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ELABORATO DI LAUREA

ASSEMBLY OF GENETIC PARTS FOR CRISPR MEDIATED INTERFERENCE AGAINST ANTIMICROBIAL RESISTANCE

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ABSTRACT

This thesis presents parts of a collaborative project designed by a team of the University of Padua that is going to participate to the iGEM competition on synthetic biology. The project aims to exploit the CRISPR interference system to contrast antimicrobial resistance (AMR). The idea we aim to develop consists in delivering a CRISPR interference system, targeting antibiotic resistance genes, by engineered bacteriophages. These phages will be modified to specifically infect one of the following hosts: *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. For a hypothetical application, the platform would be used in a synergic approach with antibiotic therapy to treat infections caused by antibiotic resistant bacteria. In particular, the thesis deals with the construction of a dCas9 and sgRNAs expression systems able to function in the above mentioned bacteria. Specifically, plasmids described in the scientific literature were engineered, mainly through PCR mutagenesis and the BioBrick RFC [10] standard assembly. The use of this standard required the mutagenesis of the dCas9 used in the project. The creation of these standardized parts, promoted by iGEM itself, aims to simplify updates of target genes and bacterial hosts for future implementations, raising a possible solution to the global issue of AMR.

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1. STATE OF THE ART

1.1 The issue of antimicrobial resistance

The antimicrobial resistance (or AMR) is a phenomenon that consists in the evolution or acquisition from the environment, through horizontal genetic transfer, of one or more genes that change the phenotype of an organism (most of the times, a microorganism is involved) from susceptible to molecules such as antibiotics, antifungals, antivirals, or antiparasitics, to resistant.
The consequence is the inefficacy in treating infections in humans, animals and crops, especially when they are caused by multi-resistant bacterial pathogens.
The World Health Organisation (WHO) has declared AMR as one of the top 10 global public health issues and according to the 2021 AR-ISS report, Italy is the second European country for AMR cases. In his review, O'Neill (O'Neill, 2014) estimated that without plans to stop the spread of the phenomenon, by 2050 10 million deaths per year would occur and the livestock will decline by 7,5%. Furthermore, the World Bank predicted a reduction in the economic output such that some of the 2030 Goals, like ending poverty, will be harder to reach.
Moreover, the production of new antimicrobials is expensive and too slow in comparison with the development of multi-drug resistant bacterial strains, and it would not stop the underlying evolutionary mechanism of the problem.
The responsibility for infections difficult to treat mainly belongs to six bacteria, which are summarized by the acronym ESKAPE: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.
The WHO has started an awareness campaign about the AMR and a responsible usage of antibiotics in health and animal care and has raised the need for more controls and data from all the nations to prevent a misuse in animal husbandry and agriculture. Many research groups are studying innovative approaches and a promising one is the use of the CRISPR/Cas9 technique. It has been already used as an antimicrobial against *S. aureus* and *E. coli* delivered by a phagemid vector (Citorik et al., 2014, Bikard et al., 2014), aiming to target the kanamycin resistance gene and the *bla* gene (coding for a beta-lactamase) respectively. Both studies revealed the specificity of the system and the re-sensitization to the two antibiotics.

1.2 The iGEM project PASTA

Given the significant impact of AMR at different levels, the team MUTANS from the University of Padua has decided to design a project aiming to counteract this global problem, especially in relation with the human health care, and in order to participate to the international iGEM (The International Genetically Engineered Machine) competition on synthetic biology. The purpose of the competition is not to find a solution with an immediate application, but to create the bases for its development and improvement.

This project, named PASTA (Phage Assisted Silencing Tool against AMR) is composed by two main parts: the first deals with the implementation of a Delivery phage particle with a modified tropism and carrying a phagemid vector (I) while the second, the Actuator, aims to clone into properly engineered plasmids a CRISPR interference expression system targeting specific genes connected to the antibiotic resistance or to the pathogenesis of *A. baumannii* ATCC 19606, *K. pneumoniae* ATCC 13883 and *P. aeruginosa* ATCC 10145 (all gram-negative bacteria). Then, there are two main options: cloning the Actuator system into the phagemid, or adding the encapsidation sequence to the dCas9-plasmids. A summary of the entire project is illustrated in Figure 1.

