Cas9, Efa3Cas9, and others, e.g., as assessed *in vitro*, in prokaryotic or eukaryotic cells, or *in vivo*. AcrIIA19 can refer to an AcrIIA19 from any organism, e.g. *Staphylococcus simulans* (IIA19-Ssim; e.g., SEQ ID NO: 7 or Accession no. WP_107591702.1) or *Staphylococcus pseudintermedius* (IIA19-Spse; e.g., SEQ ID NO: 8 or Accession no. WP_100006909.1).

[0039] The term “gene” means the segment of DNA involved in producing a polypeptide chain. It may include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

[0040] A "promoter" is defined as an array of nucleic acid control sequences that direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of transcription. The promoter can be a heterologous promoter.

[0041] An "expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular polynucleotide sequence in a host cell. An expression cassette may be part of a plasmid, viral genome, or nucleic acid fragment. Typically, an expression cassette includes a polynucleotide to be transcribed, operably linked to a promoter. The promoter can be a heterologous promoter. In the context of promoters operably linked to a polynucleotide, a "heterologous promoter" refers to a promoter that would not be so operably linked to the same polynucleotide as found in a product of nature (*e.g.*, in a wild-type organism).

[0042] "Polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. All three terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms encompass amino acid chains of any length, including full-length proteins, wherein the amino acid residues are linked by covalent peptide bonds.

[0043] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, "conservatively modified variants" refers to those nucleic acids that encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein that encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence.

[0044] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention. In some cases, conservatively modified variants of Cas9 or sgRNA can have an increased stability, assembly, or activity as described herein.

[0045] The following eight groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Glycine (G);

2) Aspartic acid (D), Glutamic acid (E);

3) Asparagine (N), Glutamine (Q);

4) Arginine (R), Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);

7) Serine (S), Threonine (T); and

8) Cysteine (C), Methionine (M)

(*see, e.g.*, Creighton, *Proteins*, W. H. Freeman and Co., N. Y. (1984)).

[0046] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0047] In the present application, amino acid residues are numbered according to their relative positions from the left-most residue, which is numbered 1 in an unmodified wild-type polypeptide sequence.

[0048] As used in herein, the terms "identical" or percent "identity," in the context of describing two or more polynucleotide or amino acid sequences, refer to two or more

sequences or specified subsequences that are the same. Two sequences that are "substantially identical" have at least 60% identity, preferably 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity, when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using a sequence comparison algorithm or by manual alignment and visual inspection where a specific region is not designated. With regard to polynucleotide sequences, this definition also refers to the complement of a test sequence. With regard to amino acid sequences, in some cases, the identity exists over a region that is at least about 50 amino acids or nucleotides in length, or more preferably over a region that is 75-100 amino acids or nucleotides in length.

[0049] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters. For sequence comparison of nucleic acids and proteins, the BLAST 2.0 algorithm and the default parameters discussed below are used.

[0050] A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

[0051] An algorithm for determining percent sequence identity and sequence similarity is the BLAST 2.0 algorithm, which are described in Altschul *et al.*, (1990) *J. Mol. Biol.* 215: 403-410. Software for performing BLAST analyses is publicly available at the National Center for Biotechnology Information website, ncbi.nlm.nih.gov. The algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word

hits acts as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a word size (W) of 28, an expectation (E) of 10, M=1, N=-2, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word size (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)).

[0052] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0053] The "CRISPR-Cas" system refers to a class of bacterial systems for defense against foreign nucleic acid. CRISPR-Cas systems are found in a wide range of eubacterial and archaeal organisms. CRISPR-Cas systems include type I, II, III, V, and VI sub-types. Wild-type type II CRISPR-Cas systems utilize the RNA-mediated nuclease, Cas9 in complex with guide and activating RNA to recognize and cleave foreign nucleic acid.

[0054] Cas9 homologs are found in a wide variety of eubacteria, including, but not limited to bacteria of the following taxonomic groups: *Actinobacteria*, *Aquificae*, *Bacteroidetes-Chlorobi*, *Chlamydiae-Verrucomicrobia*, *Chlroflexi*, *Cyanobacteria*, *Firmicutes*, *Proteobacteria*, *Spirochaetes*, and *Thermotogae*. An exemplary Cas9 polypeptide is the

Streptococcus pyogenes Cas9 polypeptide (SpyCas9). Additional Cas9 proteins and homologs thereof are described in, *e.g.*, Chylinksi, *et al.*, *RNA Biol*. 2013 May 1; 10(5): 726–737 ; *Nat. Rev. Microbiol*. 2011 June; 9(6): 467-477; Hou, *et al.*, *Proc Natl Acad Sci USA* (2013) Sep 24;110(39):15644-9; Sampson *et al.*, *Nature*. 2013 May 9;497(7448):254-7; and Jinek, *et al.*, *Science*. 2012 Aug 17;337(6096):816-21. The Cas9 protein can be nuclease defective. For example, the Cas9 protein can be a nicking endonuclease that nicks target DNA, but does not cause double strand breakage. Cas9 can also have both nuclease domains deactivated to generate "dead Cas9" (dCas9), a programmable DNA-binding protein with no nuclease activity. In some embodiments, dCas9 DNA-binding is inhibited by the polypeptides described herein.

3. Cas9 inhibitors

[0055] As set forth in the present disclosure, including the examples and sequence listing, a number of Cas9-inhibiting polypeptides have been discovered and are provided herein. Examples of exemplary Cas9-inhibiting polypeptides include proteins comprising an amino acid sequence selected from any of SEQ ID NOs: 1-8 or a fragment thereof, or an amino acid sequence substantially (e.g., at least about 50, 60, 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99%) identical to any of SEQ ID NOS: 1-8 or a fragment thereof. In some embodiments, the polypeptides, in addition to having one of the above-listed sequences, will include other amino acid sequences or other chemical moieties (e.g., detectable labels) at the amino terminus, carboxyl terminus, or both. Additional amino acid sequences can include, but are not limited to tags, detectable markers, or nuclear localization signal sequences. In some embodiments, the Cas9-inhibiting polypeptides inhibit one or more Cas9 polypeptides selected from the group consisting of SpyCas9, Efa1Cas9, and Efa3Cas9. In some embodiments, the Cas9-inhibiting polypeptide is an AcrIIA16 polypeptide. In some embodiments, the Cas9-inhibiting polypeptide is an AcrIIA17 polypeptide. In some embodiments, the Cas9-inhibiting polypeptide is an AcrIIA18 polypeptide. In some embodiments, the Cas9-inhibiting polypeptide is an AcrIIA19 polypeptide.

[0056] As used herein, a "Cas9-inhibiting polypeptide" refers to a protein that can inhibit the binding or activity of a Cas9 protein (including dCas9) through any mechanism, e.g., by inhibiting the formation or stability of a CRISPR-Cas complex (i.e., Cas9 with a guide RNA), by inhibiting its binding to a target DNA, or by inhibiting cleavage of the target DNA. A Cas9-inhibiting polypeptide could inhibit any of these activities by, e.g., 10%, 25%, 50%,

75%, 90%, or more. The function of the Cas9 protein can be assessed in one or more assays or systems, including *in vitro* (e.g., inhibiting Cas9 nuclease or DNA-binding activity) or in cells. For example, a Cas9 inhibiting polypeptide can be used to inhibit a heterologous Cas9, e.g., SpyCas9 in *Pseudomonas aeruginosa*, against bacteriophage challenge or in a self-targeting tolerance assay. They can also be used to inhibit Cas9 activity in a natural host such as *Enterococcus*. They can also be used to reduce gene editing by various Cas9 orthologs in human cell lines.

[0057] In some embodiments, the Cas9 inhibiting activity of an inhibitor is assayed in a bacteriophage plaque assay. When cells expressing Cas9 and a guide RNA are infected by bacteriophages bearing a targeted DNA sequence and protospacer adjacent motif (PAM), the infection event is prevented by Cas9, limiting the emergence of bacteriophage replicative plaques. This is compared to a bacteriophage lacking the targeted DNA sequence and to a bacteriophage infecting a strain expressing a non-targeting guide RNA, which produces normal sized colonies when used to transform the same strain. The expression of a Cas9 inhibitor, however, neutralizes Cas9 activity and leads to bacteriophage plaques. While it is believed the Cas9-inhibiting polypeptides' inhibitory activity can be measured in other ways, the above assay, presented in more detail in the Examples, is the assay for determining whether the Cas9-inhibiting polypeptide has activity.

[0058] **Table 1A** presents the amino acid sequences and accession numbers of the present Cas9-inhibiting polypeptides, and, as shown in **Table 1B**, the present Cas9-inhibiting polypeptides show a broad spectrum of activity and can inhibit a range of Cas9 proteins, including SpyCas9 (from *Streptococcus pyogenes*) and EfaCas9 from *Enterococcus*, both the CRISPR1 (SpyCas9-like) and the CRISPR3 (SauCas9-like) systems. These Cas9 families include the main families being used in human gene editing therapeutic applications. It is believed and expected that the Cas9-inhibiting polypeptides described herein will also similarly inhibit other Cas9 proteins. As such, due to their broad specificity, a single or reduced number of the present broad spectrum inhibitors could be used as a single option for gene editing "off switches" *in vivo*. Such an ability provides a significant improvement over current known inhibitors of Cas9, which are restricted to specific subtypes and would thus need to be used in combination in order to provide broad Cas9 inhibition. In particular embodiments of the invention, an AcrIIA16Lmo, AcrIIA17Efa, AcrIIA17Sga, or AcrIIA19Ssim polypeptide is used to provide broad spectrum inhibition of multiple Cas9 proteins *in vivo*, *ex vivo*, or *in vitro*.

Table 1A. Anti-CRISPR sequences

Anti-CRISPR	Strain	AA sequence	Accession Number	SEQ ID NO:
IIA16-Lmo	*Listeria monocytogenes*	MGYIGTKRSERSQDAIEDYEVPLNHFNKDLIQAFIDENEAYDT LKTKKVRLWKFVAPRAGATSWHHTGTYYNKTDHYSLEKVAD ELLQNGDEWEEQFKAYVKEEQETATSEPVFLSVIKVQIWGGS MKRPKLVGHEVVMGVKKEGWLHAVSKATQSKYKLSANKVE MQKHYSLEDYSALTKDFPEFKAQKRAINKKMKEMYN	WP_061665674.1	1
IIA16-Efa	*Enterococcus faecalis*	MGYVGKSRSVRSQIAIDNAEVPLNHITKDYILTFVTENNIDETL KNESVAMWKFVAKRHGSTSWHHVSKHYNKIDHYDLHDVAE YFSMNYDSLKNDYQNLLDQKRQAKNDLIKNLKLGIIKVQIWG GTKRYPKLEGYESVMGVVKDGWLHTVTLSNQTKYKITGNKIE EITIFELDQYDILTKKFPEFRAMKRKINKEVARLSK	WP_025188019.1	2
IIA17-Efa	*Enterococcus faecalis*	MAILNNKGEKISIDCADLISEVEEDILIFGGTFLVYAICSWREIE QVEYISDYVHADNPESYKDELTTKEYAELKEIYEKDLEELKITKN KQMNLNELLSILTIQNSIT	WP_002401839.1	3
IIA17-Sga	*Streptococcus gallolyticus*	MKISVDSEKLLNEAINDFDIFGEDFNVYAIYSYREDYDFEYISDY VDADEPTRDEFETEEDYQEVMKDFKENLDSLKFTKHKKMTIA DLVHELWEQNRIF	WP_074626943.1	4
IIA18-Sma	*Streptococcus macedonicus*	MKIDTTVTEVKENGKTYLRLLKGNEQLKAVSDKAVAGVNLFP GAKIGSFLVRQDNIVVFPDNKGEFDLDFFNLLNDNFETLVEYA KMADCLDIAFDINEKSYFNMIMWLMKNIDENWSQSPYGESF YSSKDIDWGYKPEGSLRVSDHWNFGQDGEHCPTAEPVDGW AVCKFENGKYHLIKKF	WP_099390844.1	5
IIA18-Sga	*Streptococcus gallolyticus*	MKIDTTVTEVKENGKTYLRLVEGTEQLKAISDKAMAGVNLFP GAKIDSFLVKQDSIVVFPDNKGEFDLDFFKQLDENFDTIAKYA RVATCFEEVAFDEKSYFNMIMWLMDNMDENWSQSPYGES FYSSKNIDWGYKPEGSLRVSDHWNFGENGEHCPTAEPVDG WAVCKFENGKYHLIKKF	WP_074627086.1	6
IIA19-Ssim	*Staphylococcus simulans*	MKLIVEVEETNYKNLVNYTKLTNESHNILVNRLISEYITKPYELR LDLSERYSNRDLIEFKFMLIEYCKEALQDIKELANSDEAYETDEA FEAVFRQLFEEVISNPDTVLKAFHSYTSFLEENK	WP_107591702.1	7
IIA19-Spse	*Staphylococcus pseudintermedius*	MKLIINIEDKNYKYLTELAQQDNTNIGSIVNNLIQTHITDVNES YRSVDKKELDEFSRVMQHYFHEDLASMYDVIGSDEELSTDKQ MLKVYKKLYQDVALRNGIALELFNAYKKG	WP_100006909.1	8

Table 1B. Summary of Anti-CRISPR activity

Anti-CRISPR	Inhibits Cas9	Inhibits SpCas9 in *P. aeruginosa* heterolo-gous system	Inhibits EfaCas9 in *E. faecalis* native system: CRISPR1 (Spy-like)	Inhibits EfaCas9 in *E. faecalis* native system: CRISPR3 (Sau-like)
IIA16-Lmo	Y	Y	Y	Y
IIA16-Efa	Y	ND	ND	ND
IIA17-Efa	Y	Y	Y	Y
IIA17-Sga	Y	Y	Y	Y
IIA18-Sma	Y	Y	ND	ND

IIA18-Sga	Y	ND	ND	ND
IIA19-Ssim	Y	Y	Y	Y
IIA19-Spse	Y	ND	ND	ND

ND: Not determined

4. Introduction into cells

[0059] The present disclosure provides methods of inhibiting a Cas9-polypeptide in a cell, comprising introducing any of the herein-described Cas9-inhibiting polypeptides into the cell, wherein the Cas9-inhibiting polypeptide is heterologous to the cell and is substantially (e.g., at least about 60%, 70%, 80%, 90%, 95%) identical to any one or more of the sequences shown as SEQ ID NOS: 1-8, or a fragment thereof. In some embodiments, the Cas9-inhibiting polypeptide comprises a sequence selected from SEQ ID NOS: 1-8, or a fragment thereof. In some embodiments, the polypeptide comprises a sequence selected from the group consisting of SEQ ID NO: 1, 2, 4, and 7. In some embodiements, the Cas9-inhibiting polypeptide can inhibit one or more Cas9-inhibiting polypeptides selected from the gropu consisting of SpyCas9, Efa1Cas9, and Efa3Cas9.

[0060] The Cas9-inhibiting polypeptides can be introduced into any prokaryotic or eukaryotic cell to inhibit Cas9 in that cell. In some embodiments, the cell contains Cas9 protein when the Cas9-inhibiting polypeptide is introduced into the cell. In other embodiments, the Cas9-inhibiting polypeptide is introduced into the cell and then Cas9 polypeptide is introduced into the cell.

[0061] Introduction of the Cas9-inhibiting polypeptides into the cell can take different forms. For example, in some embodiments, the Cas9-inhibiting polypeptides themselves are introduced into the cells. Any method for introduction of polypeptides into cells can be used. For example, in some embodiments, electroporation, or liposomal or nanoparticle delivery to the cells can be employed. In other embodiments, a polynucleotide encoding a Cas9-inhibiting polypeptide is introduced into the cell and the Cas9-inhibiting polypeptide is subsequently expressed in the cell. In some embodiments, the polynucleotide is an RNA. In some embodiments, the polynucleotide is a DNA.

[0062] In some embodiments, the Cas9-inhibiting polypeptide is expressed in the cell from RNA encoded by an expression cassette, wherein the expression cassette comprises a promoter operably linked to a polynucleotide encoding the Cas9-inhibiting polypeptide. In

some embodiments, the promoter is heterologous to the polynucleotide encoding the Cas9-inhibiting polypeptide. Selection of the promoter will depend on the cell in which it is to be expressed and the desired expression pattern. In some embodiments, promoters are inducible or repressible, such that expression of a nucleic acid operably linked to the promoter can be expressed under selected conditions. In some examples, a promoter is an inducible promoter, such that expression of a nucleic acid operably linked to the promoter is activated or increased. Accordingly, the present disclosure provides expression cassettes comprising a polynucleotide encoding any of the herein-described Cas9-inhibiting proteins, operably linked to a promoter.

[0063] An inducible promoter may be activated by the presence or absence of a particular molecule, for example, doxycycline, tetracycline, metal ions, alcohol, or steroid compounds. In some embodiments, an inducible promoter is a promoter that is activated by environmental conditions, for example, light or temperature. In further examples, the promoter is a repressible promoter such that expression of a nucleic acid operably linked to the promoter can be reduced to low or undetectable levels, or eliminated. A repressible promoter may be repressed by direct binding of a repressor molecule (such as binding of the trp repressor to the trp operator in the presence of tryptophan). In a particular example, a repressible promoter is a tetracycline repressible promoter. In other examples, a repressible promoter is a promoter that is repressible by environmental conditions, such as hypoxia or exposure to metal ions.

[0064] In some embodiments, the polynucleotide encoding the Cas9-inhibiting polypeptide (e.g., as part of an expression cassette) is delivered to the cell by a vector. For example, in some embodiments, the vector is a viral vector. Exemplary viral vectors can include, but are not limited to, adenoviral vectors, adeno-associated viral (AAV) vectors, and lentiviral vectors. Accordingly, the present disclosure provides vectors comprising any of the herein-described polynucleotides or expression vectors.

[0065] In some embodiments, the Cas9-inhibiting polypeptide or a polynucleotide encoding the Cas9-inhibiting polypeptide is delivered as part of or within a cell delivery system. Various delivery systems are known and can be used to administer a composition of the present disclosure, for example, encapsulation in liposomes, microparticles, microcapsules, or receptor-mediated delivery.

[0066] Exemplary liposomal delivery methodologies are described in Metselaar *et al.*, *Mini Rev. Med. Chem.* 2(4):319-29 (2002); O'Hagen *et al.*, *Expert Rev. Vaccines* 2(2):269-83

(2003); O'Hagan, *Curr. Drug Targets Infect. Disord.* 1(3):273-86 (2001); Zho *et al.*, *Biosci Rep.* 22(2):355-69 (2002); Chikh *et al.*, *Biosci Rep.* 22(2):339-53 (2002); Bungener *et al.*, *Biosci. Rep.* 22(2):323-38 (2002); Park, *Biosci Rep.* 22(2):267-81 (2002); Ulrich, *Biosci. Rep.* 22(2):129-50; Lofthouse, *Adv. Drug Deliv. Rev.* 54(6):863-70 (2002); Zhou *et al.*, *J. Inmunmunother.* 25(4):289-303 (2002); Singh *et al.*, *Pharm Res.* 19(6):715-28 (2002); Wong *et al.*, *Curr. Med. Chem.* 8(9):1123-36 (2001); and Zhou *et al.*, *Immunonmethods* (3):229-35 (1994).

[0067] Exemplary nanoparticle delivery methodologies, including gold, iron oxide, titanium, hydrogel, and calcium phosphate nanoparticle delivery methodologies, are described in Wagner and Bhaduri, *Tissue Engineering* 18(1): 1-14 (2012) (describing inorganic nanoparticles); Ding *et al.*, *Mol Ther e-pub* (2014) (describing gold nanoparticles); Zhang *et al.*, Langmuir 30(3):839-45 (2014) (describing titanium dioxide nanoparticles); Xie *et al.*, *Curr Pharm Biotechnol* 14(10):918-25 (2014) (describing biodegradable calcium phosphate nanoparticles); and Sizovs *et al.*, *J Am Chem Soc* 136(1):234-40 (2014).

