THE USE OF CRISPR/CAS SYSTEMS AS AN ALTERNATIVE TOOL TO OVERCOME ANTIBIOTIC RESISTANCE

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Introductions. Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR-associated proteins) are specific repeating DNA sequences found in the genomes of prokaryotes, such as bacteria and archaea. It is a revolutionary gene-editing technology that enables scientists to precisely modify DNA in living cells. Therefore, this tool has the potential to revolutionize many fields, including medicine, agriculture, and biotechnology, as it can lead to the development of new treatments for genetic disorders, the improvement of crop yields and resistance to pests and diseases, advancements in the production of bioactive compounds, etc.

One of the leading public health threads of the 21st century is the emergence of antibiotic-resistant strains. Antibiotic resistance (AMR) occurs when bacteria evolve mechanisms to resist the effects of antibiotics, making these drugs ineffective in treating bacterial infections. The search for new antibiotics is a complex and challenging process that is hindered by inherent difficulties. Furthermore, the rate of emergence of antibiotic resistance far exceeds the rate of development of new antibiotics, which is not sufficient to combat the increase of this problem. It is thus necessary to develop new alternative approaches, including methods of genetic manipulation, to limit antibiotic resistance in pathogens.

Therefore, the aim of this work is to analyze the potentiality of using CRISPR/Cas systems to overcome the problem of antibiotic resistance.

Materials and methods. Bikard et al. [1] assembled the pDB91 phagemid by amplifying the rinA-terS-terL region and packaging site of staphylococcal ΦNM1 followed by cloning into pC194 in *S. aureus* strain RN4220 competent cells. The plasmid pDB121, which carries the *S. pyogenes* tracrRNA, Cas9 and a minimal array containing two repeats separated by a sequence containing BsaI restriction sites used to clone crRNA guide sequences using annealed oligonucleotides, was cloned using the Gibson assembly method. Obtained pDB121 plasmids with different targeting sequences were then cloned into pDB91 [1].

Phage stocks were produced by growing RN4220 cells with a phagemid containing the desired CRISPR spacer in TSB+Cm+$CaCl_2$, followed by incubation of cultures with a concentrated ΦNM1 phage stock, centrifugation of the cell culture, and filtration of the supernatant. Recipient cells were grown in TSB with the following addition of $CaCl_2$ in exponential phase, mixed with the phage stock dilution, and plated on TSA after 1h of incubation. Survival rates were measured as the ratio of CFUs obtained with treatment over CFUs obtained without treatment. The phagemids were used to treat isolates of *S. aureus* strain USA300, which carries the methicillin resistance gene mecA and the conferring tetracycline resistance pUSA01-2 plasmid, in a mixed culture with RN cells. The following analysis included the growth curves, fluorescence measurements and flow cytometry [1].

Kim et al. [2] constructed a plasmid, pRESAFR$_{ESBL}$, that produced Cas9 protein under the BBa_J23102 promoter, tracrRNA, and crRNAs targeting TEM- and SHV-type ESBLs, by cloning crRNAs into pCAS9. *E. coli* strain that is resistant to both ampicillin (Amp) and ceftazidime (Cef) was manufactured by transformation of *E. coli* BW25113 with the plasmid pESBL isolated from *K. pneumoniae* K01-Bact-08-03094 through conjugation. The obtained ESBL strain was then transformed with either pRESAFR$_{ESBL}$ or pCas9 that expressed only Cas9, serially diluted and plated onto LB agar plates containing chloramphenicol (Clm) or Clm and Amp in combination. The re-sensitization ratio was calculated from colony-forming units after overnight incubation at 37°C [2].

