CRISPR/Cas revolution: application of the system in actinomycetes

4/20/2017

5th Methods and Techniques Lectures

Ewa Musiol-Kroll

Interfaculty Institute of Microbiology and Infection Medicine (IMIT)
Microbiology/Biotechnology
Prof. Wolfgang Wohlleben
Overview

- The CRISPR/Cas systems
  - Discovery
  - Adaptive immunity
  - Adaptation, expression and interference

- Implementation of CRISPR/Cas9

- Application of CRISPR/Cas9 for genome editing in Actinomyces
  - CRISPR/Cas9
  - CRISPR/dCas9
  - Pros and Cons of CRISPR/Cas9 implementation in Actinomyces
The acronyms CRISPR and Cas

CRISPR:
Clustered Regularly Interspaced Short Palindromic Repeats

Cas:
CRISPR-associated (genes/proteins)

http://rna.berkeley.edu/images/cas9_nature_cover.jpg
The history of CRISPR/Cas

**CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats**
Study related to *iap* (involved in isozyme conversion of alkaline phosphatase) in *E. coli*

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*ishino et al.*

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Coined “CRISPR” name, defined signature Cas genes
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Type III-B Cmr CRISPR complexes cleave RNA
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tracrRNA forms a duplex structure with crRNA in association with Cas9
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Type II CRISPR systems are modular and can be heterologously expressed in other organisms
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*Mali et al.*

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Cas9 is guided by spacer sequences and cleaves target DNA via DSBs
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*Shalem et al.*
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*Jinek et al.*
Crystal structure of Cas9 in complex with guide RNA and target DNA
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**Hsu et al., 2014**

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CRISPR/Cas

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Pourcel et al.

Identified PAM
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Hsu et al., 2014
http://crispr.u-psud.fr/crispr

Grissa et al., 2007
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- **2019**: CRISPR acts upon DNA targets
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Hsu et al., 2014
Pathogens and viruses

**Host cell**

**Innate immunity**
- Pre-existing, genetically encoded systems
- Recognize certain generic features of pathogens

**Adaptive immunity**
- Systems for specific immune response
- Development of immunological memory against previously unencountered invaders

Considered to be a feature found only in eukaryotes
Innate immunity
- Pre-existing, genetically encoded systems
- Recognize general features of pathogens

Adaptive immunity
- Systems for specific immune response
- Development of immunological memory against previously unencountered invaders

Many bacteria and archaea possess complex adaptive immune systems

Discovery of CRISPR and CRISPR associated (Cas) proteins (Ishino et al., 1987; Jansen et al., 2002)

Hypothesis formulated in 2005 (Bolotin et al., 2005; Mojica et al., 2005; Paurcel et al., 2005)

CRISPR/Cas demonstration in 2007 (Barrangou et al., 2007)
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Cas9 is guided by spacer sequences and cleaves target DNA via DSBs
Gameau et al.

Hsu et al., 2014
It was demonstrated that after viral challenge, bacteria integrated new spacers derived from phage genomic sequences.

Removal or addition of particular spacers modified the phage-resistance phenotype of the cell.

Thus, CRISPR, together with associated cas genes, provide resistance against phages, and resistance specificity is determined by spacer-phage sequence similarity.
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boosted the production of CRISPR/Cas data and CRISPR/Cas9 application
CRISPR-hits on *Science* homepage

6440 pages with hits on 09.03.2017!

6480 pages with hits on 10.03.2017!
CRISPR-hits on *Nature* homepage

2814 hits on 09.03.2017!

2829 hits on 10.03.2017!
The CRISPR/Cas locus
CRISPR locus consists of genes encoding Cas proteins, a leader sequence, and repeat and spacer sequences.

Amitai and Sorek, 2016 (Nature Reviews)
Different sets of Cas genes/proteins were identified

modified after Amitai and Sorek, 2016 (Nature Reviews)
Functional classification of Cas proteins (based on Cas signature protein)
In CRISPR array, the strain stores the immunological memory in the form of ‘spacers’— short DNA sequences originating from invading pathogens — that are interleaved with the CRISPR DNA repeats.

Locus organization

modified after Amitai and Sorek, 2016 (Nature Reviews)
Integration of CRISPR spacers
The key steps of CRISPR-Cas immunity are:

- **1) Adaptation**: insertion of new spacers into the CRISPR locus

- **2) Expression**: transcription of the CRISPR locus and processing of CRISPR RNA

- **3) Interference**: detection and degradation of mobile genetic elements by CRISPR RNA and Cas protein(s)
There are two types of spacer acquisition:

- naïve and
- primed

Both require the presence of a PAM and are dependent on the Cas1eCas2 complex.