[0068] Introduction of a Cas9-inhibiting polypeptide as described herein into a prokaryotic cell can be achieved by any method used to introduce protein or nucleic acids into a prokaryote. In some embodiments, the Cas9-inhibiting polypeptide is delivered to the prokaryotic cell by a delivery vector (e.g., a bacteriophage) that delivers a polynucleotide encoding the Cas9-inhibiting polypeptide. In some embodiments, inhibiting Cas9 in the prokaryote using a Cas9-inhibiting polypeptide of the invention could either help the phage kill the bacterium or help other phages kill it. In some embodiments, the Cas9-inhibiting polypeptide is introduced by a bacteriophage in the context of phage therapeutics, i.e., the use of bacteriophage to treat pathogenic bacterial infections, and the Cas9-inhibiting polypeptide increases the potency of the bacteriophage by inhibiting Cas9 present in the targeted bacteria.

5. Cells

[0069] A Cas9-inhibiting polypeptide as described herein can be introduced into any cell that contains, expresses, or is expected to express, Cas9. Exemplary cells can be prokaryotic or eukaryotic cells. Exemplary prokaryotic cells can include but are not limited to, those used for biotechnological purposes, the production of desired metabolites, *E. coli* and human pathogens. Examples of such prokaryotic cells can include, for example, *Escherichia coli*, *Pseudomonas* sp., *Corynebacterium* sp., *Bacillus subtitis*, *Streptococcus pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Francisella*

novicida, *Corynebacterium diphtheria*, *Enterococcus* sp., *Listeria monocytogenes*, *Mycoplasma gallisepticum*, *Streptococcus* sp., or *Treponema denticola*. Exemplary eukaryotic cells can include, for example, animal (e.g., mammalian) or plant cells. Exemplary mammalian cells include but are not limited to human, non-human primates. mouse, and rat cells. Cells can be cultured cells or primary cells. Exemplary cell types can include, but are not limited to, induced pluripotent cells, stem cells or progenitor cells, and blood cells, including but not limited to T-cells or B-cells. Accordingly, the present disclosure provides cells comprising any of the herein-described Cas9-inhibiting polypeptides, polynucleotides expression cassettes, or vectors

[0070] In some embodiments, the cells are infectious prokaryotic pathogens that possess functional CRISPR-Cas9, and the Cas9-inhibiting polypeptide is introduced to reduce the virulence of the pathogen. In some embodiments, the infectious pathogens are targeted with bacteriophage, and the Cas9-inhibiting polypeptide is introduced together with the phage to enhance the potency of the phage against the pathogen.

[0071] In some embodiments, the cells are removed from an animal (e.g., a human, optionally in need of genetic repair), and then Cas9, and optionally guide RNAs, for gene editing are introduced into the cell *ex vivo*, and a Cas9-inhibiting polypeptide is introduced into the cell. In some embodiments, the cell(s) is subsequently introduced into the same animal (autologous) or different animal (allogeneic).

[0072] In any of the embodiments described herein, a Cas9 polypeptide can be introduced into a cell to allow for Cas9 DNA binding and/or cleaving (and optionally editing), followed by introduction of a Cas9-inhibiting polypeptide as described herein. This timing of the presence of active Cas9 in the cell can thus be controlled by subsequently supplying Cas9-inhibiting polypeptides to the cell, thereby inactivating Cas9. This can be useful, for example, to reduce Cas9 "off-target" effects such that non-targeted chromosomal sequences are bound or altered. By limiting Cas9 activity to a limited "burst" that is ended upon introduction of the Cas9-inhibiting polypeptide, one can limit off-target effects. In some embodiments, the Cas9 polypeptide and the Cas9-inhibiting polypeptide are expressed from different inducible promoters, regulated by different inducers. These embodiments allow for first initiating expression of the Cas9 polypeptide followed by induction of the Cas9-inhibiting polypeptide, optionally while removing the inducer of Cas9 expression.

[0073] In some embodiments, a Cas9-inhibiting polypeptide as described herein can be introduced (e.g., administered) to an animal (e.g., a human) or plant. This can be used to control *in vivo* Cas9 activity, for example in situations in which CRISPR-Cas9 gene editing was performed *in vivo*, or in circumstances in which an individual is exposed to unwanted Cas9, for example where a bioweapon comprising Cas9 is released.

[0074] In some embodiments, a Cas9-inhibiting polypeptide as described herein can be introduced to an animal (e.g., an insect), plant, or fungus in the context of limiting the extent of a gene drive. Gene drives involve the propagation of a gene or genes through a population or species by increasing the probability that a specific allele or alleles will be transmitted to progeny. CRISPR-Cas9 can be used in gene drives, in which an integrated construct comprises the specific allele that is being propagated and comprises a guide RNA and Cas9 that enable the targeted cleavage of a homologous locus in a cell and the CRISPR-mediated transfer of the specific allele to the homologous locus. Cas9-inhibiting polypeptides could be used, e.g., to protect specific subpopulations or individuals from the effects of a gene drive, or to slow or stop the spread of a gene drive throughout a population.

[0075] Any of a large spectrum of Cas9 proteins can be inhibited by the present Cas9-inhibiting polypeptides. For example, Cas9 from *Streptococcus pyogenes*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Campylobacter jejuni*, *Francisella novicida*, *Streptococcus thermophiles*, and others can be inhibited.

6. Compositions

[0076] In some embodiments, a Cas9-inhibiting polypeptides as described herein or a polynucleotide encoding a Cas9-inhibiting polypeptide as described herein, is administered as a pharmaceutical composition. Accordingly, in some embodiments, the present disclosure provides a composition comprising any of the herein-described Cas9-inhibiting polyptides or polynucleotides encoding any of the herein-described Cas9-inhibiting polypeptide, and a pharmaceutically acceptable carrier. In some embodiments, the present disclosure provides a delivery such as a liposome, nanoparticle or other delivery vehicle as described herein or otherwise known, comprising any of the herein-described Cas9-inhibiting polypeptides or a polynucleotide encoding any of the herein-described Cas9-inhibiting polypeptides. The compositions can be administered directly to a mammal (e.g., human) to inhibit Cas9 using any route known in the art, including *e.g.*, by injection (*e.g.*, intravenous, intraperitoneal,

subcutaneous, intramuscular, or intrademal), inhalation, transdermal application, rectal administration, or oral administration.

[0077] The pharmaceutical compositions of the invention may comprise a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (see, *e.g.*, Remington's Pharmaceutical Sciences, 17th ed., 1989).

7. Examples

[0078] The following examples are offered to illustrate, but not to limit, the claimed invention.

Example 1: Broad-spectrum anti-CRISPR proteins facilitate horizontal gene transfer

Results

Novel Type II-A anti-CRISPRs (AcrIIA16-19) block SpyCas9 binding to DNA

[0079] To identify undiscovered acr genes, we first utilized the widespread acrIIA1 gene as an anchor in bioinformatic searches across genomes on NCBI (**FIG. 1A**). An AcrIIA1 homolog (41% amino acid sequence identity) was previously identified within an *L. monocytogenes* plasmid, along with an AcrIIA2 homolog that was recently characterized (AcrIIA2b.3, Jiang et al., 2019). Genomic neighbors in this locus were tested against the Type II-A Cas9 system using a SpyCas9 phage-targeting screening system in *Pseudomonas aeruginosa* (**FIG. 1B**; Borges et al., 2018; Jiang et al., 2019). Gene AWI79_RS12835 (now acrIIA16) inhibited SpyCas9 in this assay. Similarly, using acrIIA16 as the anchor gene, functional analysis of its neighbors revealed three more distinct anti-CRISPR genes (acrIIA17-19) identified in *Enterococcus, Streptococcus*, and *Staphylococcus* (**FIG. 1A**). To quantify the strength of SpyCas9 inhibition, Cas9 and the sgRNA were titrated via IPTG induction. At the lowest CRISPR-Cas expression level, all identified acrIIA genes inhibited SpyCas9, restoring phage replication to nearly the same levels as in the strain lacking CRISPR immunity (ΔCRISPR, **FIG. 1B**). However, at higher CRISPR-Cas expression levels, only AcrIIA16Lmo, AcrIIA17Sga, and control AcrIIA4 maintained inhibition against SpyCas9 (**FIG. 1B**). In agreement with this result, the AcrIIA proteins also protect against self-genome cleavage assay with similar strength (**FIG. 4B**).

[0080] To inspect the mechanism of these new AcrIIA proteins, we established a CRISPRi assay, where catalytically dead SpyCas9 (dCas9) is programmed to bind the promoter of the phzM gene. Repression of phzM halts the production of green pigment called pyocyanin, generating a yellow culture (Bondy-Denomy et al., 2015). In the presence of AcrIIA4, DNA binding by dCas9 is inhibited generating a green culture. AcrIIA16-19 all presented a similar phenotype at two dCas9 induction levels, suggesting that these new AcrIIAs inhibit SpyCas9 at the step of target DNA binding or another upstream stage (**FIG. 1C**).

acrIIA genes protect plasmids from CRISPR-targeting during conjugation

[0081] Analysis of AcrIIA16-19 distribution revealed that most orthologs are present in diverse conjugative MGEs of *Firmicutes*, with only a small percentage found in phages and other bacterial classes (**FIG. 2A, FIG. 5**). Genes adjacent to the acr loci were examined for presence of prophage or plasmid genes. Interestingly, acrIIA16, 17, and 19 exist primarily in non-phage MGEs including plasmids or ICEs. We reasoned that these Acr proteins could be suited to offer protection to conjugative elements (**FIG. 2A**).

[0082] To investigate AcrIIA activity during plasmid conjugation, we tested the ability of Cas9 to target a plasmid when an AcrIIA protein is expressed either in the recipient or by the conjugating element. Conjugation efficiency (and thus, Cas9 targeting efficiency) was assessed through an antibiotic resistance marker on the conjugative element. Previously reported *E. faecalis* strains (Hullahalli et al., 2017) were used for this assay, with acrIIA genes individually expressed from an *E. faecalis* promoter native to the acr locus. *E. faecalis* encodes two distinct endogenous Type II-A CRISPR-Cas variants – CRISPR1, which is 52% identical to SpyCas9 and CRISPR3, which is ~32% identical to SauCas9 (**FIG. 6A**). When acrIIA16, 17, and 19 were pre-expressed in recipient cells, all inhibited CRISPR1 robustly, and CRISPR3 to a lesser degree (**FIGS. 2B, 6B**). acrIIA4 only inhibited CRISPR1 activity, which encodes a Cas9 that has a similar PAM-interacting domain to SpyCas9 (**FIG. 2B**).

[0083] We then sought to investigate whether AcrIIA proteins could function during plasmid conjugation when acrIIA genes were expressed from the conjugating CRISPR-targeted plasmid. acrIIA16-17 and acrIIA19 were indeed protective against CRISPR1 plasmid targeting when produced during conjugation, while acrIIA17 orthologs provided modest protection against CRISPR3 (**FIGS. 2C, 6C**). Oddly, plasmids expressing certain acr genes did not produce detectable transconjugants (e.g. acrIIA17Efa when challenged with CRISPR1 and acrIIA4/acrIIA19Ssim against CRISPR3), but this was independent of

CRISPR-targeting (**FIG. 6D**), for a reason that is unknown. We conclude that acrIIA genes are able to inhibit both CRISPR-Cas9 systems during plasmid conjugation in *E. faecalis* and can enhance HGT by >1 order of magnitude when pre-expressed in recipient cells.

AcrIIA16-19 proteins interact with SpyCas9

[0084] To further investigate the mechanism of inhibition of the new AcrIIAs, we purified one homolog of AcrIIA16-19 to directly test their effect on SpyCas9 activity (**FIG. 7A**). *In vitro* cleavage experiments revealed that purified AcrIIA16-19 proteins do not inhibit SpyCas9-mediated DNA cleavage, while the positive control AcrIIA2b.3 does (**FIG. 3A**). Given that all the AcrIIA purified proteins did not inhibit SpyCas9 activity *in vitro*, we considered that the cellular environment may be essential for their function. Immunoprecipitation of SpyCas9 from bacteria co-expressing each AcrIIA protein demonstrated that AcrIIA16-19 interact with SpyCas9-sgRNA (**FIG. 3B**). The absence of any other stoichiometric, co-purifying proteins suggests a direct interaction between Cas9 and the Acr proteins (**FIG. 3B**, right gel). Interestingly, SpyCas9 co-purified with AcrIIA17-19 does not perform DNA cleavage, although SpyCas9 co-purified with AcrIIA16Lmo is not inhibited (**FIG. 3C**). The failure of AcrIIA16Lmo to inhibit SpyCas9 *in vitro* is likely due to its low expression level, as visualized in the input western blot (**FIG. 3B**).

[0085] In conducting the immunoprecipitation experiments (above), we noticed that SpyCas9 expressed in our strain of *Pseudomonas aeruginosa* exhibited a series of degradation products when blotted for the C-terminal Myc tag (**FIG. 3D**). Upon closer inspection of the GST-Acr pulldowns, enriched SpyCas9 fragments co-immunoprecipitated with AcrIIA16-19 appear to be different from those of AcrIIA4, suggesting a distinct binding mechanism. To test this, we immunoprecipitated AcrIIA16-19 from *P. aeruginosa* expressing Apo-SpyCas9 without sgRNA, a complex previously reported to be only a weak AcrIIA4 binding partner (Shin et al., 2017). AcrIIA16-17 and AcrIIA19 co-purified with Apo-SpyCas9, while AcrIIA4 shows weak binding (comparing the relative amount of AcrIIA4 to Cas9). Interestingly, AcrIIA18 does not appear to interact with Apo-SpyCas9 (**FIGS. 3D, 7C**). These results suggest that AcrIIA16, 17, and 19 have distinctive SpyCas9 interacting sites from AcrIIA4, with AcrIIA18 additionally displaying a unique binding profile.

Discussion

[0086] Numerous strategies continue to be developed for identification of Acrs, with a remarkably diverse range of disclosed inhibition mechanisms. Here, we employed a “guilt-

by-association" bioinformatics approach to discover new acr genes in various MGEs. Given the reported coexistence of acrIIA1 with other acrs, it is an effective anchor gene to utilize in searches of acr loci (Rauch et al., 2017; Jiang et al., 2019; Osuna et al., 2019). The acr genes reported here are found in plasmids and ICEs, as well as some prophages, and other uncharacterized elements. These Cas9 inhibitors successfully protect phage DNA during infection and plasmid DNA during conjugation. AcrIIA16-19 interact with SpyCas9 via novel binding mechanisms compared to AcrIIA4 and AcrIIA2, to inhibit target DNA binding and cleavage *in vitro* and *in vivo*. Finally, the new AcrIIA proteins, e.g., AcrIIA16Lmo, AcrIIA16Efa, AcrIIA17Sga, and AcrIIA19Ssim, displayed broad-spectrum inhibition of Type II-A Cas9 orthologs.

[0087] It is of high clinical relevance to find acrIIA genes in *E. faecalis*, where the spread of antibiotic resistance genes is frequently promoted through plasmid transfer despite the presence of host-encoded CRISPR-Cas systems. This work opens the door to the identification of more acr genes in this organism. Previous work has shown that multidrug resistant *E. faecalis* strains are more likely to lack CRISPR-Cas9 but can acquire MGEs with protospacer matches due to low levels of Cas9 expression, and tolerate those plasmids transiently (Palmer and Gilmore, 2010; Hullahalli et al. 2017; Hullahalli et al. 2018). Our results suggest that these complex interactions have an additional layer and that a state of plasmid self-targeting could be stabilized for some time prior to potential CRISPR-Cas or spacer loss. We demonstrated that AcrIIA proteins not only could enhance the spread of a given antibiotic resistance plasmid, but it also limits the hosts ability to limit the acquisition of other MGEs.

[0088] With the increasing use of CRISPR-Cas systems for various genome editing applications, the discovery and characterization of natural inhibitors that regulate a variety of Cas9 orthologs via different mechanisms remains critical. The broad-spectrum inhibitors are attractive as practical regulators of multiple distinct Cas9 proteins.

Methods

Microbes

[0089] *Escherichia coli* (DH5α, XL1Blue, NEB 10-beta, or NEB turbo) were routinely cultured in lysogeny broth (LB) at 37 °C supplemented with antibiotics at the following concentrations: gentamicin (30 μg/mL), carbenicillin (100 μg/mL), kanamycin (25 μg/mL),

chloramphenicol (25 μg/mL), erythromycin (300 μg/mL) or tetracycline (10 μg/mL). *Pseudomonas aeruginosa* (PAO1) was cultured in LB medium at 37 °C with supplemented antibiotics for plasmid maintenance: gentamicin (50 μg/mL) or carbenicillin (250 μg/mL). For maintaining multiple plasmids in the same *P. aeruginosa* strain, antibiotic concentrations were adjusted to 30 μg/mL gentamicin and 100 μg/mL carbenicillin. All *Enterococcus faecalis* strains (C173, OG1RF, T11RF, T11RFΔCas9) were cultured in brain-heart-infusion (BHI) medium at 37 °C, unless otherwise mentioned. Antibiotics were used in the following concentrations: spectinomycin (500 μg/mL), streptomycin (500 μg/mL), rifampicin (50 μg/mL), fusidic acid (25 μg/mL), chloramphenicol (15 μg/mL) or erythromycin (50 μg/mL).

Construction of P. aeruginosa and E. faecalis strains

[0090] *P. aeruginosa* heterologous type II-A system was generated as previously described (Borges et al., 2018) under "construction of PAO1::SpyCas9 expression strain," with sgRNA integrated into the bacterial genome using the mini-CTX2 vector (Hoang et al., 2000) or expressed from multi-copy episomal plasmid pMMB67HE-PLac for *in vivo* assays, and plasmid pHERD30T-PBad for *in vitro* assays. All acr candidate genes were synthesized as gene fragments (Twist Biosciences) and cloned using Gibson Assembly into plasmids of *P. aeruginosa* vectors pHERD30T or pMMB67HE, and *E. faecalis* vectors pKH12 or pMSP3535 (gifts from Kelli L. Palmer and Gary Dunny RRID:Addgene_46886 respectively). Plasmids were electroporated into PAO1 (Choi et al., 2006) for all *P. aeruginosa* strains, and *E. faecalis* strains C173, OG1RF, T11RF and T11RFΔCas9 using previously published protocols (Bhardwaj et al., 2016). All strains and plasmids constructed and used in this study are listed in **Table 2**.

Bacteriophage plaque assays in P. aeruginosa

[0091] Plaque assays were performed as previously described (Borges et al, 2018; Jiang et al. 2019) with sgRNA designed to target Pseudomonas phage JBD30. The PLac promoter driving chromosomally integrated SpyCas9 and sgRNA, or pMMB67HE-sgRNA was induced with titrating levels of IPTG (0.1, 1, 10 mM) and the PBad promoter driving pHERD30T-acr with 0.1% arabinose. One representative plate for each candidate were imaged using Gel Doc EZ Gel Documentation System (Bio-Rad) and Image Lab software.

Self-genome targeting and CRISPRi assay in P. aeruginosa

[0092] Strains with chromosomally integrated WT SpyCas9 or dCas9 are programmed with pMMB67HE-sgRNA to target the PAO1 chromosomal phzM gene promoter in the presence of pHERD30T-acr. Cultures were grown overnight in LB supplemented with appropriate antibiotics for plasmid maintenance and 0.1% arabinose to pre-induce anti-CRISPR expression. Overnight cultures are diluted in 1:100 LB supplemented with inducers 0.1% arabinose and IPTG (0.01, 0.1, 0.25, 1, 10 mM to titrate CRISPR strength) in a 96-well Costar plate (150 µL/well) for self-targeting survival analysis or glass tubes (3 mL) for CRISPRi, in triplicates. Self-genome targeting was assayed by measuring bacterial growth curves for 16-24 hours in Synergy H1 microplate reader (BioTek, using Gen5 software) at 37 °C with continuous shaking, and data displayed as the mean OD600 of at least three biological replicates ± standard deviation (error bars) as a function of time. For CRISPRi, cells were grown for 20-24 hours with continuous shaking. Next, pyocyanin was extracted and quantified as previously described (Bondy-Denomy et al., 2015). Data are displayed as the mean OD520 of at least three biological replicates ± standard deviation (error bars) and representative pictures are shown.