Tagliaferri et al. [3] had used the Type II CRISPR-Cas9 system to control AMR by insertion of bla$_{TEM-1}$ targeting sgRNA into the plasmid pSB1C3 containing the *Streptococcus pyogenes* CRISPR-Cas9 locus. Tests were carried out on the non-AMR *E. coli BL21* model strain, followed by three other clinical *Enterobacteriaceae* samples—*E. coli* 189A, *Enterobacter hormaechei* 4962 and *Klebsiella variicola* 68AI isolated from bacteremia patients. *E. coli* BL21 was transformed with both pSB1C3 and pSB1A2 containing the bla$_{TEM-1}$ gene for Amp resistance by the heat-shock method, and the clinical isolates received the CRISPR-Cas9 plasmid pSB1C3 by electroporation. Bacteria were then cultivated in LB+Clm medium at 37°C with and without Amp with the following growth kinetics recording in every hour for 24 h [3].

Results and discussion. After treatment of exponentially growing USA300 and RN cells mixed 1:1 with pDB121::mecA and non-targeting pDB121 cells were plated on a non-selective, oxacillin-containing or chloramphenicol-containing media to measure the proportion of cells receiving the phagemid treatment or the proportion of USA300Φ cells in the population relatively (Fig. 1. a). As a result, treatment with phagemid::mecA successfully reduced the proportion of USA300 from 50% to 0.4%, and so proved to be selectively killing methicillin-resistant strains within a mixed population [1, 4].

As for tetracycline-resistant plasmids, in all cases when pUSA01, pUSA02 or both were targeted, cell death was not observed, but more than 99.99 % of the cells became sensitive to tetracycline, the majority of them due to the loss of pUSA02 (Fig. 1. b) [1].

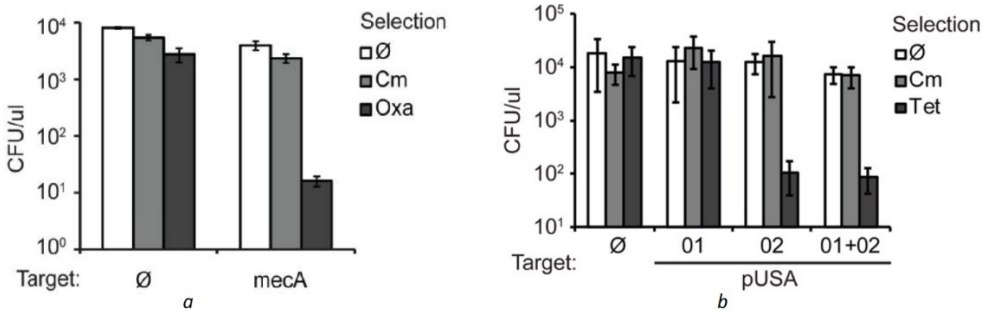
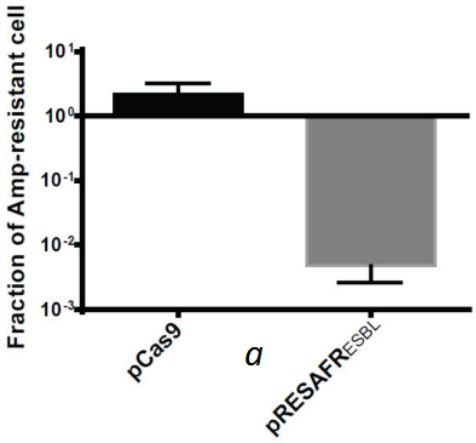


Fig. 1. Targeting antibiotic resistance genes and plasmids in an MRSA strain: a – the numbers of colony-forming units detected on different media after treatment with pDB121::mecA and pDB121⌀, b - the numbers of colony-forming units after treatment of USA300Φ with pDB121 lysates targeting plasmids pUSA01-2 individually or in combination [1].

The transformation of pRESAFR$_{ESBL}$ into the ESBL *E. coli* strain containing pESBL led to the death of more than 99% of cells upon treatment with Amp (Fig. 2. a), suggesting that the presence of targeting plasmid caused the ampicillin sensitivity in previously resistant cells. In addition, although resistance to Cef is mediated by CTX-M β-lactamases with different target sequences, tests against several antibiotics showed the obtained sensitivity to Cef in cells with pRESAFR$_{ESBL}$ (Fig. 2. b) indicating that constructed plasmid affected other resistance genes. Multiple ESBL genes are frequently carried together in a single plasmid and, therefore, the double-stranded break in the target sequence of TEM and SHV induced clearance of the whole plasmid, sensitizing the ESBL strain to different antibiotics [2].