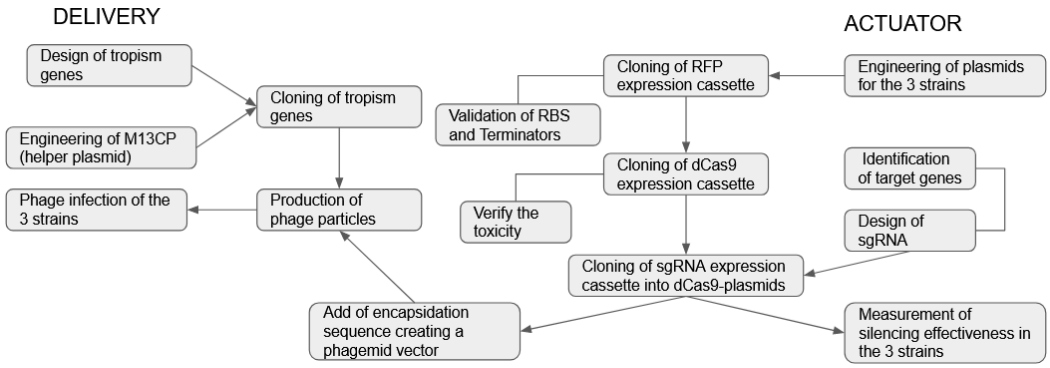


FIGURE 1| Overview of the PASTA project, composed of the "Actuator" and "Delivery" parts. The "Actuator" consists in preparing plasmids (capable of replicating in the three bacterial strains of interest) engineered in order to express a RFP marker and to contain genetic parts (a cassette for dCas9 and specific RNA guides) to silence resistance genes. The plasmids, transformed into phagemids and bearing the RNA guides for the CRISPRi mechanism, will be used together with a helper plasmid to produce phage particles capable of infecting the three bacteria of interest and to silence their resistance genes.

One of the main challenges was to design the platform described above exploiting standard biological parts. Indeed, iGEM promotes the use of a standard assembly, the BioBrick RFC [10], according to which the so called BioBricks are joined through two restriction enzymes (EcoRI and XbaI, SpeI and PstI) that flank each genetic sequence or cassette. The four restriction sites are comprised respectively in the Prefix (22 bp) and Suffix (21 bp) (Figure 2). This type of standard was the primarily one applied in the project. Of course, all the chosen parts, including the plasmid's source, had to be devoid of other sites for the four REs used in the assembly: the iGEM Registry provides already compatible and well described Parts. Some of them have the status "In stock", meaning that they can be requested to iGEM.

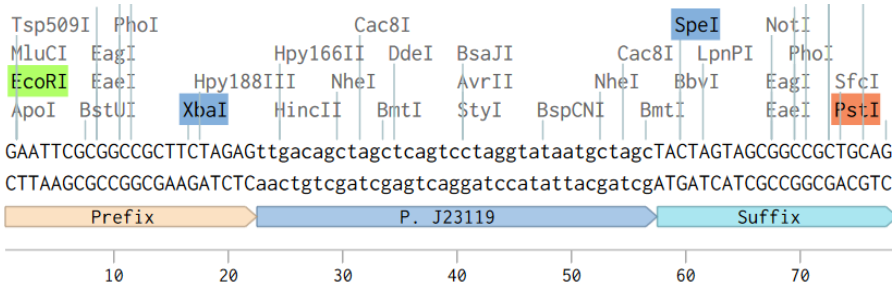


FIGURE 2| Example of a genetic Part between Prefix and Suffix. XbaI and SpeI are isocaudamers. J23119 is a Promoter often used upstream a sgRNA for a CRSPR/Cas or a CRISPRi system.