Conjugation assay in E. faecalis

[0093] Protospacers perfectly matching to indicated spacers in CRISPR1 or CRISPR3 array (**FIG. 6A**) were synthesized as complementary oligonucleotides (IDT) and cloned into pKH12 (Hullahalli et al., 2017) to generate the targeted conjugative plasmid. The promoter region of the of acr loci in *E. faecalis* (nucleotide sequence 350 bp upstream) was synthesized (Twist Bioscience) and cloned upstream the acr genes of the targeted pKH12 conjugative plasmid or pMSP3535. The derivatives of pKH12 were introduced into the C173 donor strain as the transferring plasmid, and pMSP3535 into OG1RF, T11RF or T11RFΔCas9 to pre-express the Acr proteins in recipient cells.

[0094] Conjugation mating experiments were performed as described by Price et al., 2016, except for the following adjustments. Diluted cultures of plasmid-donor and recipient strains were grown to OD600 0.9-1.0, after which 100 µL of donor strain was mixed with 900 µL of OG1RF recipient strains or 500 µL donor with 500 µL of T11RF recipients. Resuspended pellets were plated on Mixed Cellulose Ester filter membranes (Advantec #A020H047A) on BHI agar plates without selection and incubated overnight at 37 °C. The next day, mated cells were collected by washing the filter membrane with 1.5 mL of 1X PBS and 10-fold serial

dilutions were plated or spotted on BHI agar plates supplemented with antibiotics to quantify donor (spectinomycin, streptomycin and chloramphenicol), recipient (rifampicin and fusidic acid, and erythromycin for pMSP353 containing strains) or transconjugant (rifampicin, fusidic acid and chloramphenicol, with erythromycin for pre-expressed Acr strains) populations. Plates were incubated for 48 to 72 hours at 30 °C to allow colonies to develop. Plates with 30 to 300 colonies were used to calculate CFU/mL and conjugation frequency was determined by dividing the number of transconjugants over donors. For plates with spotted dilutions, the fold reductions in transconjugants were qualitatively derived by examining at least three replicates of each experiment. Plate images were acquired as above in the section "bacteriophage plaque assays in *P. aeruginosa*" and a representative picture is shown.

Expression and purification of anti-CRISPR proteins

[0095] N-terminally 6xHis-tagged Acr proteins were purified from *E. coli* BL21 following the protocol in Osuna et al., 2019 under "Cas9 and anti-CRISPR protein expression and purification". AcrIIA16 lysate was incubated with HiTrap Heparin HP affinity column (GE #17040601), while AcrIIA2b.3, IIA17, IIA18 and IIA19 were incubated with Ni-NTA Agarose Beads (Qiagen). All elutions were dialyzed by SEC using ENrich SEC 650 10 x 300 Column (Bio-Rad #780-1650) to remove imidazole.

Cleavage assays using purified proteins

[0096] Lyophilized crRNA was resuspended, complexed with tracrRNA in Nuclease-free Duplex Buffer following protocol from IDT, and incubated with SpyCas9 (NEB) at room temperature for 15 mins to form SpyCas9-RNP. All reactions were carried out in 1X MST Buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 20 mM MgCl2, 5 mM DTT, 5% Glycerol, 0.05% Tween-20 [v/v]). 25nM SpyCas9-RNP was incubated with 250 nM of Acr protein for 1 h on ice. DNA substrate linearized by NheI digestion was added to a final concentration of 2 nM and the reaction was allowed to cut for 0, 5, 10 and 30 mins, at each timepoint the reaction was quenched in warm Quench Buffer (50 mM EDTA, 0.02% SDS) followed by heating at 95 °C for 10 mins. Products were analyzed on 1% agarose gel and stained with SYBR Safe.

Co-immunoprecipitation of SpyCas9-3xMyc and GST-Acr

[0097] Chromosomally integrated SpyCas9 and pHERD30T-sgRNA for guide-loaded Cas9 or empty vector for apo-Cas9 were expressed off the PBad promoter, and pMMB67HE-GST-AcrIIA expressed of PLac in P. aeruginosa PAO1 strain. Saturated overnight cultures were diluted 1:100 the next morning in a total volume of 50 mL, induced with 0.3% arabinose and 1 mM IPTG at OD600 0.3-0.4, and harvested at OD600 1.8-2.0 by centrifugation at 6,000 x g for 10 mins at 4 °C. Cell pellets were flash frozen on dry ice, resuspended in 1 mL lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 20 mM $MgCl_2$, 0.5% NP40, 5% Glycerol [v/v], 5 mM DTT, and 1 mM PMSF), lysed by sonication (20 s pulse for 4 cycles with cooling on ice between cycles, and lysates were clarified by centrifugation at 14,000 x g for 10 mins at 4 °C. For input samples, 10 µL lysates were added in 3X volume of 4X Laemmli Sample Buffer. Using a magnetic stand, Anti-c-Myc Magnetic Beads #88842 or Gluthathione Magnetic Agarose Beads #78601 (Thermo Fisher Scientific) were prewashed with 1 mL of cold wash buffer (50 mM Tris-Cl pH 7.4, 150mM NaCl, 20mM $MgCl_2$), and remaining lysate were added to bead slurry in a volume ratio of 20:1 for Myc or 40:1 for GST followed by overnight incubation at 4 °C with end-over-end rotation. Beads were washed five times using a magnetic stand at room temperature with 1mL of cold wash buffer with addition of 5mM DTT, gradual decreasing concentrations of detergent NP40 (0.5%, 0.05%, 0.01%, 0.005%, 0) and glycerol (5%, 0.5%, 0.05%, 0.005%, 0). Bead-bound proteins were resuspended in 100 µLof final wash buffer without detergent and glycerol. For analysis, 10 µL of beads-bound protein were added to equal volume of 4X Laemmli Sample Buffer. Samples were analyzed on 4-20% SDS-Page gel and stained with Coomassie (Bio-Safe Coomassie Stain, Bio-Rad).

Immunoblotting

[0098] Protein samples were separated by SDS-Page using 4-20% gel (Mini-PROTEAN TGX Precast Gels, Bio-Rad) and transferred in 1X Tris/Glycine Buffer (Bio-Rad) with 20 % Methanol onto 0.2 µm Immun-Blot PVDF Membrane (Bio-Rad). Blots were probed with the following antibodies diluted 1:5000 in 1X TBS-T containing 5% nonfat dry milk: mouse anti-Myc (Cell Signaling Technology #2276, RRID:AB_331783), rabbit anti-GST (Cell Signaling Technology #2625, RRID:AB_490796), mouse anti-E. coli RNA Polymerase β (BioLegend #663903, RRID:AB_2564524), HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology #sc-2005, RRID:AB_631736) and HRP-conjugated goat anti-rabbit IgG (Bio-Rad #170-6515, RRID:AB_11125142). Blots were developed using Clarity ECL Western

Blotting Substrate (Bio-Rad), and chemiluminescence was detected on an Azure c400 Biosystems Imager.

Cleavage assays using SpyCas9-3xMyc tagged pull downs

[0099] DNA substrate linearized by NheI digestion was added into beads-bound protein slurry to a final concentration of 1.5 nM and the reaction was allowed to react for 1, 5, 10 and 30 mins in the thermomixer at 25 °C with gentle shaking 1000 rpm. At each timepoint, the reaction was quenched in warm Quench Buffer (50 mM EDTA, 0.02% SDS), followed by heating at 95 °C for 10 mins. Products were analyzed on 1% agarose gels stained with SYBR Safe.

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Table 2A. Bacterial strains used in the study

Name	Bug	Strain	Genotype	Plasmid	Plasmid Link	Resistance
bCM018	*P. aeruginosa*	PAO1	tn7pLac::spyCas9	pUC18-Tn7-lac		Gent 50
bCM022	*E. coli*	DH5a	pUC-pLac::spyCas9	pUC18-Tn7-Lac::spyCas9		Gent 30
bCM037	*E. coli*	DH5a	mini-CTX2-pLac	pCM5	benchling.com/s/seq-pcO0qZtbMvAln67bq7hT	Tet 10

bCM038	*E. coli*	DH5a	AcrIIA4	pCM6	benchling.com/s/seq-lQdlb5g575NZufBXnRVa	Gent 30
bCM039	*P. aeruginosa*	PAO1	tn7pLac::spyCas9	pUC18-Tn7-lac		
bCM040	*E. coli*	DH5a	ctxpLac::sgRNA-BsaIsites	pCM7	benchling.com/s/seq-hBDIwnJjRKiliQbKZT67	Tet 10
bCM041	*E. coli*	DH5a	ctx2pLac::sgRNA-JBD30	pCM8	benchling.com/s/seq-MxNZDuWiktc0YjbWfy20	Tet 10
bCM045	*P. aeruginosa*	PAO1	tn7pLac::spyCas0, ctx2pLac::sgRNA-BsaIsites			
bCM046	*P. aeruginosa*	PAO1	tn7pLac::spyCas0, ctx2pLac::sgRNA-JBD30			
bCM047	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, pMMB67HE-EV	pMMB67HE ev		Carb 250
bCM048	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, pMMB67HE-sgJBD30	pMMB67HE::sgCas9_JBD30	benchling.com/s/seq-QmcCNgobGcvdfaHjOKpM	Carb 250
bCM049	*P. aeruginosa*	PAO1	tn7pLac::spyCas9 , pMMB67HE-EV, pHERD30T-EV	pMMB67HE::ev, pHERD30T::ev		Gent 30, Carb 250
bCM051	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, pMMB67HE::sgJBD30, pHERD30T-EV	pMMB67HE::sgCas9_JBD30, pHERD30T::ev		Gent 30, Carb 250
bCM052	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, pMMB67HE::sgJBD30, AcrIIA4	pMMB67HE::sgCas9_JBD30, pCM6		Gent 30, Carb 250
bCM053	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, ctx2pLac::sgBsaI, pHERD30T-EV	pHERD30T::ev		Gent 50
bCM055	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, ctx2pLac::sgJBD30, pHERD30T-EV	pHERD30T::ev		Gent 50
bCM056	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, ctx2pLac::sgJBD30, AcrIIA4	pCM6		Gent 50
bCM068	*P. aeruginosa*	PAO1	tn7pLac::dSpyCas9			
bCM079	*E. coli*	DH5a	AcrIIA16_Lmo	pCM28	benchling.com/s/seq-buYB9nI2BRCcxsF1a9S0	Gent 30
bCM085	*E. coli*	DH5a	sgRNA-PhzM5	pCM11	benchling.com/s/seq-35qjyzqBYltrtNN23kiP	Tet 10
bCM100	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, ctx2pLac::sgJBD30, AcrIIA16_Lmo	pCM28		Gent 50
bCM153	*E. coli*	DH5a	AcrIIA16_Lmo	pCM38	benchling.com/s/seq-sCfcUF2qBXtWQvZ6y2II	Carb 100
bCM154	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, pMMB67HE::sgJBD30, AcrIIA16_Lmo	pMMB67HE::sgJBD30, pCM28		Gent 30, Carb 100
bCM155	*E. coli*	Turbo	AcrIIA17_Efa	pCM45	benchling.com/s/seq-1mMtrBikmxsTt3mKTO8g	Gent 30
bCM156	*E. coli*	Turbo	AcrIIA17_Sga	pCM46	benchling.com/s/seq-be1xgfFVSFxPgHogpOmd	Gent 30
bCM159	*E. coli*	Turbo	AcrIIA16_Lmo	pCM48	benchling.com/s/seq-WvAhUZYIlPB9dvVf3Vst	Kan 25
bCM160	*E. coli*	Turbo	AcrIIA18_Sma	pCM49	benchling.com/s/seq-SndExMkJ6QkLUqiltibE	Gent 30
bCM173	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, pMMB67HE::sgJBD30, AcrIIA17_Efa	pMMB67HE::sgJBD30, pCM45		Gent 30, Carb 100
bCM174	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, pMMB67HE::sgJBD30, AcrIIA17_Sga	pMMB67HE::sgJBD30, pCM46		Gent 30, Carb 100
bCM176	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, pMMB67HE::sgJBD30, AcrIIA18_Sma	pMMB67HE::sgJBD30, pCM49		Gent 30, Carb 100
bCM178	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, ctx2pLac::sgJBD30, AcrIIA17_Efa	pCM45		Gent 50
bCM180	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, ctx2pLac::sgJBD30, AcrIIA18_Sma	pCM49		Gent 50

bCM196	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, ctx2pLac::sgJBD30, AcrIIA17_Sga	pCM46		Gent 50
bCM198	*P. aeruginosa*	PAO1	tn7pBAD::spyCas9, 30T::sgJBD30, pMMB67HE::ev	30T::sgJBD30, pMMB67HE::ev		Gent 30, Carb 100
bCM200	*P. aeruginosa*	PAO1	tn7pBAD::spyCas9, 30T::sgJBD30, AcrIIA4	30T::sgJBD30, pMMB67HE::AcrIIA4		
bCM202	*P. aeruginosa*	PAO1	tn7pBAD::spyCas9, 30T::sgJBD30	pHERD30T::sgJBD30		Gent 50
bCM235	*E. coli*	DH5a	AcrIIA18_Sma	pCM78	benchling.com/s/seq-SHYFRiGAcmTRiXyOxsnQ	Kan 25
bCM236	*E. coli*	BL21	AcrIIA17_Sga	pCM70		Kan 25
bCM239	*E. coli*	BL21	AcrIIA16_Lmo	pCM48		Kan 25
bCM246	*E. coli*	BL21	AcrIIA18_Sma	pCM78		Kan 25
bCM248	*E. coli*	EC1000	pKH12			Chlo 25
bCM249	*E. faecalis*	OG1RF	wt			Rif 50
bCM250	*E. faecalis*	T11RF	wt			Rif 50
bCM251	*E. faecalis*	T11RF	Δcas9			Rif 50
bCM252	*E. faecalis*	C173	wt			Spec 500, Strep 500
bCM262	*P. aeruginosa*	PAO1	tn7pLac::spyCas9, pMMB67HE::sgPhzm5	pCM110		Carb 250
bCM263	*P. aeruginosa*	PAO1	tn7pLac::dCas9, pMMB67HE::sgPhzm5	pCM110		Carb 250
bCM271	*E. faecalis*	C173	pKH12	pKH12		Spec 500, Strep 500, Chlo 15
bCM272	*E. coli*		pMSP3535	pCM116		Erm 300
bCM273	*E. faecalis*	C173	pKH12::CR3S6	pCM118	benchling.com/s/seq-wj3NsaNMGeaj0jVTCNYa	Spec 500, Strep 500, Chlo 15
bCM283	*E. coli*	Inoue	pKH CR1S96	pCM117	benchling.com/s/seq-IplfloODeopBvE3xxuFZ	Chlo 25
bCM284	*E. coli*	Inoue	pKH CR3S6	pCM118	benchling.com/s/seq-wj3NsaNMGeaj0jVTCNYa	Chlo 25
bCM285	*E. faecalis*	C173	pKH12::CR1S96	pCM117	benchling.com/s/seq-IplfloODeopBvE3xxuFZ	Spec 500, Strep 500, Chlo 15
bCM313	*E. faecalis*	OG1RF	EV	pCM116		Rif, Fus, Erm 50
bCM319	*E. faecalis*	T11RF	EV	pCM116		Rif, Fus, Erm 50
bCM320	*E. faecalis*	OG1RF	Efae promoter, AcrIIA16_Lmo	pCM125		Rif, Fus, Erm 50
bCM321	*E. faecalis*	T11RF	Efae promoter, AcrIIA16_Lmo	pCM125		Rif, Fus, Erm 50
bCM322	*E. faecalis*	OG1RF	Efae promoter, AcrIIA4	pCM126		Rif, Fus, Erm 50
bCM323	*E. faecalis*	OG1RF	Efae promoter, AcrIIA17_Efa	pCM127		Rif, Fus, Erm 50
bCM324	*E. faecalis*	OG1RF	Efae promoter, AcrIIA17_Sga	pCM128		Rif, Fus, Erm 50
bCM325	*E. faecalis*	OG1RF	Efae promoter, AcrIIA19_Ssim	pCM129		Rif, Fus, Erm 50
bCM328	*E. faecalis*	T11RF	Efae promoter, AcrIIA4	pCM126		Rif, Fus, Erm 50
bCM329	*E. faecalis*	T11RF	Efae promoter, AcrIIA17_Efa	pCM127		Rif, Fus, Erm 50
bCM330	*E. faecalis*	T11RF	Efae promoter, AcrIIA17_Sga	pCM128		Rif, Fus, Erm 50

bCM331	*E. faecalis*	T11RF	Efae promoter, AcrIIA19_Ssim	pCM129		Rif, Fus, Erm 50
bCM337	*E. faecalis*	C173	pKH-CR1 :: Efae promoter, AcrIIA16_Lmo	pCM135		Strep, Spec 50, Chlo 15
bCM338	*E. faecalis*	C173	pKH-CR1 :: Efae promoter, AcrIIA17_Efa	pCM137		Strep, Spec 50, Chlo 15
bCM339	*E. faecalis*	C173	pKH-CR1 :: Efae promoter, AcrIIA17_Sga	pCM138		Strep, Spec 50, Chlo 15
bCM340	*E. faecalis*	C173	pKH-CR1 :: Efae promoter, AcrIIA19_Ssim	pCM139		Strep, Spec 50, Chlo 15
bCM341	*E. faecalis*	C173	pKH-CR3 :: Efae promoter, AcrIIA16_Lmo	pCM142		Strep, Spec 50, Chlo 15
bCM342	*E. faecalis*	C173	pKH-CR3 :: Efae promoter, AcrIIA4	pCM143		Strep, Spec 50, Chlo 15
bCM343	*E. faecalis*	C173	pKH-CR3 :: Efae promoter, AcrIIA17_Efa	pCM144		Strep, Spec 50, Chlo 15
bCM344	*E. faecalis*	C173	pKH-CR3 :: Efae promoter, AcrIIA17_Sga	pCM145		Strep, Spec 50, Chlo 15
bCM347	*E. coli*	BL21	AcrIIA19_Ssim	pCM132		Kan 25
bCM348	*E. coli*	XL1-blue	VEGFA2 protospacer	pCM133		Gent 10
bCM350	*E. faecalis*	C173	pKH-CR1 :: Efae promoter, AcrIIA4	pCM136		Strep, Spec 50, Chlo 15
bCM352	*E. faecalis*	C173	pKH-CR3 :: Efae promoter, AcrIIA19_Ssim	pCM146		Strep, Spec 50, Chlo 15
bCM353	*E. coli*	Turbo	JBD30 IIA protospacer	pCM149	benchling.com/s/seq-rEeZCqLFhUskCR0FBvwm	Gent 10
bCM358	*P. aeruginosa*	PAO1	tn7pBAD::spyCas9, sgJBD30, GST-AcrIIA4	30T-gJBD30, pMMB67HE::GST-AcrIIA4		Gent30, Carb100
bCM359	*P. aeruginosa*	PAO1	tn7pBAD::spyCas9, sgJBD30, GST-AcrIIA2b.3	30T-gJBD30, pMMB67HE::GST-AcrIIA2b.3		Gent30, Carb100
bCM361	*P. aeruginosa*	PAO1	tn7pBAD::spyCas9, sgJBD30, GST-AcrIIA16_Lmo	30T-gJBD30, pCM150	benchling.com/s/seq-RIu4DGKrPLZ02ASg6eD5	Gent30, Carb100
bCM362	*P. aeruginosa*	PAO1	tn7pBAD::spyCas9, sgJBD30, GST-AcrIIA7_Sga	30T-gJBD30, pCM151	benchling.com/s/seq-GN6tS6v9oPKA4OJ1r4g9	Gent30, Carb100
bCM363	*P. aeruginosa*	PAO1	tn7pBAD::spyCas9, sgJBD30, GST-AcrIIA18_Sma	30T-gJBD30, pCM152	benchling.com/s/seq-T3XKs8xXQ0oMEIj27XDZ	Gent30, Carb100
bCM364	*P. aeruginosa*	PAO1	tn7pBAD::spyCas9, sgJBD30, GST-AcrIIA19_Ssim	30T-gJBD30, pCM153	benchling.com/s/seq-qu45PDP2tf9rPGkVPLCh	Gent30, Carb100
bCM365	*P. aeruginosa*	PAO1	tn7pBAD::spyCas9, pHERD30T-EV	pHERD30T-EV		Gent50
bCM366	*P. aeruginosa*	PAO1	tn7pBAD::spyCas9, sgJBD30, GST-EV	30T-gJBD30, pMMB67HE::GST-EV		Gent30, Carb100
bCMP3-A1	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-EV, pHERD30T-EV	pHERD30T-EV		Gent30, Carb100
bCMP3-A2	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-sgJBD30, pHERD30T-EV	pHERD30T-EV		Gent30, Carb100
bCMP3-A3	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-sgJBD30, pHERD30T-AcrIIA4	pCM6		Gent30, Carb100
bCMP3-A4	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-sgJBD30, pHERD30T-AcrIIA16_Lmo	pCM28		Gent30, Carb100