The Cas1eCas2 complex recognizes the CRISPR and likely prepares it for spacer integration.

- Naïve spacer acquisition occurs when there is no previous information about the target in the CRISPR.
- Primed spacer acquisition requires a spacer in the CRISPR locus that matches the target DNA and the presence of Cas3 and the Cascade complex.
- Primed acquisition results in insertion of more spacers from same mobile genetic element.

*PAM: Protospacer Adjacent Motif*
Functional classification of Cas proteins (based on Cas signature protein)
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modified after Rath et al., 2015
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Expression and interference

Step 2 is the expression: transcription of the CRISPR locus and processing of CRISPR RNA
Step 3 is the interference: detection and degradation of mobile genetic elements by CRISPR RNA and Cas protein(s)
In Type I systems, the pre-crRNA is processed by Cas5 or Cas6
DNA target interference requires Cas3 in addition to Cascade and crRNA
While the Type I system (Cas3) cleaves and degrades one strand of the DNA, Type II (Cas9) generates double-stranded DNA breaks via its nuclease activities (cleavage of the target and non-target strand by RuvC and HNH domain of Cas9).

The compactness of Cas9 and programmability of double-stranded DNA breaks.
Functional classification of Cas proteins (based on Cas signature protein)

Single protein!!!!!
From prokaryotic defense to powerful biomolecular toolbox

Cas9 compactness

Cas9 programmability of double-stranded DNA breaks

Repair of DSB

Scientific breakthrough of CRISPR/Cas9

“All-in-One” protein

Introducing DSB at a defined position in the genome

Endogenous DNA-repair machinery

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From prokaryotic defense to powerful biomolecular toolbox

Scientific breakthrough of CRISPR/Cas9 (in particular as genome editing tool for eukaryotes)

Cas9 compactness
Cas9 programmability of double-stranded DNA breaks
Repair of DSB

Endogenous DNA-repair machinery

NHEJ
Nonhomologous end-joining (NHEJ)
Indel mutation
Premature stop codon

predominant in eukaryotic cells/organisms

HDR
Homology-directed repair (HDR)
Precise gene editing

predominant in prokaryotic cells/bacteria
(NHEJ complementation by Ku&LigD in bacteria)
The powerful biomolecular toolbox

Best known application: the development of Type II system into powerful genetic tool for engineering of eukaryotic cells and since recently prokaryotic cells

The system was simplified for practical use: Jinek and coworkers fused the processed version of the tracrRNA (=trans-activating crRNA) and crRN from Streptococcus pyogenes into a chimeric RNA termed “single-gude RNA” (sgRNA)

Combining crRNA and tracrRNA (=trans-activating crRNA) into a single guide RNA

Unlike Cas3, which degrades the target, Cas9 produces a single double-stranded break in the DNA-important feature for gene-editing

sgRNA

(= combination of crRNA and tracrRNA (=trans-activating crRNA) to a single guide RNA)
https://youtu.be/LcKwkQb_1fk
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sgRNA (= combination of crRNA and tracrRNA (=trans-activating crRNA) to a single guide RNA)
Application in *Actinomyces*

Example 1: CRISPR/Cas9 based genome engineering
CRISPR/Cas9 for actinomycetes

High-Efficiency Multiplex Genome Editing of *Streptomyces* Species Using an Engineered CRISPR/Cas System

Ryan E. Cobb,†‡ Yajie Wang,† and Huimin Zhao§†‡§

†Department of Chemical and Biomolecular Engineering, ‡Institute for Genomic Biology, §Departments of Chemistry, Biochemistry and Bioengineering, Center for Biophysics and Computational Biology, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801, United States

CRISPR-Cas9 Based Engineering of Actinomycetal Genomes

Yaojun Tong,† Pep Charusanti,†‡ Lixin Zhang,‖ Tilmann Weber,§†‡ and Sang Yup Lee§†‡§

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‖Department of Bioengineering, University of California, San Diego, La Jolla, California 92093, United States
§Metabolic and Biomolecular Engineering National Research Laboratory, Department of Chemical and Biomolecular Engineering (BK21 Plus Program), Center for Systems and Synthetic Biotechnology, Institute for the BioCentury, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 305-701, Republic of Korea
§Chinese Academy of Sciences, Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Beijing 100190, China
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The temperature sensitive pGM1190 was used for cloning of Cas9

Dr. Günther Muth
Interfaculty Institute of Microbiology and Infection Medicine (IMIT)
Microbiology/Biotechnology

The plasmid backbone
The system is based on Jinek et al. (2012)

- **Cas9** is a **nuclease**, an enzyme specialized for cutting DNA. It has two active cutting sites (HNH and RuvC), one for each strand of the DNA's double helix.