This thesis focuses on the preparation of the expression cassettes for a d(Sp)Cas9 protein and the sgRNA and their assembly into plasmids able to replicate in *A. baumannii* and *K. pneumoniae* (2). To take the final decisions regarding which Promoters, RBSs and Terminators could be suitable, it was necessary to find information in literature, and to integrate it with experimental evidences obtained during the laboratory activities.

1.3 The CRISPR interference method

As previously mentioned, the idea behind the project is to inactivate antibiotic resistance gens by CRISPR interference and to accomplish that we need to transfer the genetic mechanism of CRISPRi within bacteria.
The well-studied CRISPR/Cas system has been exploited for genome editing over the years. The proteins associated with the different classes of CRISPR, the so-called Cas, have domains with nuclease activity (Figure 3.A). Class 2 includes various types where a single protein with many domains, such as Cas9 in type II (the simplest), performs nuclease activity against a target DNA sequence once it has bound a guide RNA (crRNA) which is complementary to the target DNA itself. The crRNA must be previously activated by a trans-activating RNA (trascRNA) in order to bind Cas9. The subsequent engineering of the mechanism led to the creation of a single RNA sequence necessary for the function of Cas9, called single guide RNA (sgRNA). Furthermore, a short proto-spacer adjacent motif (PAM) must be present at the 3' end of the target, determining the binding and cleavage specificity of Cas9. The CRISPR/Cas system is naturally present in bacteria as an adaptive immune system, and crRNAs are encoded from genetic clusters in the bacterial genome and used to target exogenous DNA previously encountered by the bacterium. Therefore, there are several bacterium-specific isoforms of Cas9, the most used being the one isolated from *Streptococcus pyogenes* (SpCas9), which specifically requires a 5'-NNG-3' PAM sequence. Point mutations of the PAM totally prevent the binding of the Cas9.
Within the project, it was decided to exploit a CRISPRi system, in which the two nuclease domains of the Cas9, precisely the SpCas9, are mutated and inactivated. Thus, the obtained dead Cas9 (dCas9) binds DNA in the same way the wild-type does, but the dCas9:sgRNA complex performs the transcription repression (Figure 3.B) or the modulation of the expression of the target (not shown here).

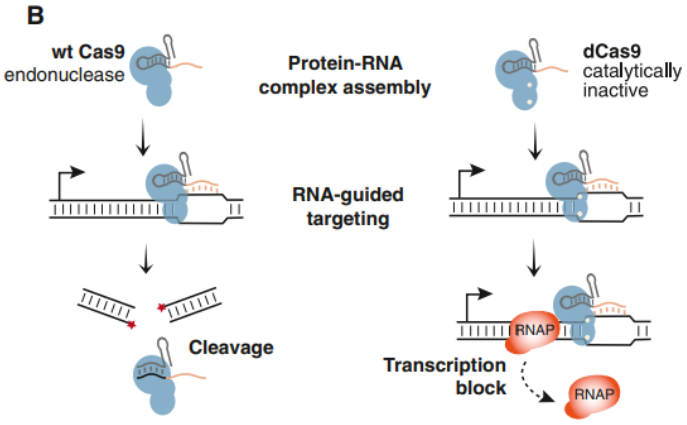


FIGURE 3| B) Binding and cleavage of the target sequence by the wild-type Cas9:sgRNA complex (left). Repression of RNA polymerase (RNAP) progression along the DNA by the dCas9:sgRNA complex (right). The two white points represent the D10A and H841A mutations. (Qi et al., 2013)

The decision to opt for a dCas9 is explained by the possibility of re-sensitizing bacteria to antibiotics by silencing resistance or pathogenesis genes, such as those related to biofilm formation. Furthermore, by targeting non-essential genes, the selective pressure that leads to the formation of bacteria escaping the CRISPRi mechanism is lower than a CRISPR/Cas9, since the main cause of death of the bacterium would remain the antibiotic. Obviously, evolution of resistant strains it is still possible, but in the case of mutations that occur on the complementary sequence of the guide RNA, the latter can be easily and quickly replaced in the host vector to silence new genes, after the characterization of the novel strain.