bCMP3-A5	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-sgJBD30, pHERD30T-AcrIIA17_Efa	pCM45		Gent30, Carb100
bCMP3-A6	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-sgJBD30, pHERD30T-AcrIIA17_Sga	pCM46		Gent30, Carb100
bCMP3-A7	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-sgJBD30, pHERD30T-AcrIIA18_Sma	pCM49		Gent30, Carb100
bCMP3-A8	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-sgJBD30, pHERD30T-AcrIIA19_Ssim	pCM83		Gent30, Carb100
bCMP3-C2	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-sgPhzM5, pHERD30T-EV	pHERD30T-EV		Gent30, Carb100
bCMP3-C3	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-sgPhzM5, pHERD30T-AcrIIA4	pCM6		Gent30, Carb100
bCMP3-C4	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-sgPhzM5, pHERD30T-AcrIIA16_Lmo	pCM28		Gent30, Carb100
bCMP3-C5	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-sgPhzM5, pHERD30T-AcrIIA17_Efa	pCM45		Gent30, Carb100
bCMP3-C6	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-sgPhzM5, pHERD30T-AcrIIA17_Sga	pCM46		Gent30, Carb100
bCMP3-C7	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-sgPhzM5, pHERD30T-AcrIIA18_Sma	pCM49		Gent30, Carb100
bCMP3-C8	*P. aeruginosa*	PAO1	tn7pLac::SpCas9, pMMB67HE-sgPhzM5, pHERD30T-AcrIIA19_Ssim	pCM83		Gent30, Carb100
bCMP3-E2	*P. aeruginosa*	PAO1	tn7pLac::dCas9, pMMB67HE-sgPhzM5, pHERD30T-EV	pHERD30T-EV		Gent30, Carb100
bCMP3-E3	*P. aeruginosa*	PAO1	tn7pLac::dCas9, pMMB67HE-sgPhzM5, pHERD30T-AcrIIA4	pCM6		Gent30, Carb100
bCMP3-E4	*P. aeruginosa*	PAO1	tn7pLac::dCas9, pMMB67HE-sgPhzM5, pHERD30T-AcrIIA16_Lmo	pCM28		Gent30, Carb100
bCMP3-E5	*P. aeruginosa*	PAO1	tn7pLac::dCas9, pMMB67HE-sgPhzM5, pHERD30T-AcrIIA17_Efa	pCM45		Gent30, Carb100
bCMP3-E6	*P. aeruginosa*	PAO1	tn7pLac::dCas9, pMMB67HE-sgPhzM5, pHERD30T-AcrIIA17_Sga	pCM46		Gent30, Carb100
bCMP3-E7	*P. aeruginosa*	PAO1	tn7pLac::dCas9, pMMB67HE-sgPhzM5, pHERD30T-AcrIIA18_Sma	pCM49		Gent30, Carb100
bCMP3-E8	*P. aeruginosa*	PAO1	tn7pLac::dCas9, pMMB67HE-sgPhzM5, pHERD30T-AcrIIA19_Ssim	pCM83		Gent30, Carb100

Table 2B. Plasmids used in the study

Name	Background	Genotype	Plasmid Link
pCM005	CTX2	mini-CTX2-lac	benchling.com/s/seq-pcO0qZtbMvAln67bq7hT
pCM006	pHERD30T	AcrIIA4	benchling.com/s/seq-lQdlb5g575NZufBXnRVa
pCM007	pCM5	sgRNA-SpCas9-BsaI	benchling.com/s/seq-hBDlwnJjRKiliQbKZT67
pCM008	pCM5	sgRNA-SpCas9-JBD30	benchling.com/s/seq-MxNZDuWiktc0YjbWfy20
pCM011	pCM7	sgRNA-SpCas9-PhzM5	benchling.com/s/seq-35qjyzqBYltrtNN23kiP
pCM028	pHERD30T	AcrIIA16_Lmo	benchling.com/s/seq-buYB9nl2BRCcxsF1a9S0
pCM038	pMMB67HE	AcrIIA16_Lmo	benchling.com/s/seq-sCfcUF2qBXtWQvZ6y2ll
pCM045	pHERD30T	AcrIIA17_Efa	benchling.com/s/seq-1mMtrBikmxsTt3mKTO8g
pCM046	pHERD30T	AcrIIA17_Sga	benchling.com/s/seq-be1xgfFVSFxPgHogpOmd
pCM048	pET28	AcrIIA16_Lmo	benchling.com/s/seq-WvAhUZYIIPB9dvVf3Vst

pCM049	pHERD30T	AcrIIA18_Sma	benchling.com/s/seq-SndExMkJ6QkLUqiltibE
pCM070	pET28	AcrIIA17_Sga	benchling.com/s/seq-skz7bOaQ4KAP7Tr3REL5
pCM078	pET28	AcrIIA18_Sma	benchling.com/s/seq-SHYFRiGAcmTRiXyOxsnQ
pCM083	pHERD30T	AcrIIA19_Ssim	benchling.com/s/seq-OCDuuwHPzkXZN5aldGNo
pCM096	pKH12	pKH12-EV	benchling.com/s/seq-XFN4NQULYef2kGAJ7yyx
pCM116	pMSP3535	pMSP3535-EV	benchling.com/s/seq-wc9nrxP5AGXwMrjpGLh7
pCM117	pKH12	CR1-S96	benchling.com/s/seq-lplfloODeopBvE3xxuFZ
pCM118	pKH12	CR3-S6	benchling.com/s/seq-wj3NsaNMGeaj0jVTCNYa
pCM125	pMSP3535	Efae promoter, AcrIIA16_Lmo	benchling.com/s/seq-c1KLI447GcniB235u7LK
pCM126	pMSP3535	Efae promoter, AcrIIA4	benchling.com/s/seq-hJRjaEPXXvflABZBluB2
pCM127	pMSP3535	Efae promoter, AcrIIA17_Efa	benchling.com/s/seq-oBRwnqeuPpxdma8YN4mp
pCM128	pMSP3535	Efae promoter, AcrIIA17_Sga	benchling.com/s/seq-plp8eWnTCaH76ZoeIlBB
pCM129	pMSP3535	Efae promoter, AcrIIA19_Ssim	benchling.com/s/seq-rp9c2mHHlfnQQAQTiE1S
pCM132	pET28	AcrIIA19_Ssim	benchling.com/s/seq-HzkjlqsohGNkRABi3PWS
pCM133	pHERD30T	VEGFA protospacer	benchling.com/s/seq-Xzlp1bxKBqjD8leQ9JFa
pCM135	pCM117	CR1, Efae promoter, AcrIIA16_Lmo	benchling.com/s/seq-wWWMmOIl5Xeph364ZrE8
pCM136	pCM117	CR1, Efae promoter, AcrIIA4	benchling.com/s/seq-pe8IibQxvpYkPwDfuwgH
pCM137	pCM117	CR1, Efae promoter, AcrIIA17_Efa	benchling.com/s/seq-pqGFn4SjC1D3KASJhCDy
pCM138	pCM117	CR1, Efae promoter, AcrIIA17_Sga	benchling.com/s/seq-DcGbk3dNnUhxvPK5q428
pCM139	pCM117	CR1, Efae promoter, AcrIIA19_Ssim	benchling.com/s/seq-qshXPjlcxj7u9NVbWqL2
pCM142	pCM118	CR3, Efae promoter, AcrIIA16_Lmo	benchling.com/s/seq-5sPKYJ8SAccqqjWRLvEi
pCM143	pCM118	CR3, Efae promoter, AcrIIA4	benchling.com/s/seq-xDqEBSG98omFjlAzhsSh
pCM144	pCM118	CR3, Efae promoter, AcrIIA17_Efa	benchling.com/s/seq-bTzslUxZXAOkckfJOdqb
pCM145	pCM118	CR3, Efae promoter, AcrIIA17_Sga	benchling.com/s/seq-an430GQD9XSgMfVyd0Gj
pCM146	pCM118	CR3, Efae promoter, AcrIIA19_Ssim	benchling.com/s/seq-oXTWcnWk46PW0mQlaZjr
pCM149	pHERD30T	JBD30 IIA protospacer	benchling.com/s/seq-rEeZCqLFhUskCR0FBvwm
pCM150	pMMB67HE	GST-AcrIIA16_Lmo	benchling.com/s/seq-Rlu4DGKrPLZ02ASg6eD5
pCM151	pMMB67HE	GST-AcrIIA17_Sga	benchling.com/s/seq-GN6tS6v9oPKA4OJ1r4g9
pCM152	pMMB67HE	GST-AcrIIA18_Sma	benchling.com/s/seq-T3XKs8xXQ0oMElj27XDZ
pCM153	pMMB67HE	GST-AcrIIA19_Ssim	benchling.com/s/seq-qu45PDP2tf9rPGkVPLCh
Name	Background	Genotype	Plasmid Link
pCM005	CTX2	mini-CTX2-lac	benchling.com/s/seq-pcO0qZtbMvAln67bq7hT
pCM006	pHERD30T	AcrIIA4	benchling.com/s/seq-lQdlb5g575NZufBXnRVa
pCM007	pCM5	sgRNA-SpCas9-BsaI	benchling.com/s/seq-hBDlwnJjRKiliQbKZT67
pCM008	pCM5	sgRNA-SpCas9-JBD30	benchling.com/s/seq-MxNZDuWiktc0YjbWfy20
pCM011	pCM7	sgRNA-SpCas9-PhzM5	benchling.com/s/seq-35qjyzqBYltrtNN23kiP
pCM028	pHERD30T	AcrIIA16_Lmo	benchling.com/s/seq-buYB9nl2BRCcxsF1a9S0
pCM038	pMMB67HE	AcrIIA16_Lmo	benchling.com/s/seq-sCfcUF2qBXtWQvZ6y2ll
pCM045	pHERD30T	AcrIIA17_Efa	benchling.com/s/seq-1mMtrBikmxsTt3mKTO8g

[0100] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

INFORMAL SEQUENCE LISTING

AcrIIA16 protein sequences

SEQ ID NO:1
IIA16-Lmo
Listeria monocytogenes
MGYIGTKRSERSQDAIEDYEVPLNHFNKDLIQAFIDENEAYDTLKTKKVRLWKFVAP
RAGATSWHHTGTYYYNKTDHYSLEKVADELLQNGDEWEEQFKAYVKEEQETATSEP
VFLSVIKVQIWGGSMKRPKLVGHEVVMGVKKEGWLHAVSKATQSKYKLSANKVE
MQKHYSLEDYSALTKDFPEFKAQKRAINKKMKEMYN

SEQ ID NO:2
IIA16-Efa
Enterococcus faecalis
MGYVGKSRSVRSQIAIDNAEVPLNHITKDYILTFVTENNIDETLKNESVAMWKFVAK
RHGSTSWHHVSKHYNKIDHYDLHDVAEYFSMNYDSLKNDYQNLLDQKRQAKNDLI
KNLKLGIIKVQIWGGTKRYPKLEGYESVMGVVKDGWLHTVTLSNQTKYKITGNKIE
EITIFELDQYDILTKKFPEFRAMKRKINKEVARLSK

AcrIIA17 protein sequences

SEQ ID NO:3
IIA17-Efa
Enterococcus faecalis
MAILNNKGEKISIDCADLISEVEEDILIFGGTFLVYAICSWREIEQVEYISDYVHADNPE
SYKDELTTKEYAELKEIYEKDLEELKITKNKQMNLNELLSILTIQNSIT

SEQ ID NO:4
IIA17-Sga
Streptococcus gallolyticus
MKISVDSEKLLNEAINDFDIFGEDFNVYAIYSYREDYDFEYISDYVDADEPTRDEFET
EEDYQEVMKDFKENLDSLKFTKHKKMTIADLVHELWEQNRIF

AcrIIA18 protein sequences

SEQ ID NO:5
IIA18-Sma
Streptococcus macedonicus
MKIDTTVTEVKENGKTYLRLLKGNEQLKAVSDKAVAGVNLFPGAKIGSFLVRQDNI
VVFPDNKGEFDLDFFNLLNDNFETLVEYAKMADCLDIAFDINEKSYFNMIMWLMKN
IDENWSQSPYGESFYSSKDIDWGYKPEGSLRVSDHWNFGQDGEHCPTAEPVDGWAV
CKFENGKYHLIKKF

SEQ ID NO:6
IIA18-Sga
Streptococcus gallolyticus

MKIDTTVTEVKENGKTYLRLVEGTEQLKAISDKAMAGVNLFPGAKIDSFLVKQDSIV
VFPDNKGEFDLDFFKQLDENFDTIAKYARVATCFEEVAFDEKSYFNMIMWLMDNM
DENWSQSPYGESFYSSKNIDWGYKPEGSLRVSDHWNFGENGEHCPTAEPVDGWAV
CKFENGKYHLIKKF

AcrIIA19 protein sequences

SEQ ID NO:7
IIA19-Ssim
Staphylococcus simulans
MKLIVEVEETNYKNLVNYTKLTNESHNILVNRLISEYITKPYELRLDLSERYSNRDLIE
FKFMLIEYCKEALQDIKELANSDEAYETDEAFEAVFRQLFEEVISNPDTVLKAFHSYT
SFLEENK

SEQ ID NO:8
IIA19-Spseu
Staphylococcus pseudintermedius
MKLIINIEDKNYKYLTELAQQDNTNIGSIVNNLIQTHITDVNESYRSVDKKELDEFSRV
MQHYFHEDLASMYDVIGSDEELSTDKQMLKVYKKLYQDVALRNGIALELFNAYKK
G

WHAT IS CLAIMED IS:

1. A method of inhibiting a Cas9 polypeptide in a cell, the method comprising,

introducing a Cas9-inhibiting polypeptide into a cell, wherein:

the Cas9-inhibiting polypeptide is heterologous to the cell, and

the Cas9-inhibiting polypeptide is substantially (e.g., at least 60%, 70%, 80%, 90%, 95%) identical to any one or more of SEQ ID NOS: 1-8;

thereby inhibiting the Cas9 polypeptide in the cell.

2. The method of claim 1, comprising contacting the Cas9-inhibiting polypeptide with a Cas9 polypeptide in the cell.

3. The method of claim 1 or 2, wherein the Cas9-inhibiting polypeptide comprises the amino acid sequence of any one of SEQ ID NOS: 1-8.

4. The method of claim 3, wherein the Cas9-inhibiting polypeptide comprises the amino acid sequence of SEQ ID NO: 1, 2, 4 or 7.

5. The method of any one of claims 1 to 4, wherein the cell comprises an expression cassette comprising a promoter operably linked to a polynucleotide encoding the Cas9 polypeptide.

6. The method of any one of claims 1 to 5, wherein the cell comprises the Cas9 polypeptide before the introducing of the Cas9-inhibiting polypeptide.

7. The method of claim 6, wherein the promoter is inducible and the method comprises contacting the cell with an agent or condition that induces expression of the Cas9 polypeptide in the cell prior to the introducing of the Cas9-inhibiting polypeptide.

8. The method of any one of claims 1 to 5, wherein the cell comprises the Cas9 polypeptide after the introducing of the Cas9-inhibiting polypeptide.

9. The method of claim 8, wherein the promoter is inducible and the method comprises contacting the cell with an agent or condition that induces expression of the Cas9 polypeptide in the cell after the introducing of the Cas9-inhibiting polypeptide.

10. The method of any one of claims 1 to 9, wherein the introducing of the Cas9-inhibiting polypeptide comprises expressing the Cas9-inhibiting polypeptide in the cell from an expression cassette that is present in the cell and is heterologous to the cell, wherein the expression cassette comprises a promoter operably linked to a polynucleotide encoding the Cas9-inhibiting polypeptide.

11. The method of claim 10, wherein the promoter is an inducible promoter and the introducing of the Cas9-inhibiting polypeptide comprises contacting the cell with an agent that induces expression of the Cas9-inhibiting polypeptide.

12. The method of any one of claims 1 to 11, wherein the introducing of the Cas9-inhibiting polypeptide comprises introducing an RNA encoding the Cas9-inhibiting polypeptide into the cell and expressing the Cas9-inhibiting polypeptide in the cell from the RNA.

13. The method of any one of claims 1 to 12, wherein the introducing of the Cas9-inhibiting polypeptide comprises inserting the Cas9-inhibiting polypeptide into the cell or contacting the cell with the Cas9-inhibiting polypeptide.

14. The method of any of claims 1 to 13, wherein the cell is a eukaryotic cell.

15. The method of claim 14, wherein the cell is a mammalian cell.

16. The method of claim 15, wherein the cell is a human cell.

17. The method of any one of claims 14 to 16, wherein the cell is a blood cell or an induced pluripotent stem cell.

18. The method of any one of claims 15 to 17, wherein the method occurs *ex vivo*.

19. The method of claim 18, wherein the cells are introduced into a mammal after the introducing of the Cas9-inhibiting polypeptide.

20. The method of claim 19, wherein the cells are autologous to the mammal.

21. The method of any one of claims 1 to 13, wherein the cell is a prokaryotic cell.

22. The method of claim 21, wherein the introducing of the Cas9-inhibiting polypeptide comprises introducing a polynucleotide encoding the Cas9-inhibiting polypeptide into the cell using bacteriophage, and expressing the Cas9-inhibiting polypeptide in the cell from the polynucleotide.

23. The method of any one of claims 1 to 22, wherein the Cas9 polypeptide is SpyCas9, Efa1Cas9, or Efa3Cas9.

24. A cell comprising a Cas9-inhibiting polypeptide, wherein the Cas9-inhibiting polypeptide is heterologous to the cell and the Cas9-inhibiting polypeptide is substantially (e.g., at least 60%, 70%, 80%, 90%, 95%) identical to any one or more of SEQ ID NOS: 1-8.

25. The cell of claim 24, wherein the cell is a eukaryotic cell.

26. The cell of claim 25, wherein the cell is a mammalian cell.

27. The cell of claim 26, wherein the cell is a human cell.

28. The cell of claim 24, wherein the cell is a prokaryotic cell.

29. A polynucleotide comprising a nucleic acid encoding a Cas9-inhibiting polypeptide, wherein the Cas9-inhibiting polypeptide is substantially (e.g., at least 60%, 70%, 80%, 90%, 95%) identical to any one or more of SEQ ID NOS: 1-8.

30. The polynucleotide of claim 29, wherein the Cas9-inhibiting polypeptide inhibits a Cas9 polypeptide selected from the group consisting of SpyCas9, Efa1Cas9, and Efa3Cas9.

31. The polynucleotide of claim 29 or 30, wherein the polynucleotide is RNA.

32. The polynucleotide of claim 29 or 30, wherein the polynucleotide is DNA.

33. An expression cassette comprising the polynucleotide of claim 32, operably linked to a promoter.

34. The expression cassette of claim 33, wherein the promoter is heterologous to the polynucleotide encoding the Cas9-inhibiting polypeptide.

35. The expression cassette of claim 33 or 34, wherein the promoter is inducible.

36. A vector comprising the expression cassette of any one of claims 33 to 35.

37. The vector of claim 36, wherein the vector is a viral vector.

38. A bacteriophage comprising the expression cassette of any one of claims 33 to 35.

39 . An isolated Cas9-inhibiting polypeptide, wherein the Cas9-inhibiting polypeptide is substantially (e.g., at least 60%, 70%, 80%, 90%, 95%) identical to any one or more of SEQ ID NOS:1-8.

40. The isolated Cas9-inhibiting polypeptide of claim 39, wherein the Cas9-inhibiting polypeptide inhibits one or more Cas9 polypeptides selected from the group consisting of SpyCas9, Efa1Cas9, and Efa3Cas9.

41. A pharmaceutical composition comprising the polynucleotide of any one of claims 29 to 32 or the polypeptide of claim 39 or 40.