- The tracrRNA and spacer RNA were combined into a "single-guide RNA" molecule

- In presence of Cas9, DNA-target recognition and cutting occurs
The target strain and genes

- The target *Actinomyces* was *Streptomyces coelicolor* - the producer of:
  - undecylprodigiosin *(red)*
  - actinorhodin *(blue)* -> target compound

- Target genes:
  - *actI-ORF1*: encodes actinorhodin polyketide beta-ketoacyl synthase subunit gene
  - *actVB*: Actinorhodin polyketide dimerase flavin reductase (last step of the biosynthesis)
• **actI-ORF1**: encodes actinorhodin polyketide beta-ketoacyl synthase subunit gene

• **actVB**: Actinorhodin polyketide dimerase flavin reductase (last step of the biosynthesis)

The “homing device” sgRNA (=crRNA and the associated tracrRNA) was cloned with the 20nt target sequence within the sgRNA scaffold was inserted into NcoI and SnaBI sites

The StuI site was used for insertion of additional homologues regions and/or the “Scalid expression cassette (synthesized by GenScript) to complement the NHEJ pathway
sgRNA prediction tools

- sgRNA Scorer
- EuPaGDT Eukaryotic Pathogen gRNA Design Tool
- CRISPR-RT CRISPR RNA-Targeting
- Breaking-Cas
- CRISPR-DO
- CRISPR/Cas9 target online predictor
- CCTop
- ProtospacerWB Protospacer Workbench
- COSMID CRISPR Off-target Sites with Mismatches, Insertions, and Deletions
- CRISPR-P
- CRISPy
- CGAT CRISPR Genome Analysis Tool
- CLD CRISPR library designer
- CRISPOR
- Off-Spotter
- PhytoCRISP-Ex
- SSC Spacer Scoring for CRISPR
- CT-Finder
- CRISPETa CRISPR Paired Excision Tool
- Cas-Database
- CasFinder
- WU-CRISPR
- CRISPy-web

- Cas-Designer
- CRISPRscan
- CRISPR-ERA CRISPR-mediated Editing, Repression, and Activation
- Azimuth
- SSFinder
- CROP-IT CRISPR/Cas9 Off-target Prediction and Identification Tool
- CRISPR multitargeter
- CRISPRseek
- DESKGEN
- flyCRISPR
- CRISPRdirect
- CRISPR design
- sgRNAcas9
- Benchling
- Cas-OFFinder
- GT-Scan Genome Target Scan
- CRISPRer
- CasOT
- CHOPCHOP
- E-CRISP
- Cas9 Design
- CRISPR gRNA Design tool

https://omictools.com/crispy-tool
CRISPy-web: An online resource to design sgRNAs for CRISPR applications
Blin et al., 2016

http://crispy.secondarymetabolites.org/#/input
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Blin et al., 2016
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actIORF1 (SCO5087)

or

actVB (SCO5092)
actIORF1 (SC05087)

GTGGCTCGAAGGAGGCTCGA
AGCTCGATCAAGTCTGATGCT
GAAGCGCAGAGTCGATCTCA
CCCCTCGCCCTACCGGTTCAC
GCAGCGATATCTGCTGCTGT
CTGCAACGCCTACCACATGA
TGAGCCAGTCTCCAGAATCTC
AGGAGGCTCGAAGGCGGATA

...
Table S3 sgRNAs used in this study.

<table>
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<tr>
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<th>PAM</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>ActIorf1-1 NT</td>
<td>GGGCTCGAGAGAGGTCCGA</td>
<td>AGG</td>
<td>Gene deletion/expression control</td>
</tr>
<tr>
<td>ActIorf1-2 T</td>
<td>AGCTCGATCAAGTCTGATG</td>
<td>CGG</td>
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<td>ActIorf1-3 T</td>
<td>GAAGCGCAGAGTCTGCACTCA</td>
<td>CGG</td>
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<tr>
<td>ActIorf1-4 T</td>
<td>CCCCTCGCCCTACCCTCCAC</td>
<td>AGG</td>
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<tr>
<td>ActIorf1-5 T</td>
<td>GCAGGAGTCATCTGCTGCTGT</td>
<td>CGG</td>
<td>Gene deletion</td>
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<tr>
<td>ActIorf1-6 T</td>
<td>CTGCAACGCCCTACCCTGATGA</td>
<td>CGG</td>
<td>Gene deletion</td>
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<tr>
<td>ActIorf1-7 NT</td>
<td>TGAGCAGTCCCCAGAACTGC</td>
<td>CGG</td>
<td>Gene expression control</td>
</tr>
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<td>ActIorf1-8 NT</td>
<td>AGGAGGCTCGAGGCGGATA</td>
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<tr>
<td>Actvb-1 NT</td>
<td>TGGCCGCAACTGTCCGACAC</td>
<td>CGG</td>
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<td>Actvb-2 NT</td>
<td>CGGCATCTCCTCGAATCTCCCT</td>
<td>AGG</td>
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<td>Actvb-3 T</td>
<td>TCCCCGTTGTCTCCGACAGTTG</td>
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<td>Actvb-4 T</td>
<td>ACTGGTCTGCTGCTGGCTAGTA</td>
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<td>Actvb-5 NT</td>
<td>ATCTTCGAACCTCCCTAGGCC</td>
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<td>Actvb-6 NT</td>
<td>GTCCCGGAGACATTCTCTGGT</td>
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sgRNA for actVB and actI-ORF1