1.4 sgRNA design

The sgRNA is composed of a 3' constant region, the scaffold, and a 5' variable region, corresponding to 17-24 nt complementary to the target DNA (Figure 4).

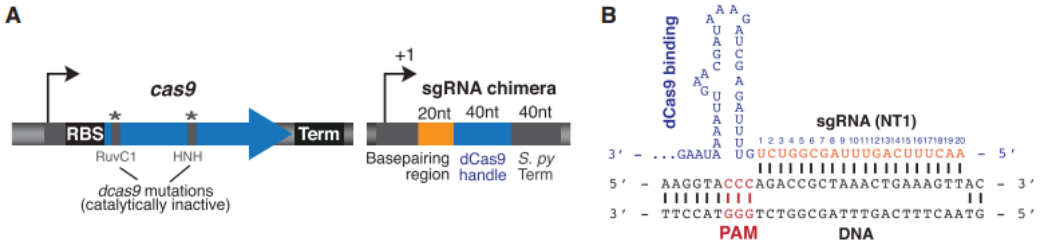


FIGURE 4| A) Example of the composition of a sgRNA: the basepairing region of about 20 nt, the scaffold composed by 40 nt dCas9 handle, the hairpin that binds the dCas9, and 42 nt of the terminator from *S. pyogenes*. B) sgRNA:DNA complex downstream the PAM sequence. (Qi et al., 2013)

Given an annotated bacterial genome, if the goal is gene repression, the sgRNA has to bind either the non-template (NT; or coding) DNA (so the sgRNA coding sequence should be equal to the template DNA upstream the PAM, Figure 4.B) or the promoter. But in the case of the described project there was insufficient knowledge on the regions upstream of the genes of interest. Furthermore, the silencing efficacy generally depends on the binding distance of the guide from the transcription start site (TSS), in an inversely proportional manner. The

greater the distance from the TSS, the greater the likelihood of truncated protein formation (Figure 5.B). Another important feature is that the required GC [%] content is between 30 and 70.

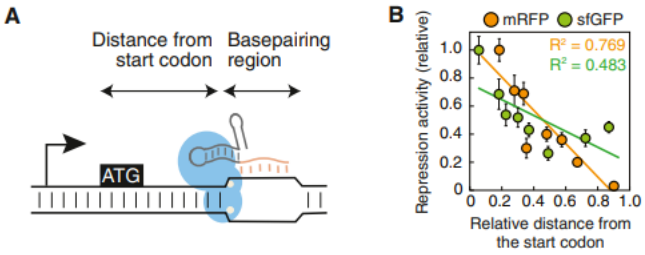


FIGURE 5| A) Binding of dCas9 to NT DNA of a coding sequence. B) In general, it is optimal that the guide binds by the 20% of the coding region. (Qi et al., 2013)

One of the most critical difficulties in using CRISPR/Cas, and thus CRISPRi, is the design of a specific sgRNA to prevent a high number of off-target sites. Cui et al (2018) reviewed a list of tools for on and off-target predictions in the proper design of sgRNA, such as CHOPCHOP (https://chopchop.cbu.uib.no/) and Cas-OFFinder (http://www.rgenome.net/cas-offinder/) respectively. It is important that there are no single base mutations and therefore no mismatches with the target DNA in the first 12-7 nt at 3' end (seed region) of the guide; otherwise, the effectiveness and specificity of the system decrease. A further option is to target the same gene with two guides simultaneously, hence with two dCas9:sgRNA complexes. According to Anderson et al., (2021), two guides must be at least 40 nt apart to not interfere with each other.