42. A delivery vehicle comprising the polynucleotide of any one of claims 29 to 32 or the polypeptide of claim 39 or 40.

43. The delivery vehicle of claim 42, wherein the delivery vehicle is a liposome or nanoparticle.

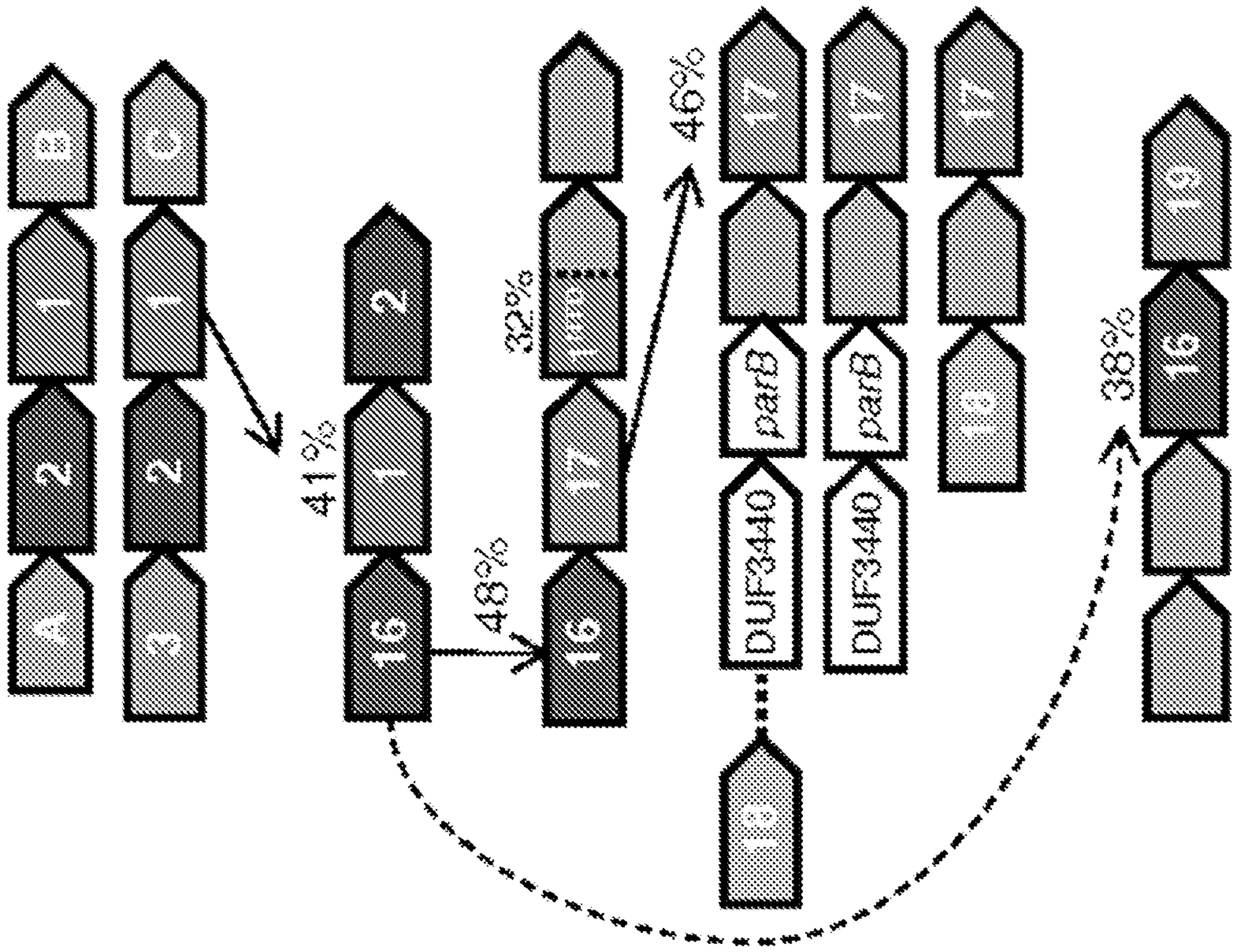


FIG. 1A

Listeria monocytogenes
prophages

Listeria monocytogenes
plasmid

Enterococcus faecalis
plasmid/ICE

Streptococcus prophages

S. gallolyticus

S. agalactiae

S. macedonicus

Staphylococcus simulans
MGE

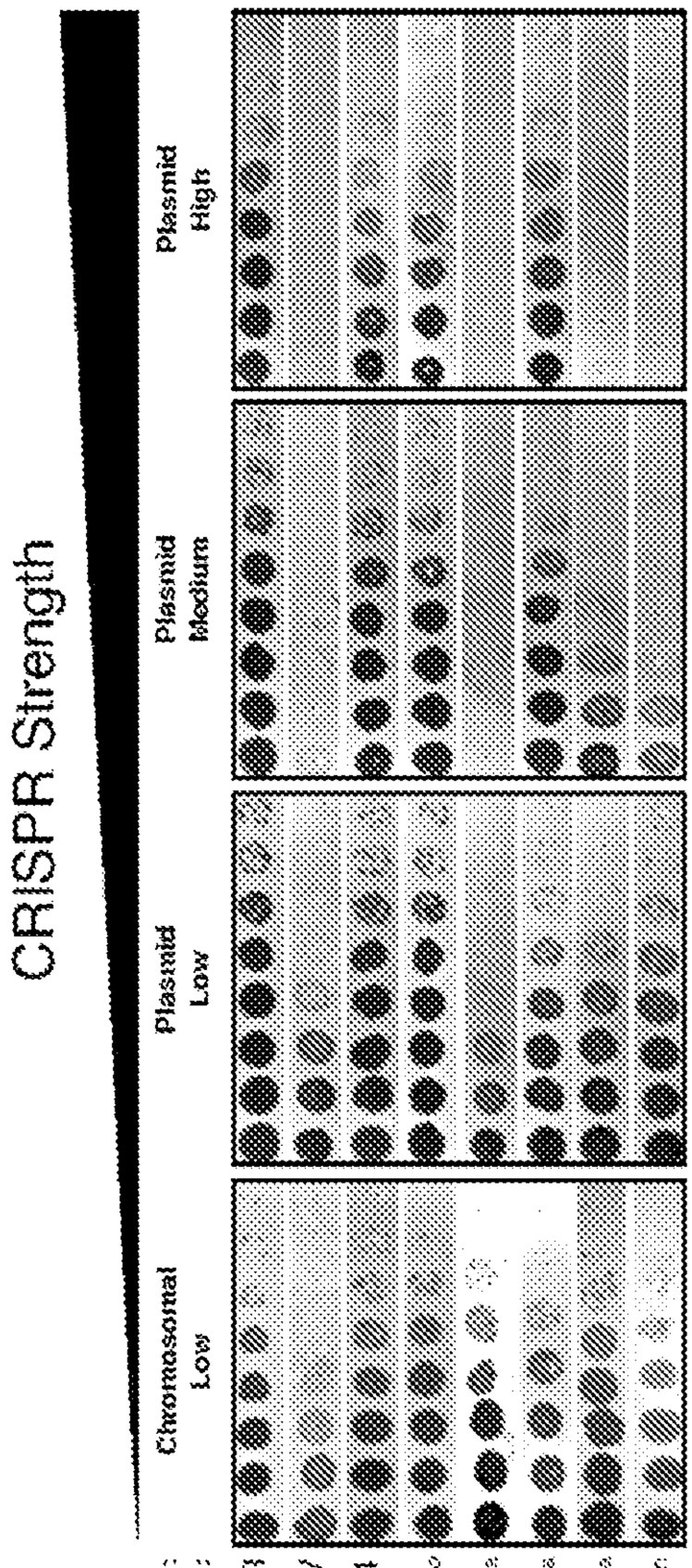

CRISPR Strength
Plasmid High
Plasmid Low
Plasmid Medium
Chromosomal Low
sgRNA expression: Induction Level:
ΔCRISPR
EV
AcrIIA4
HA16Lmo
HA17era
HA17Sga
HA18Sma
HA19Sam
10-fold serial dilutions of phage

FIG. 1B

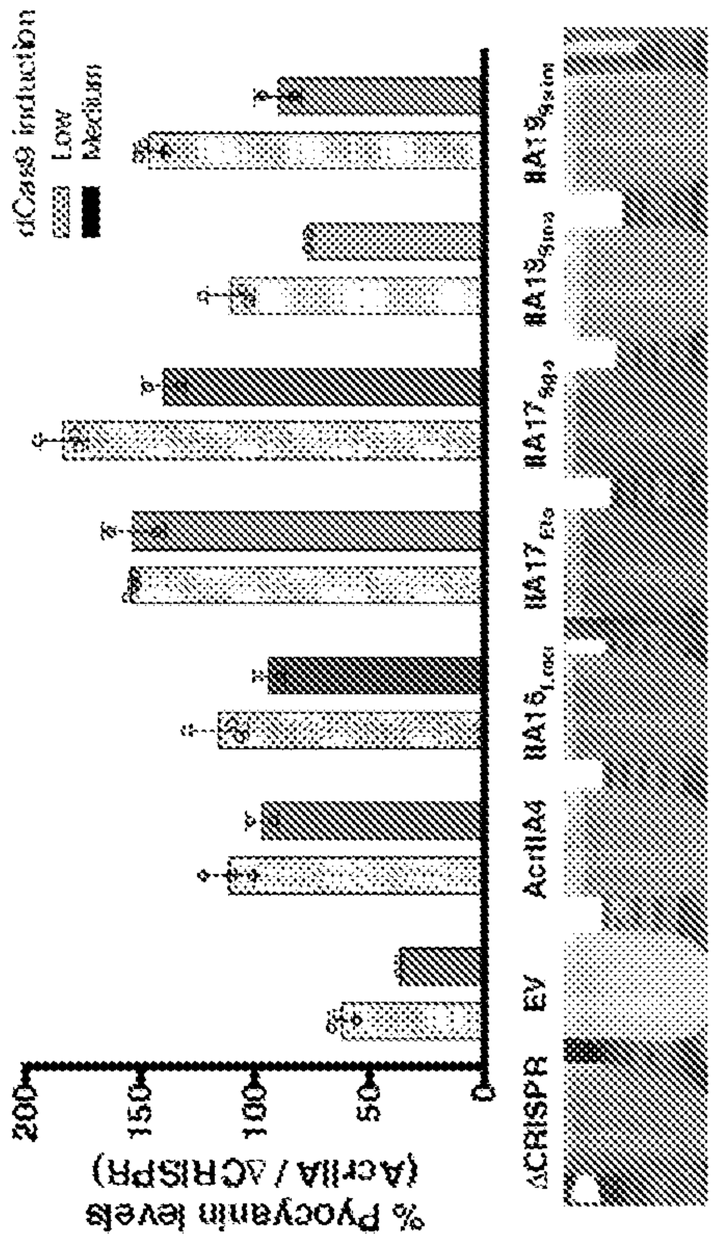

dCas9 Inhibition
dCas9 induction
Low
Medium
% dCas9 Inhibition
HA17Ete
HA17Sag
HA19Sma
HA19Sam
% Pyocyanin levels (AcrIIA / ΔCRISPR)
200
150
100
50
0
ΔCRISPR
EV
AcrIIA4
HA16Lmo

FIG. 1C

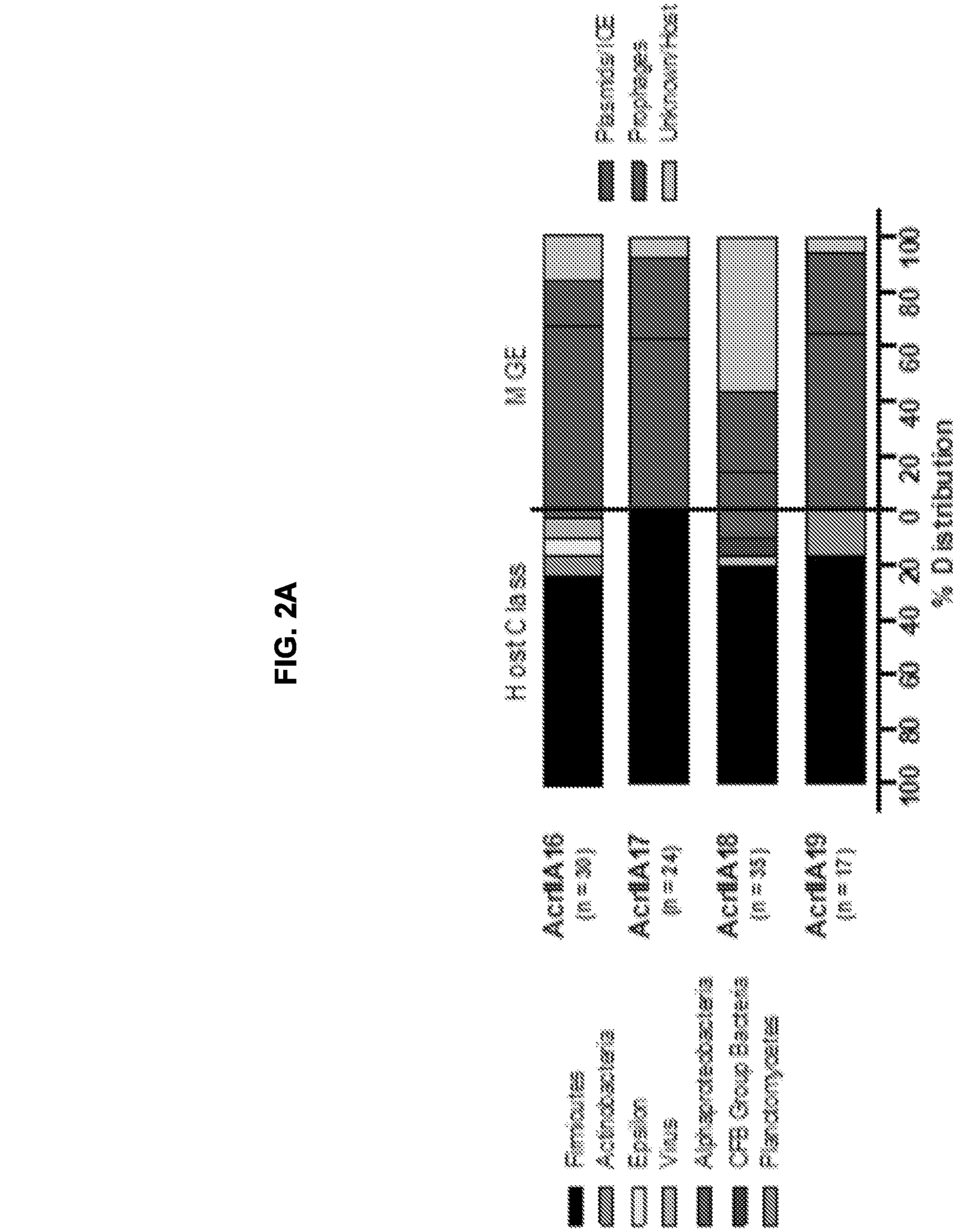

Enterobacteriaceae
Acinetobacter
Staphylococcus
Viridans group Streptococci
Anaerobes
Pseudomonas
Host C is s2
Host C is s1
Host C is s3
0
20
40
60
80
100
% Distribution
AcMAA16
(n = 201)
AcMAA17
(n = 249)
AcMAA18
(n = 151)
AcMAA19
(n = 177)

FIG. 2A

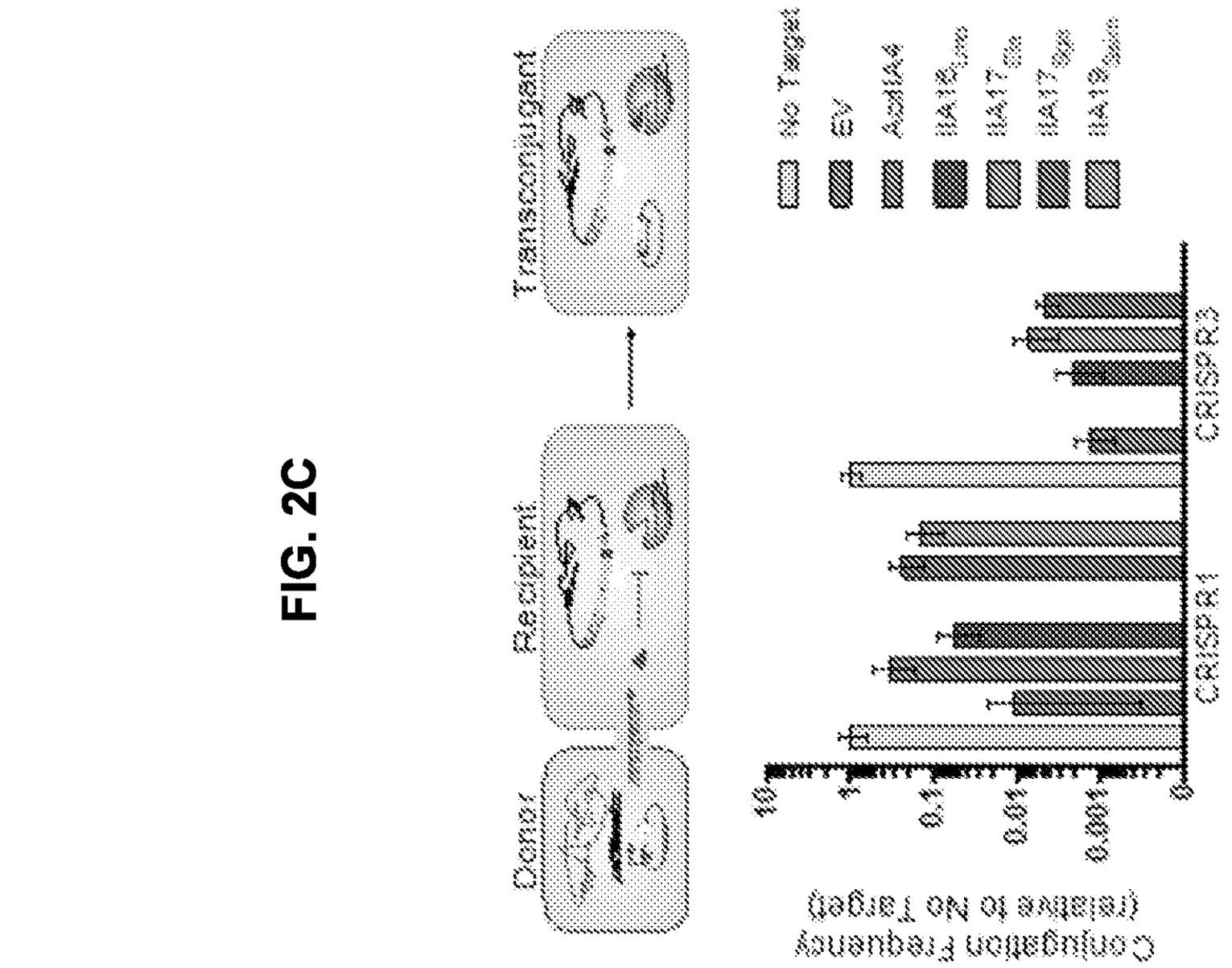

Transcriptagent
Recipient
Donor
No Target
EV
AsiAA
IIA18Long
IIA17Short
IIA17Long
IIA18Short
Conjugation Frequency
(relative to No Target)
10
1
0.1
0.01
0.001
0
CRISPR1
CRISPR3