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<td>Actvb-3</td>
<td>TTCCGGGTGTCGCAAGAGCG</td>
<td>CGG</td>
<td>Gene deletion</td>
</tr>
<tr>
<td>Actvb-4</td>
<td>ACTGATCTGCTGCAAT</td>
<td>CGG</td>
<td>Gene deletion</td>
</tr>
<tr>
<td>Actvb-5</td>
<td>ATCTTCAACTGCCGAGACGC</td>
<td>AGG</td>
<td>Gene deletion</td>
</tr>
<tr>
<td>Actvb-6</td>
<td>GTCCCGAGCATCCCTCCTGT</td>
<td>CGG</td>
<td>Gene deletion</td>
</tr>
</tbody>
</table>

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Including homologous arms
Successful deletion/ mutant genotype

Control PCRs resulted in correct bands
Inactivation actVB and actl-ORF1

Phenotype and genotype were confirmed

Tong et al., 2015
Application
Example 2
Sometimes the repression of gene expression is preferred over gene deletion...

active Cas9 = Cas9
- endonuclease activity
- gRNA-dependent targeting of DNA

deadCas9 = dCas9
- NO endonuclease activity
- possessing the ability to target DNA depending on gRNA

- D10A, Asp10 → Ala10
- H840A, His840 → Ala840
Reversible repression

**active Cas9 = Cas9**
- endonuclease activity
- gRNA-dependent targeting of DNA

**deadCas9 = dCas9**
- NO endonuclease activity
- possessing the ability to target DNA depending on gRNA

Based on previous studies (e.g. Jinek et al., 2012)

- **D10A, Asp10 → Ala10**
- **H840A; His840 → Ala840**

The **CRISPRi system** is used to control the expression of target gene(s)

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One (actlorf1) of the previously used genes was targeted.
**sgRNA design**

The act cluster, ~22 kbp

Template strand

actIorf1

Nontemplate strand

and = sgRNA

modified after Tong et al., 2015
Reversible repression actl-ORF1

The act cluster, ~22 kbp

Template strand

actlorf1

Nontemplate strand

actlORF1

CRISPRi-based silencing of actlorf1

CRISPR/Cas

modified after Tong et al., 2015
The CRISPR/Cas-plasmids are available from Weber/Sang Yup-Lee´s-lab and Günther Muth/Wohlleben´s-lab

Tong et al., 2015
Only limited number of applications in actinomycetes and consequently limited knowledge about its effects

Off-target effects leading to undesired rearrangements in the genome (e.g. undesired deletions when targeting genes of duplicated sec. metabolite clusters)
Unexpected rearrangements

- Whole genome sequencing of selected mutants and sequence analysis
- NON-precise deletion (e.g. 37 kb were lost)

Tong et al., 2015
Disadvantages of *Actinomyces* CRISPR/Cas

- Only limited number of applications in actinomycetes and consequently limited knowledge about its effects

- Off-target effects leading to undesired rearrangements in the genome (e.g. undesired deletions when targeting genes of duplicated sec. metabolite clusters)

- Eventually, whole genome sequencing to confirm the mutant genotype

- Stability(?)/ temperature sensitive replicon
Advantages of Actinomyces CRISPR/Cas9 and/or CRISPR/dCas9

- Highly efficient system for genome engineering (including homologues regions resulted in almost 100% efficiency)
  - Deletion
  - Insertion
  - Sequence replacements
  - Libraries of deletions of a target gene
  - Reversible control of gene expression using dCas9

- Not only genes, but whole gene cluster can be deleted (development of “super-hosts” or “minimal-hosts”)
- One-plasmid-based system
- Easy elimination of the temperature sensitive plasmid
- Relatively easy to handle (design of sgRNA)
- NHEJ can be reconstructed by complementation with Ku and/or LigD
THANK YOU!