2. MATERIALS AND METHODS

2.1 PCR Mutagenesis

The Quick Protocol for Q5® Site-Directed Mutagenesis Kit (from New England Biolabs™) was used for the PCR mutagenesis of the EcoRI site inside the dCas9 coding sequence carried by the plasmid J116-dCas9_3k3. The used protocol includes some points that may depend on different specific cases and operator's choices, and only those specific cases and choices are explicitly reported below, as well as any optimizations and variations made to them during the transformation phase of the *E. coli* TOP10 chemocompetent cells.

2.1.1 PCR reaction setup

The Forward and Reverse Primers used for the PCR (5.1 section of this document) were designed on Benchling (www.benchling.com). They are non-overlapping and the point mutation, responsible for creating a mismatch between the Template DNA and a cytosine at the position 13 5'-end of the Primer itself, is present in the Forward Primer.
The reagents necessary for the PCR reaction have been aliquoted following the amounts indicated in the protocol (Q5 Hot Start High-Fidelity 2X Master Mix, Forward and Reverse Primer, Nuclease-free Water and the Template DNA) with the final reaction volume being 25 μl. In particular, the Template DNA was obtained from a colony inoculated on 5 ml LB+kanamicyn starting from the stock at -80°C of *E.coli* harboring the plasmid J116-dCas9_3k3. The inoculum was incubated at 37°C 220 rpm O/N. The next day, after using the QIAprep® Spin Miniprep Kit (the first centrifugation was performed at 4°C and the final eluition was made with 30 μl of Nuclease free water), the concentration of the sample DNA and $\frac{260}{280}$ and $\frac{260}{230}$ ratios were measured by the NanoDrop (Thermo Scientific™).
Table 1 shows the PCR steps with any specifications.

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25 Cycles	98°C	10 seconds
	Tm*	20 seconds
	72°C	3:30 minutes**
Final Extension	72°C	2 minutes
Hold	4°C	

TABLE 1| Cycling Conditions. * The Tm of 65°C suggested by NEB Tm Calculator™ did not work, therefore it has been tested a gradient of lower temperatures: 63°C, 60°C and 58°C (as recommended in the Troubleshooting section of the protocol);
**30 seconds/kb (in this case, 7kb).

2.1.2 Gel electrophoresis

The PCR products were analyzed on a 1% agarose gel electrophoresis where 15 μl of each sample mixed with 5 μl of loading dye (Thermo Scientific™ TriTrack 6X) were loaded. The expected band was about 7 kb, which then had to be compared with the ladder (Thermo Scientific™ GeneRuler 1 kb).

2.1.3 KLD reaction setup and transformation

The resulting positive sample with the best quality was selected for setting up the KLD reaction (Kinase, Ligase and DpnI enzymes) with 2X KLD Reaction Buffer, 10X KLD Enzyme Mix and 10X KLD Enzyme Mix, and after a 5-minute incubation, the transformation was executed with the following instructions:
-add 10 μl of KLD reaction product to the 100 μl of TOP10 competent *E. coli* cells inside a 2 mL tube;
-incubate on ice for 30 min, heat shock at 42°C for 1 min, another 1-minute incubation on ice;
-add 1 ml of sterile, pre-warmed LB and incubate the tube at 37 °C and 220 rpm for 1 hour.
After the transformation, 100 μl of the bacterial solution have been plated on a pre-warmed LB agar plate with kanamycin, while the rest of the volume was centrifuged at 10000 rpm for 1 min; about 1 ml of the resulting supernatant was discarded and the pellet was resuspended in the remaining liquid, thus concentrating the cells. The entire volume has been plated on another pre-warmed LB agar plate with kanamycin and the two plates were incubated at 37 °C O/N or until colonies appeared.

2.1.4 Sequencing

The mutagenesis has been controlled by sequencing. One grown colony has been selected from the plate with the highest concentration, two from the second plate, all three were inoculated in falcons containing 5 ml LB+kanamycin and O/N incubated at 37°C and 220 rpm. The next day, 750 μl were taken from each inoculum to prepare a stock with 250 μl of 80% glycerol at -80°C of each colony. Lastly, the miniprepped DNAs have been obtained from the inoculums and sequenced to verify that the base substitution had taken place (thanks to the FWD_dCas9_c primer, 5.1 section of this document, binding the DNA template 486 nt upstream of the mutation of interest).