FIG. 2C

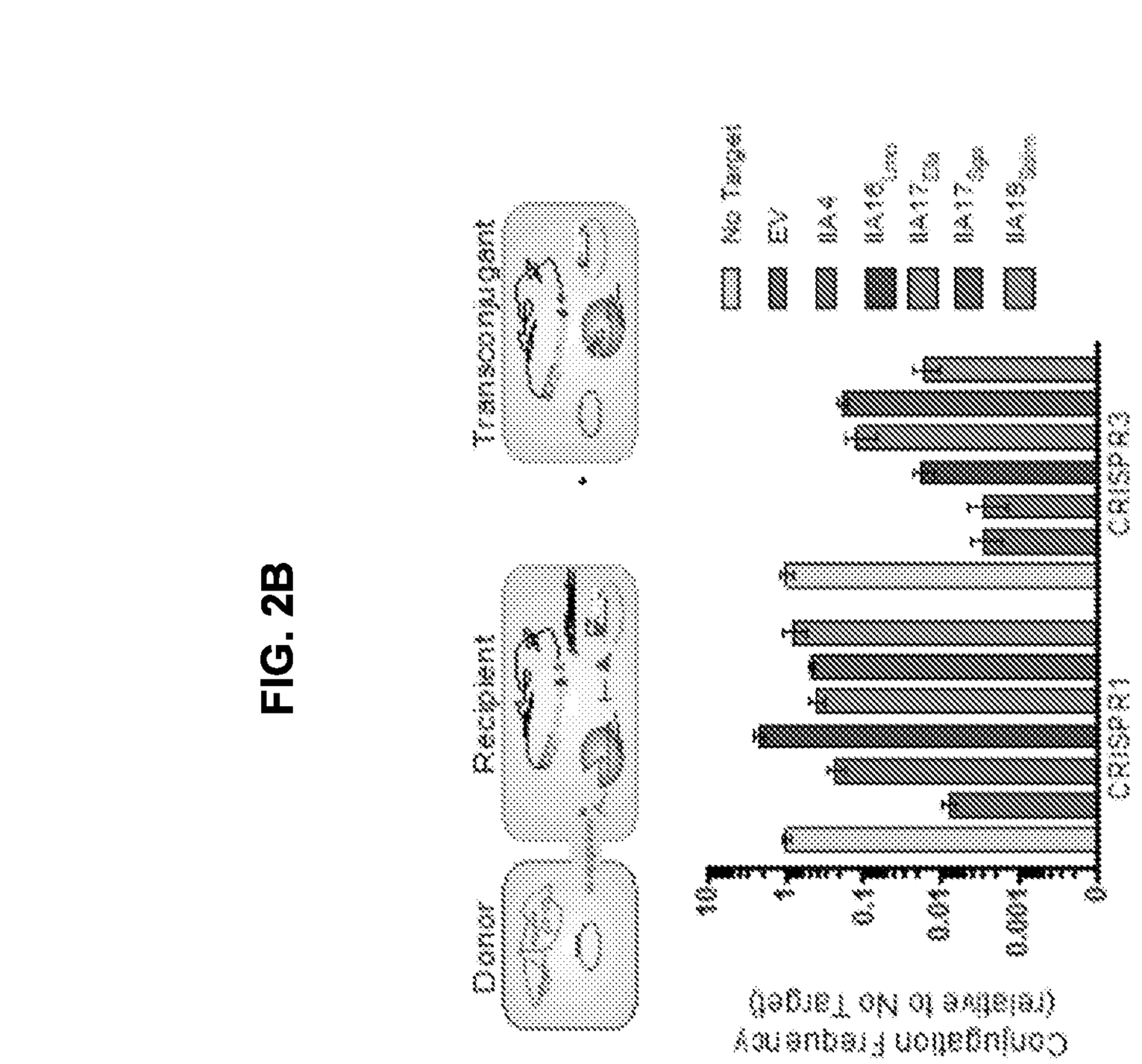

Transcriptagent
Recipient
Donor
No Target
EV
IIA4
IIA18Long
IIA17Short
IIA17Long
IIA18Short
Conjugation Frequency
(relative to No Target)
10
1
0.1
0.01
0.001
0
CRISPR3
CRISPR1

FIG. 2B

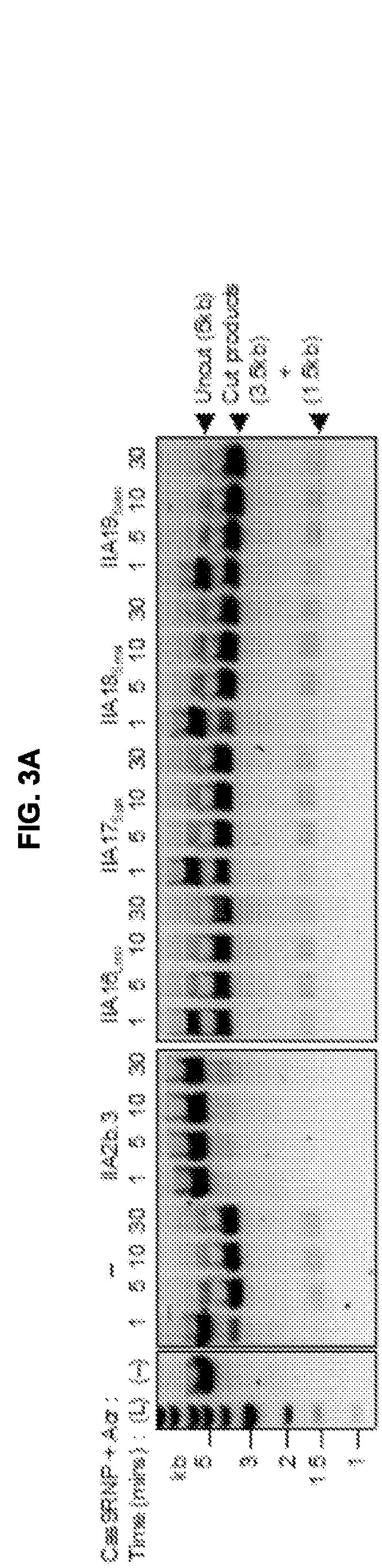

IIA2a.3
IIA19Δ2aa
IIA19
IIA17
Time (min): 0 (-) 1 5 10 30
Cas9RNP + As :
1% Agarose – SYBR
Uncut (5.8 kb)
Cut products (3.8kb) + (1.8kb)
IP: SpCas9-3xMyc
PAO1 WT
GST-Acr
GST-EV
IIA19Δ2aa
IIA19
IIA17
IIA16
4-20% SDS-Page – Coomassie
GST
GST-IIA16Δ2aa 80kD
GST-IIA17Δ2aa 39kD
GST-IIA19Δ2aa 42kD
GST 27kD
Cas9-3xMyc 160kD
α-E.coli RNA polymerase 160kD
(loading control)
4-20% SDS-Page – Immunoblot
Input
0
100
25
50
75
100
125

FIG. 3A

FIG. 3B

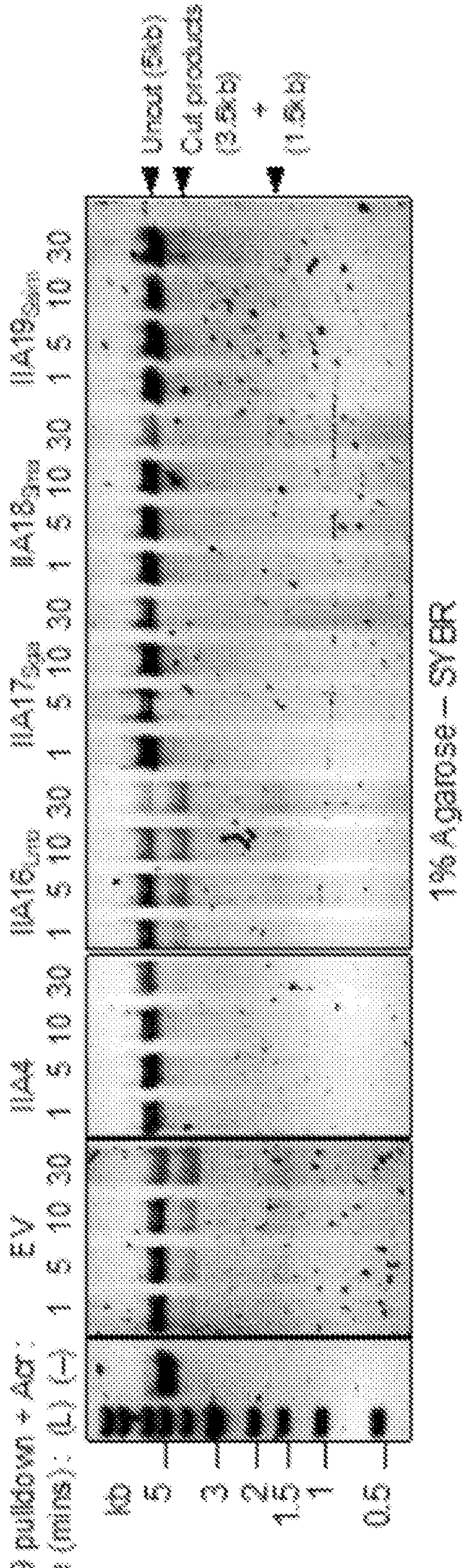

Unbound (Sub)
Cut products (2,3,4a,b)
(2,4a,b) +
(1,4a,b)
IIA17Δ/Δ
IIA18Δ/Δ
IIA4
IIA16Δ/Δ
IIA19Δ/Δ
5 10 30 1 5 10 30 1 5 10 30
EV
1% Agarose – SYBR
Cas9 cutting: (-) (+)
Time (min):
0 5 10 15 30 60

FIG. 3C

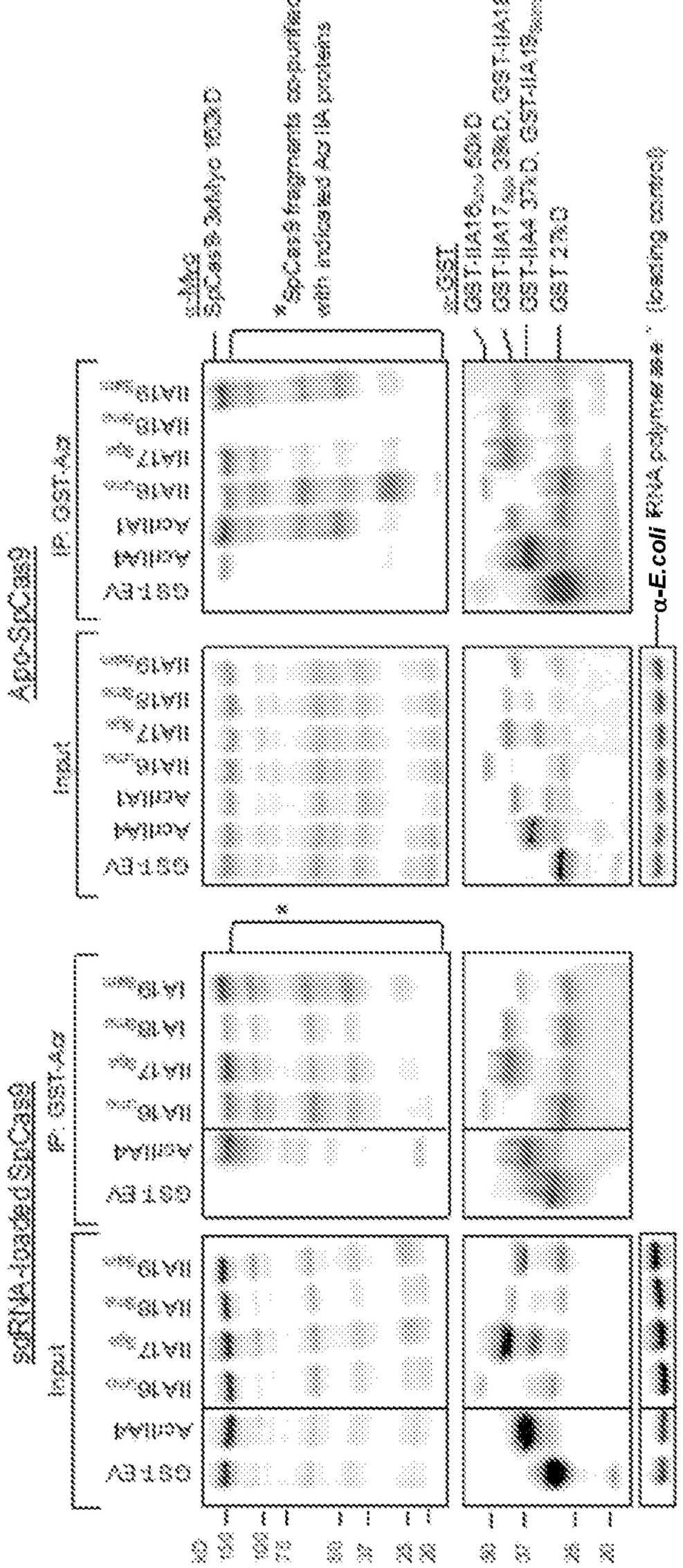

α-E.coli RNA polymerase (loading control)
4-20% SDS-PAGE – Immunoblot
Ago-SpCas9
IP: GST-Ago
Input
soRNA-loaded SpCas9
IP: GST-Ago
Input
GST
GST-IIA19Δ/Δ 60kD
GST-IIA17Δ/Δ 38kD, GST-IIA19Δ/Δ 40kD
GST-IIA4 37kD, GST-IIA19Δ/Δ 40kD
GST 27kD
*SpCas9 fragments co-purified with truncated Ago IIA proteins
SpCas9 (apo) 160kD
GST
IIA19Δ/Δ
IIA18Δ/Δ
IIA17Δ/Δ
IIA4
IIA16Δ/Δ
Ago1
Ago3
Ago4
GST
0 5 10 30 1 5 10 30 1 5 10 30

FIG. 3D

FIG. 4A

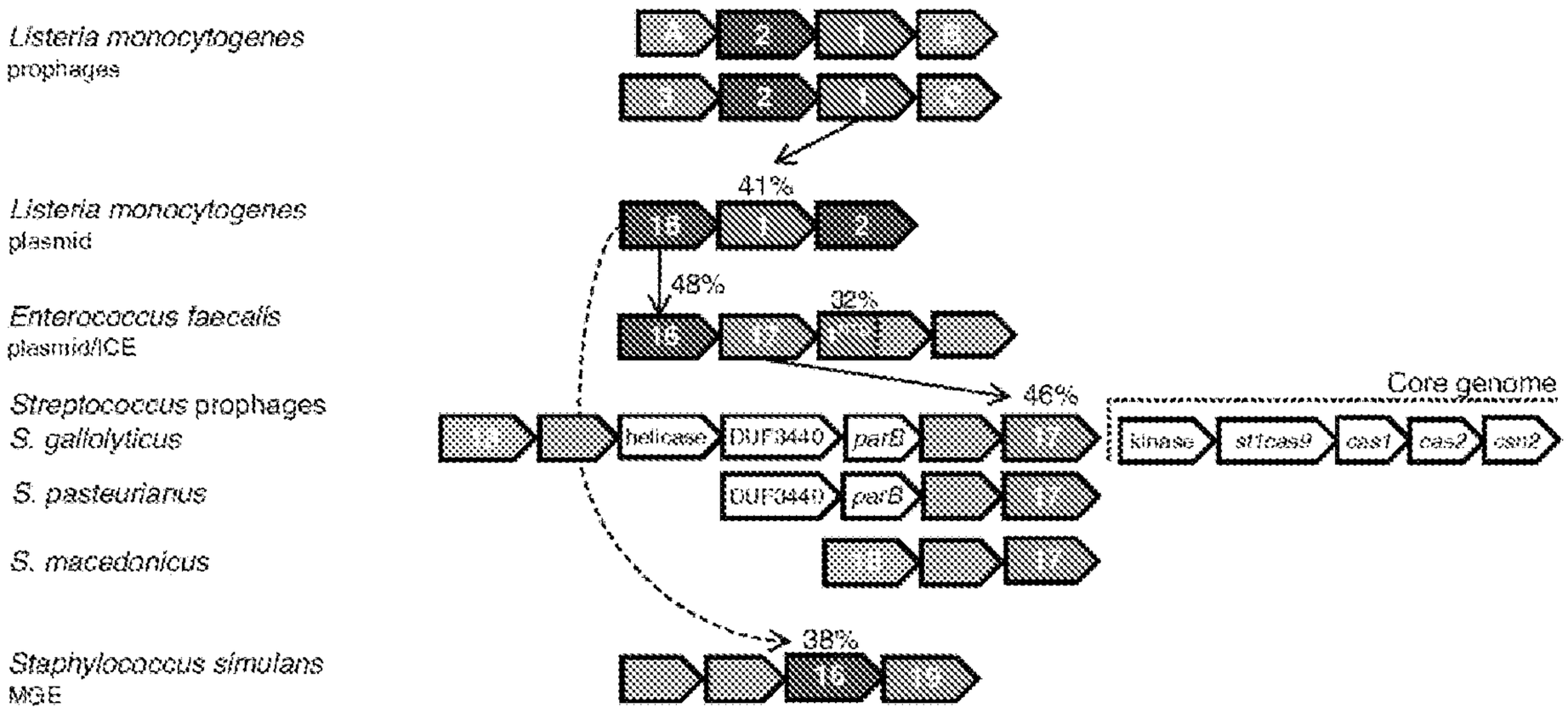
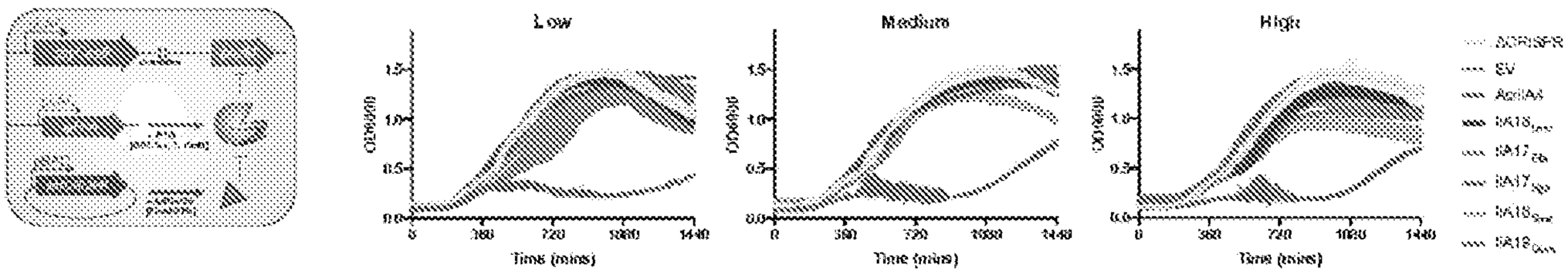

Listeria monocytogenes prophages
Listeria monocytogenes plasmid
Enterococcus faecalis plasmid/ICE
Streptococcus prophages
S. gallolyticus
S. pasteurianus
S. macedonicus
Staphylococcus simulans MGE
41%
48%
32%
46%
38%
helicase
DUF3440
parB
kinase
sf1cas9
cas1
cas2
csn2
Core genome