Antibiotics	Diameter of inhibition zone (mm)			
	E. coli BW25113	*E. coli* BW25113 (pESBL)		
		None	pCas9	pRESAFR$_{ESBL}$
Chloramphenicol (34 μg)	17	18	-	-
Ceftazidime (1 μg)	18	-	-	20.0±0.63
Ampicillin (10 μg)	15	-	-	16.6±1.02

b

Fig 2. Re-sensitization to antibiotics of *E. coli* carrying ESBL plasmids by the ReSAFR system: a – the numbers of the antibiotic-resistant cell fraction for cells with pCas9 and pRESAFR$_{ESBL}$ obtained by comparing the colony-forming units (CFU) after treatment with ampicillin and the CFU before treatment, b - diameters of the inhibition zones after treatment with several antibiotics [2].

Transformation of pSB1C3 containing CRISPR-Cas9 system successfully reversed AMR in the model TEM+/CRISPR+ strain (Fig. 3. a, b.) by reduction of pSB1A2 in 99.995% of cells [3].

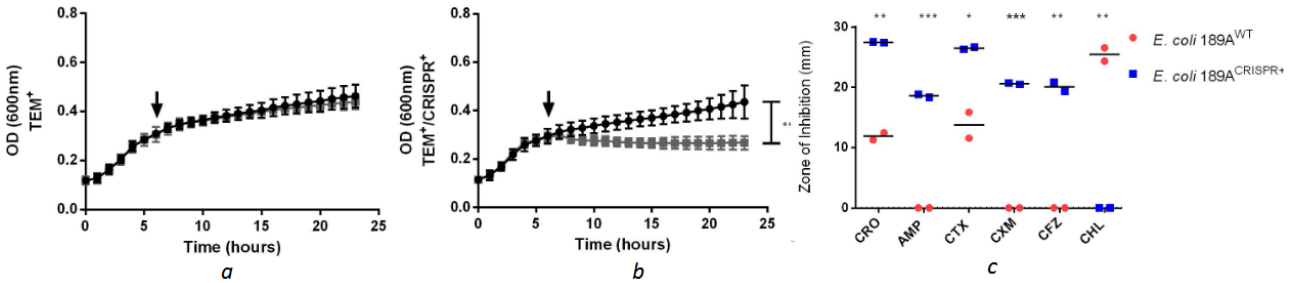


Fig. 3. Effect of the CRISPR-Cas9-based interference with the blaTEM–1 gene in different strains: a – growth of *E. coli* BL21 containing the blaTEM–1 resistance gene in the presence (gray curve) or absence (black curve) of ampicillin, b – growth of *E. coli* BL21 containing blaTEM–1 and CRISPR-Cas9, c – measurement of inhibition zones showing re-sensitization to several antibiotics in *E. coli* 189A [3].

In the case of clinical isolate *E. coli* 189A, re-sensitization to Amp, cefazolin (Cfz), cefuroxime (Cxm), ceftriaxone (Cro), and cefotaxime (Ctx) (Fig. 3. c) was observed as a consequence of complete plasmid clearance. On the contrary, *E. hormaechei* 4962 and *K. variicola* 68AI showed lower efficiency of re-sensitization with some plasmid retention, due to damages in CRISPR-Cas9 locus and the presence of other, not targeted, resistance genes [3, 5].

Conclusions. Based on the results of the conducted analysis, CRISPR/Cas systems were determined to be a tool with immense potential for treating antibiotic resistance. The results of several studies have shown that these systems can selectively recognize and re-sensitize antibiotic-resistant bacteria by targeting the resistance genes and editing their sequence, followed by plasmid clearance, as well as destroy them through chromosomal damage. Additionally, CRISPR/Cas systems can be used to eliminate bacterial infections in a less harmful way to healthy cells than traditional antibiotics, as they can be targeted to specific bacteria, leaving the healthy microbiome intact. Therefore, this alternative tool can be utilized to bring some respite to the problem of antibiotic resistance.

References:

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