FIG. 4B


CRISPR Strength
Low
Medium
High
Time (mins)
ΔCRISPR
EV
AcrIIA4

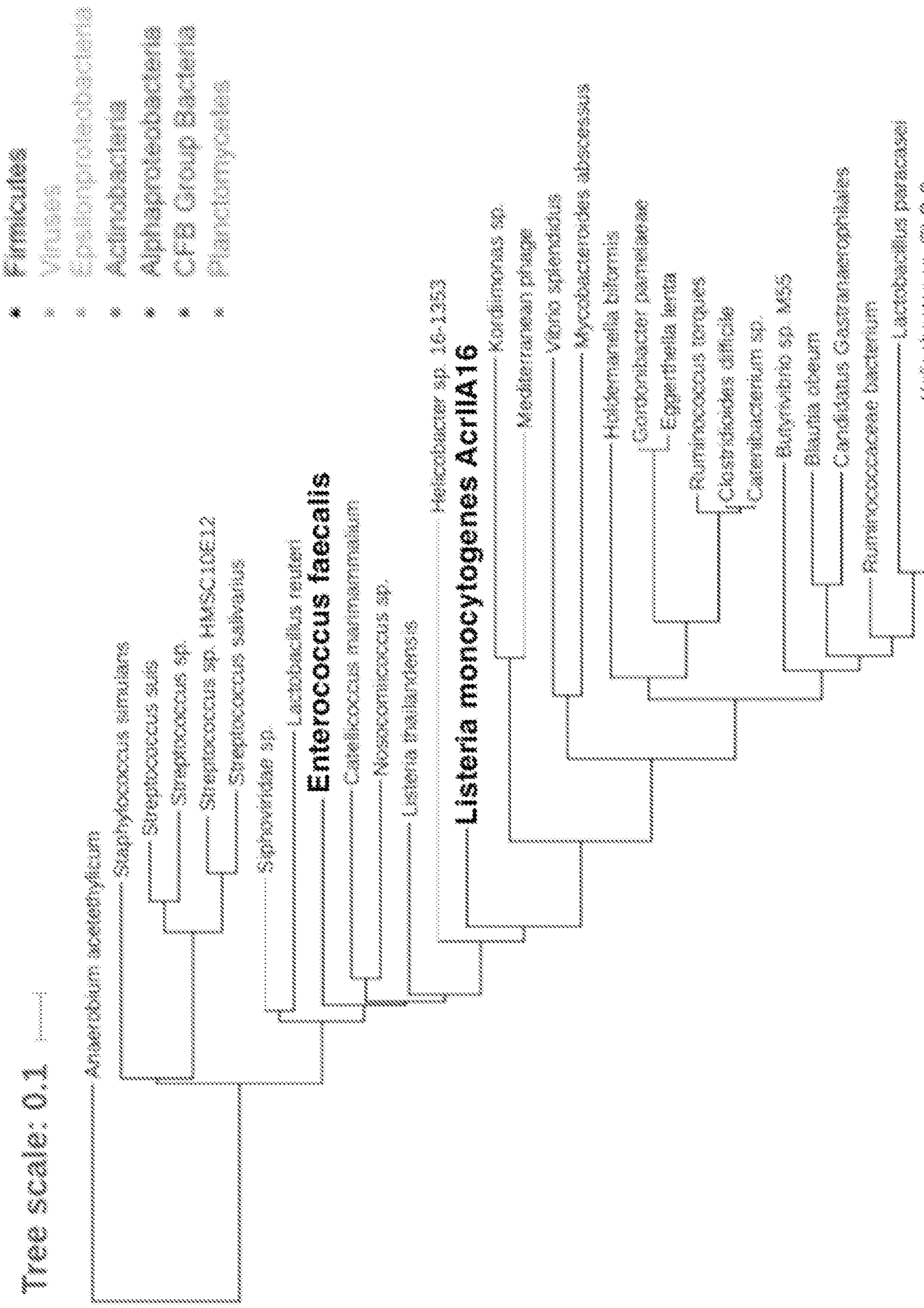

Firmicutes
Virulent
Saprophytic
Actinobacteria
Aphanizomenon
Cr8 Group B
Planktothrix
Enterococcus faecalis
Lactobacillus rhamnosus
Streptococcus salivarius
Streptococcus sp.
Streptococcus sp.
Streptococcus suis
Streptococcus uberis
Staphylococcus simiae
Anaerotruncus colihominis
Clostridium sp.
Ruminococcus torques
Clostridium difficile
Coprobacillus sp.
Eggerthella lenta
Coprococcus eutactus
Blautia obeum
Ruminococcus bicirculans
Candidatus Caccomonas
Ruminococcus bromii
Lactobacillus mucosae
Weissella sp. 16-1353
Listeria monocytogenes AcrIIA16
Kandleria vitulina
Weissella oryzae
Vibrio splendidus
Mycobacterium abscessus
Haloferax lucentense
Haloferax volcanii
Halorubrum sp. 16-1353
Kandleria sp.
Weissella cibaria
Vibrio splendidus
Mycobacterium abscessus
Haloferax lucentense
Haloferax volcanii
Halorubrum sp. 16-1353
Lactobacillus mucosae
Weissella sp. 16-1353
Halorubrum sp. CJO-3
0.1 scale: 0.1

FIG. 5A

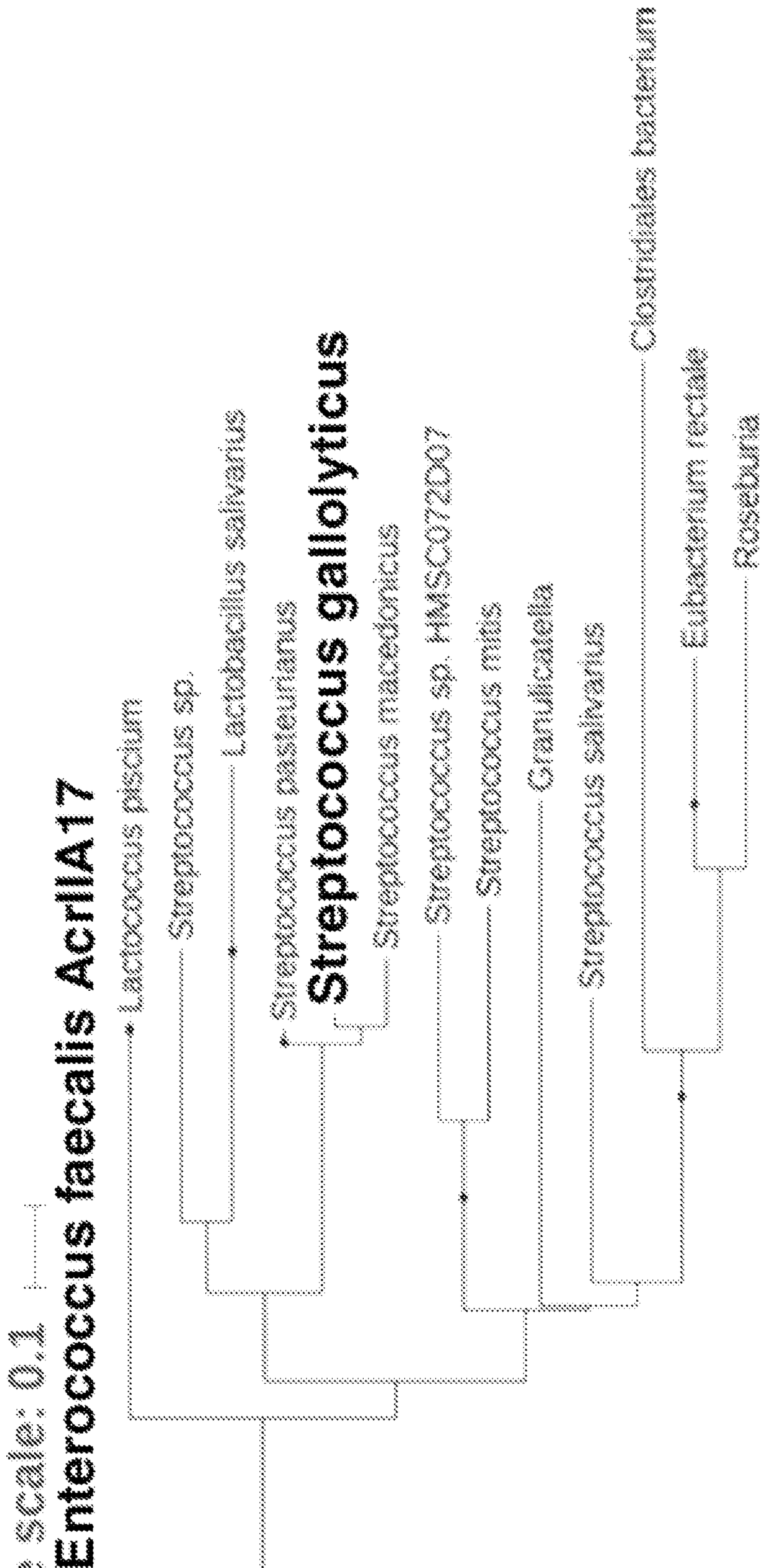

Enterococcus faecalis AcrIIA17
Streptococcus macedonicus
Streptococcus salivarius
Streptococcus pasteurianus
Lactococcus piscium
Streptococcus sp.
Lactobacillus mitis
Streptococcus sp.
Granulicatella adiacens
Streptococcus salivarius
Clostridium tetani
Eubacterium rectale
Roseburia
Streptococcus
Tree scale: 0.1

FIG. 5B

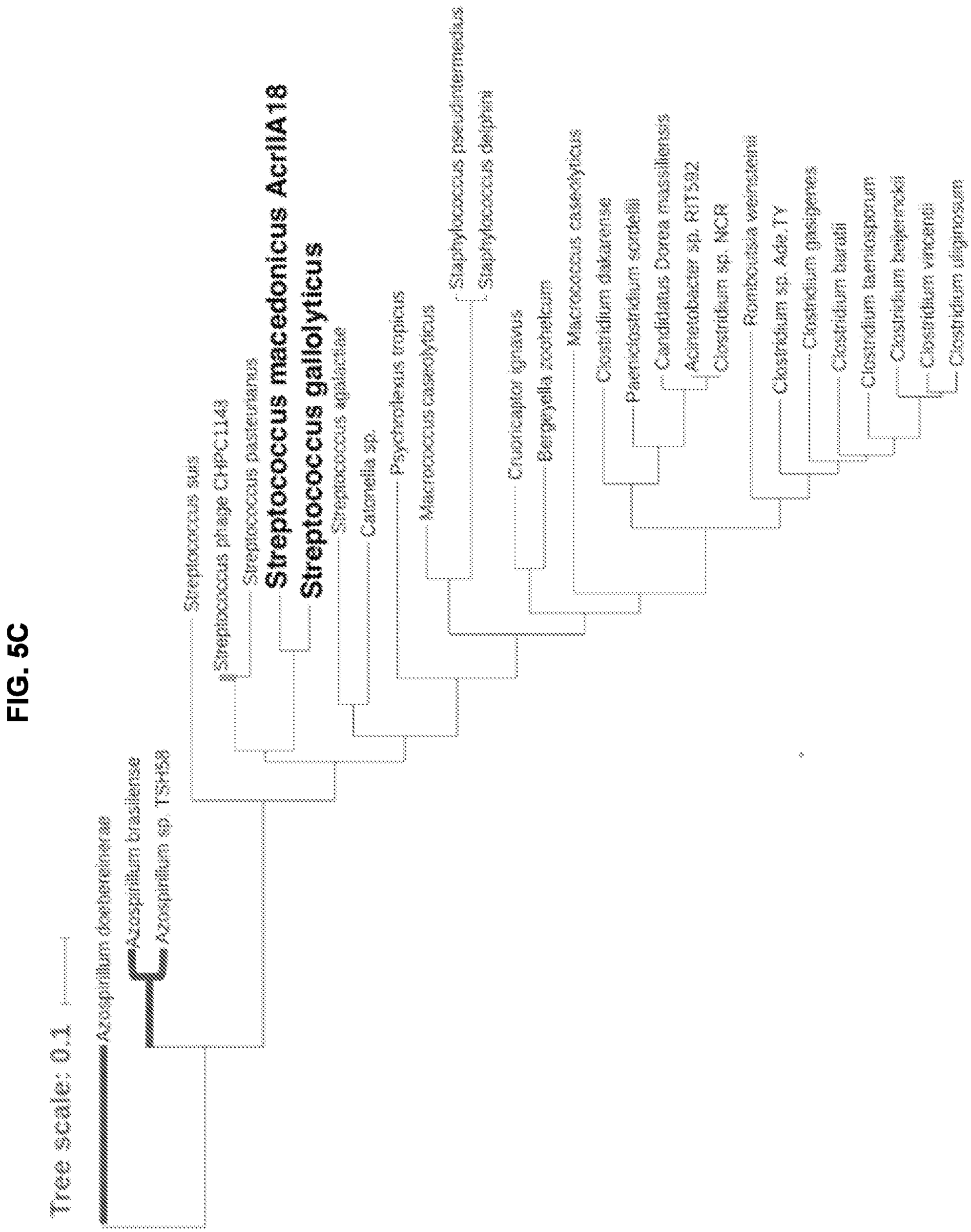

Streptococcus suis
Streptococcus phage CHPC1143
Streptococcus oralis
Streptococcus macedonicus AcrIIA18
Streptococcus gallolyticus
Streptococcus equinus
Catenella sp.
Peptostreptococcus tropinis
Marvinbryantia formatexigens
Staphylococcus pseudintermedius
Staphylococcus delphini
Oxalobacter sp.
Bergeyella zoohelcum
Macrococcus caseolyticus
Clostridium difficile
Parvimonas micra
Candidatus Cloacimonas acidaminovorans
Acinetobacter sp. RUH2
Oscillibacter sp. MCA
Romboutsia ilealis
Clostridium sp. Ade.TY
Clostridium perfringens
Clostridium botulinum
Clostridium tetani
Clostridium beijerinckii
Clostridium thermocellum
tree scale: 0.1
Azospirillum brasilense
Azospirillum sp. TSA2S
Azospirillum doebereinerae

FIG. 5C

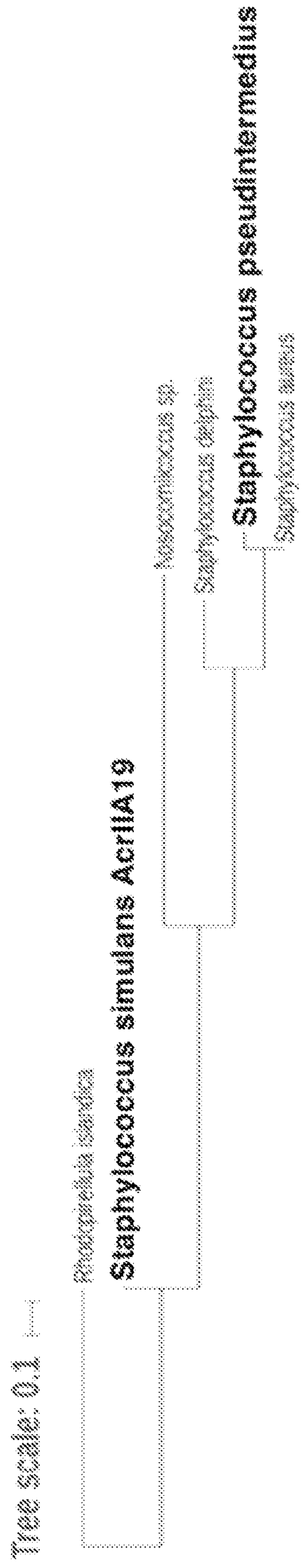

Staphylococcus pseudintermedius
Staphylococcus simulans AcrIIA19
Tree scale: 0.1

FIG. 5D

FIG. 6A

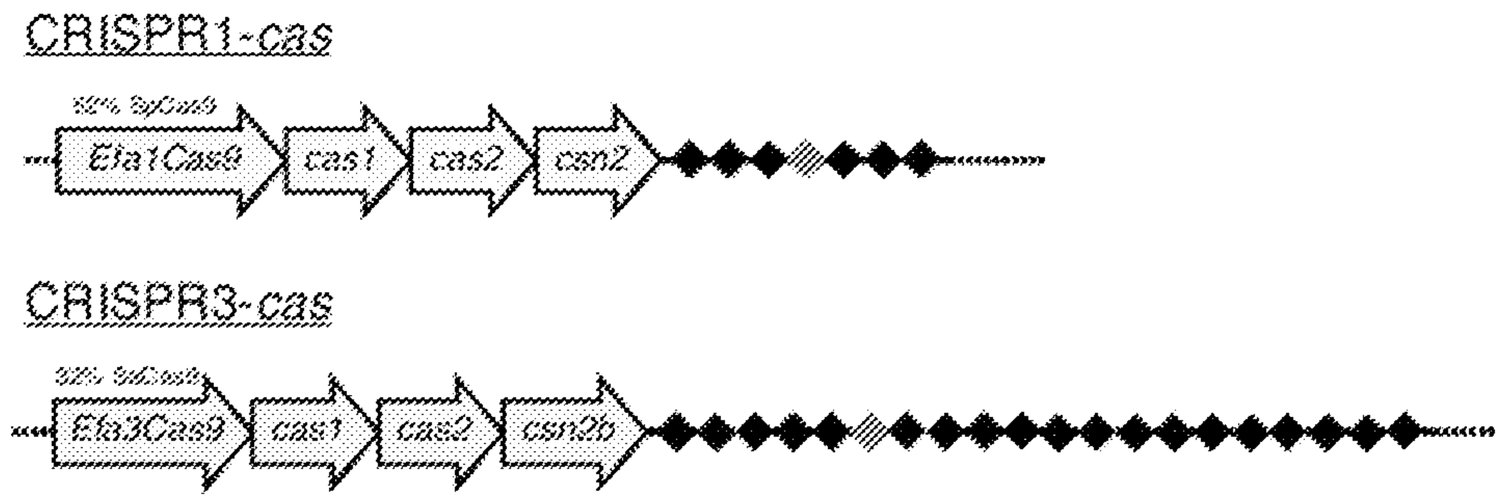


FIG. 6B

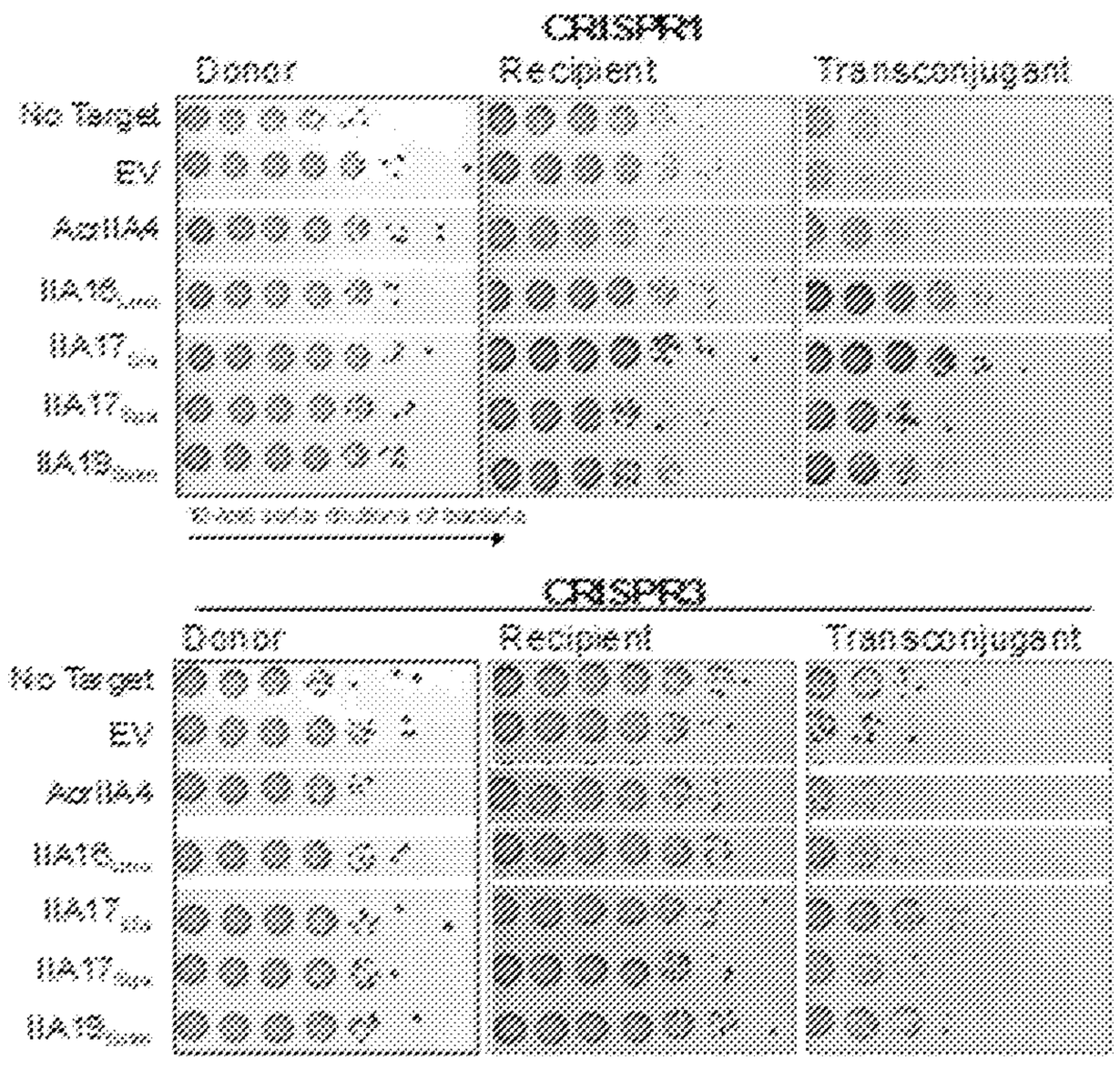
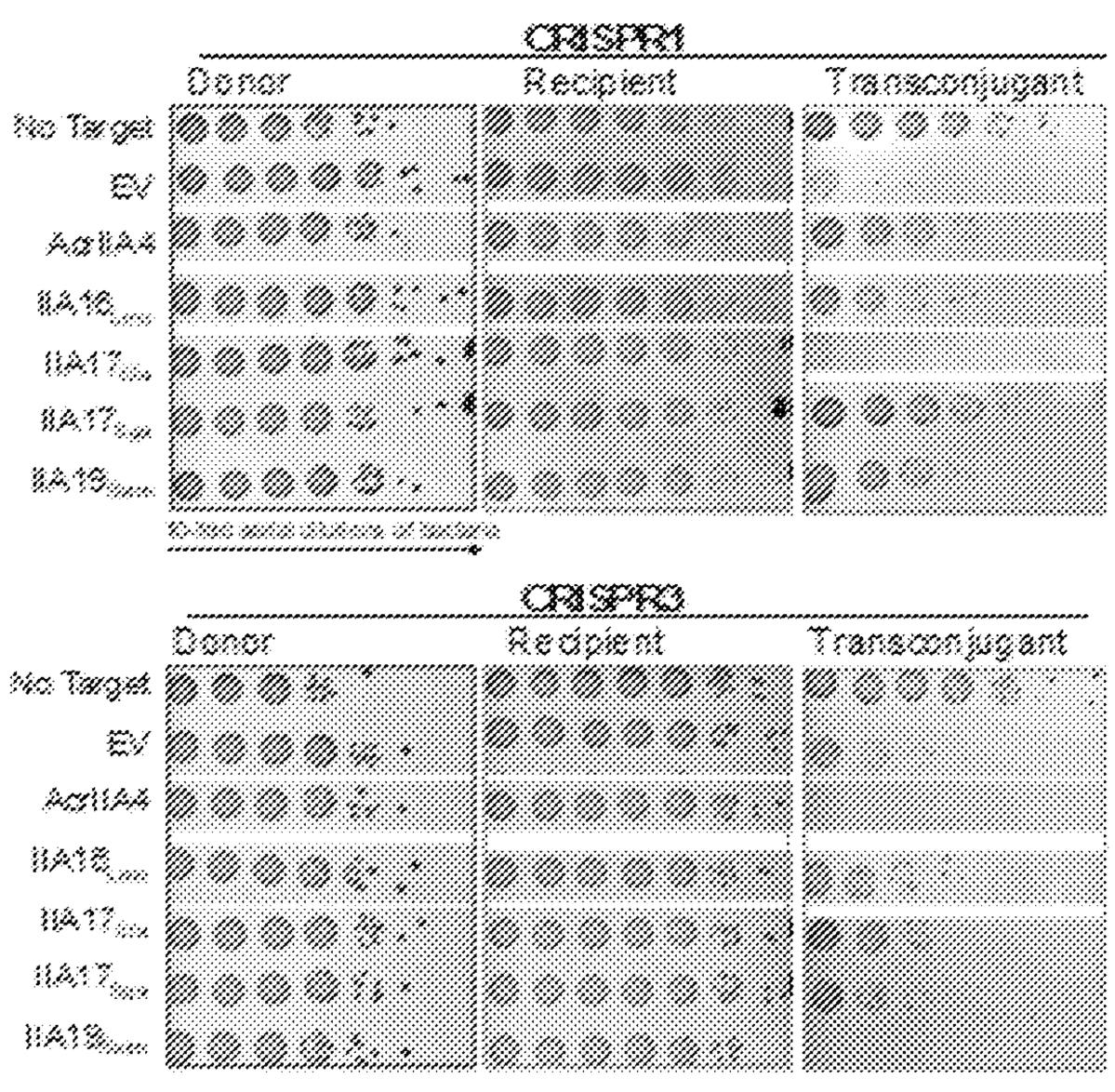


FIG. 6C



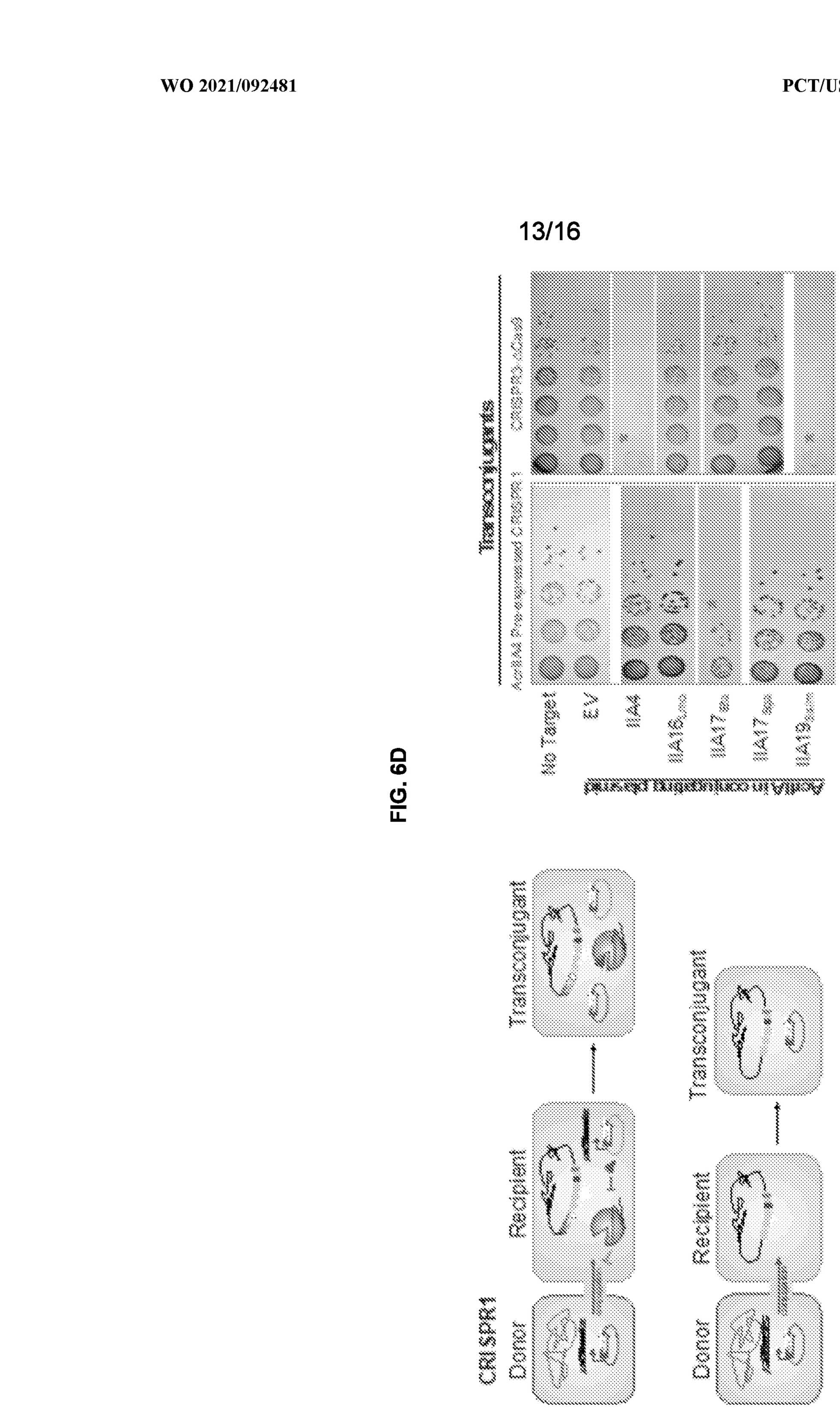
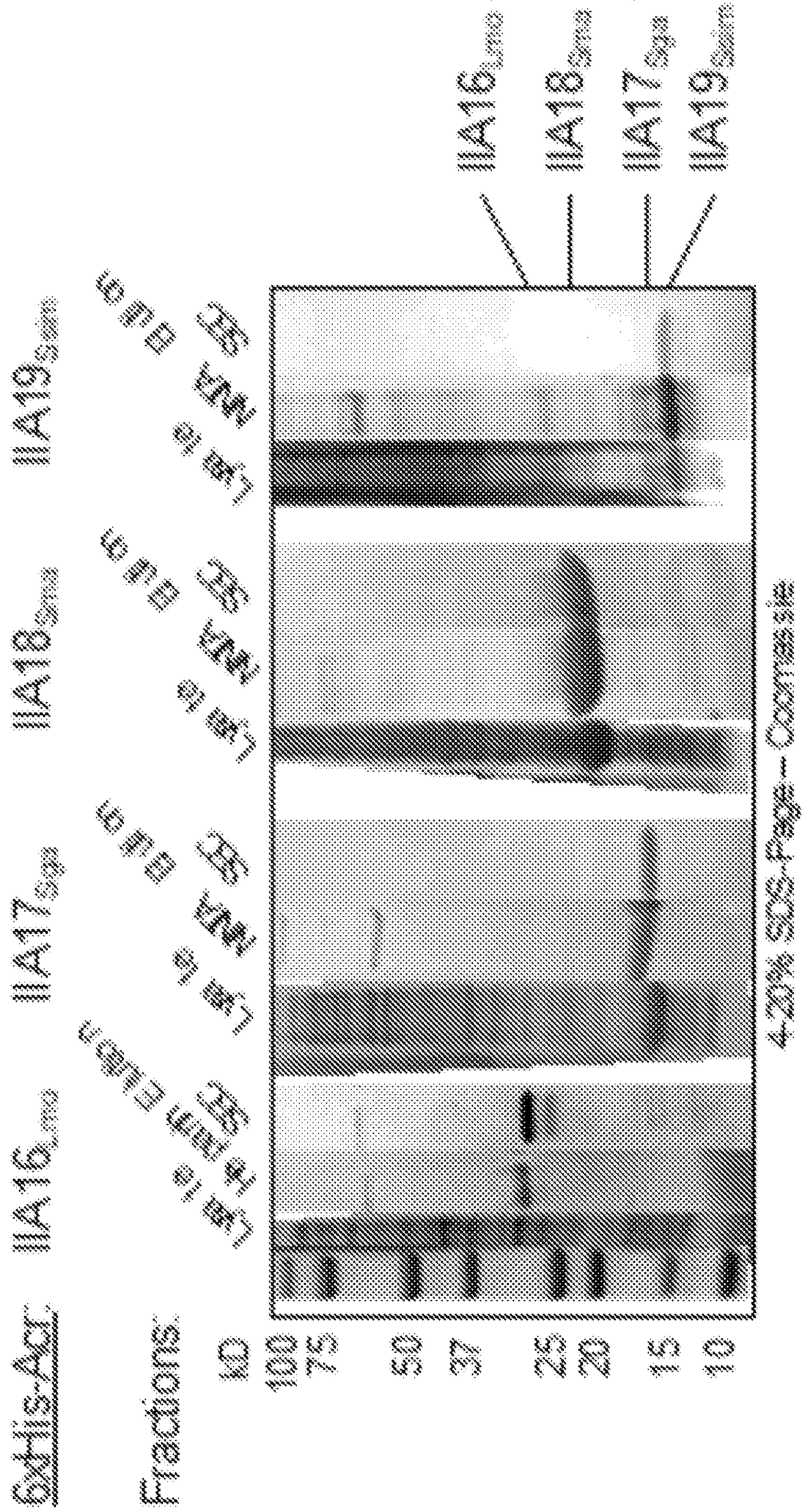

Transcriptome
Acetbul Pre-exposure Chlamydia Chlamydia Co-cultured
Cell Type Target
E7
IIA4
IIA16 sec
IIA17 sec
IIA17 sec
IIA19 sec
Acetabularia cell type target
Transplant
Transplant
Recipient
Donor
CRISPR
Recipient
Donor

FIG. 6D

FIG. 7A


IIA16 sec
IIA18 sec
IIA17 sec
IIA19 sec
4-20% SDS-PAGE – Coomassie
90
100
55
37
22
25
15
10
Fractions
Exhis-sec

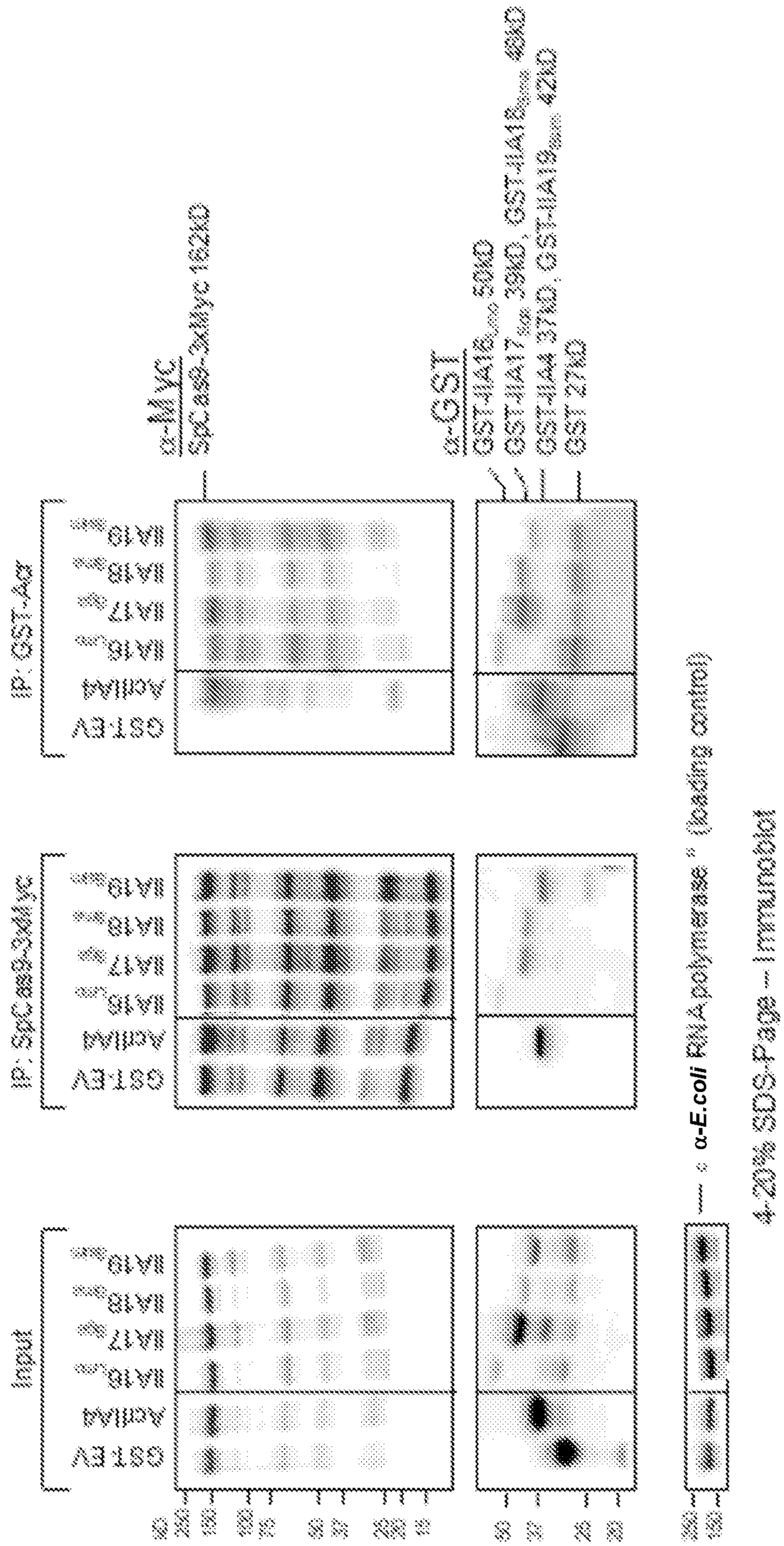

IP: GST-Ago
GST-EV
Ago1
Ago2
Ago3
Ago4
α-Myc
Ago1-4-Myc 180kD
α-GST
GST-Ago1 95kD
GST-Ago2 98kD
GST-Ago3 97kD
GST-Ago4 97kD
GST 27kD
IP: Ago1-4-Myc
GST-EV
Ago1
Ago2
Ago3
Ago4
4-20% SDS-PAGE – Immunoblot
α-E.coli RNA polymerase σ (loading control)
Input
GST-EV
Ago1
Ago2
Ago3
Ago4

FIG. 7B

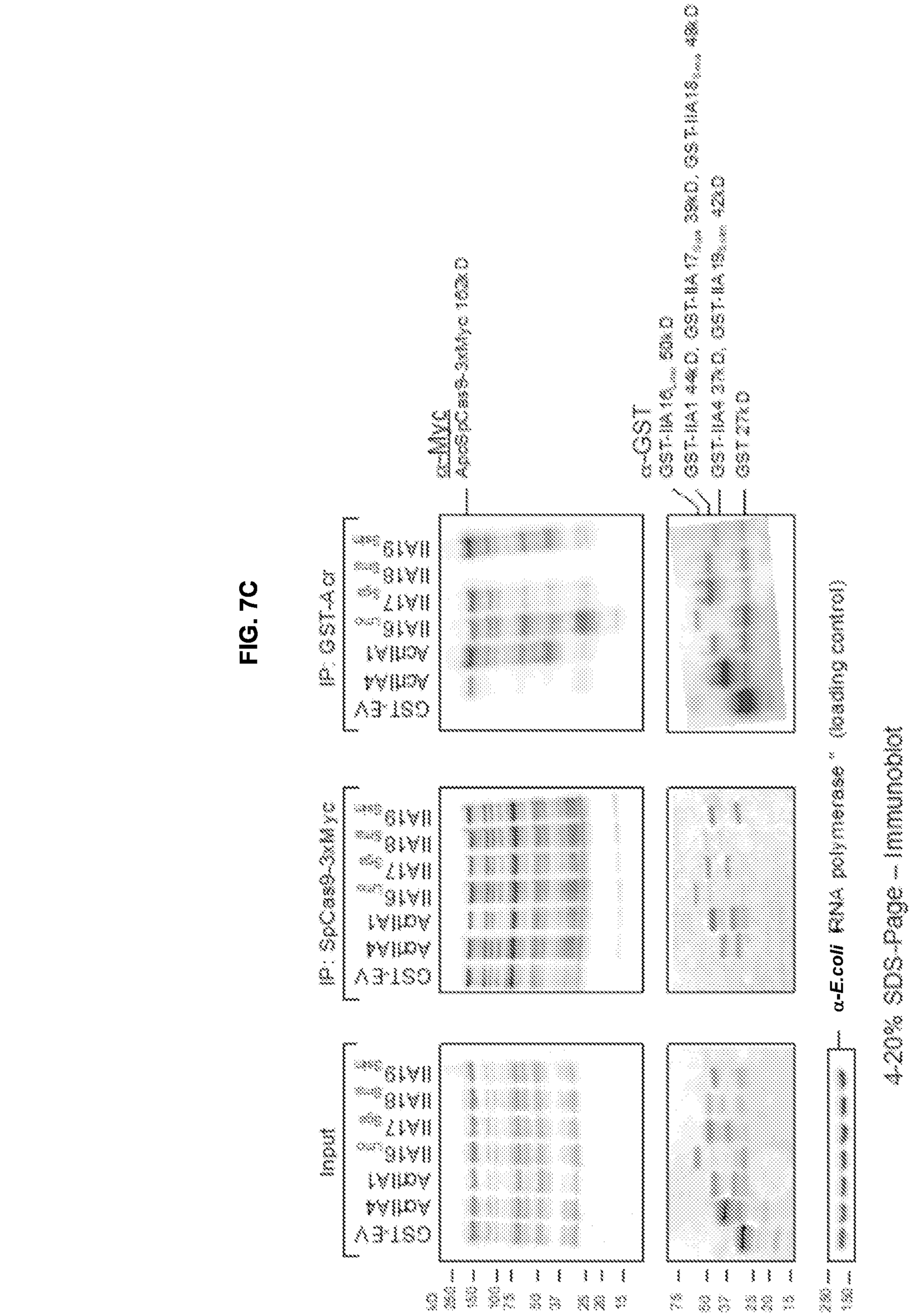

α-Cas9
α-GST
GST-IIA16 60kD
GST-IIA1 44kD, GST-IIA17 39kD, GST-IIA19 42kD
GST-IIA4 27kD, GST-IIA18 42kD
GST 27kD
IP: SpCas9-3xMyc
IP: GST-Acr
GST-Acr
AcrIIA4
AcrIIA1
IIA16
IIA17
IIA18
IIA19
α-E.coli RNA polymerase " (loading control)
Input
SDS-Page – Immunoblot
260
160
110
80
60
50
40
30
25
20
15
10

FIG. 7C