2.2 Cloning dCas9 cassette into pSGAb-km

The dCas9_m expression cassette (insert) was cloned into pSGAb-I13507_iGEM (backbone), creating the pSGAb-dCas9_m plasmid.
First, the DNA templates are obtained following the same steps described in point 2.1.1 of this document (plasmid J116-dCas9_3k3) starting from the stocks of *E. coli* containing the plasmids pSGAb-I13507_iGEM and J116-dCas9_m_3k3.

2.2.1 DNA Digestions, Gel electrophoresis and Gel extraction

Both plasmids are digested simultaneously with EcoRI and PstI enzymes (Directional cloning). The reference protocol is the one for Fast Digestion of Different DNA provided by Thermo Scientific™ for all enzymes of the FastDigest type. In the specific case, the indications on double digestions of plasmid DNA are followed. The reagent aliquots are: 14 µL nuclease-free, 2 µL DNA (up to 1 µg), 2 µL 10X FastDigest Green Buffer, 1 µL FastDigest enzyme 1, 1 µL FastDigest enzyme 2 (20 µL Total volume).
The reaction is carried out in a thermocycler for 10 min at 37°C, subsequently for 5 min at 80°C.
The reaction products were run in gel as in point 2.1.2, but for that the total volume of both samples was loaded without adding the loading dye, which is already present in the digestion buffer.
The expected bands are about 4kb for the dCas9_m cassette and about 6kb for pSGAb-I13507_iGEM. The bands are extracted using the QIAquick® Gel Extraction Kit.

2.2.2 Ligation, transformation and screening

The ligation was performed adding 1 µl of 10X T4 DNA Ligase Buffer and 1 µl of T4 DNA Ligase, 5 Weiss U/µL #EL0011 (by Thermo Scientific™) to 20-40 ng of vector DNA and the appropriate amount of insert DNA (calculated according to the ligation ratios) in a final total volume of 10 µl. The reaction takes place at 16°C O/N.
The following day, the heat inactivation of the T4 DNA Ligase enzyme is carried out at 65°C for 10 min and the transformation is done following the instructions in point 2.1.3 (without previously plating 100 µl).
The grown colonies are inoculated in 5ml LB+kanamycin and incubated at 37°C O/N 220 rpm. The next day, after using the QIAprep® Spin Miniprep Kit, the concentration of the sample DNA and the $\frac{260}{280}$ and $\frac{260}{230}$ ratios were measured by the NanoDrop (Thermo Scientific™).
The screening of the ligation has been made by digestion of the miniprepped plasmid with the same enzymes and the expected band on gel electroforesis were about 4kb for the dCas9_m (the insert) and about 6kb for the vector pSGAb-I13507_iGEM. The miniprepped DNA was also sequenced for a further confirmation with the primer FW_seq_pSGAb_Acineto (Supplemental Material).

2.3 sgRNA design tools

This section reports the general search settings in the sgRNA design tools for each target gene of the ATCC 19606 and 13883 strains. Furthermore, the parameters of interest (in the case of a CRISPRi system) are indicated.

Benchling settings:
- select the Target Region (the CDS of interest) inside the uploaded bacterial genome;

- use the preset; settle Genome on “GCA_002136865_EnsemblRelease_53 (Acinetobacter baumannii)” or “KPBAA1705_01152013 (Klebsiella pneumoniae ATCC BAA-1705)”;
- search for a guide with the highest On and Off-Target Scores (above 60.0 is better), and nearest to the TSS of the gene (within the first 20% of the sequence); if the coding sequence is on the positive strand (5’-3’), the guide must be on the strand (3’-5’), otherwise the opposite.

CHOPCHOP settings:
- paste the Target sequence (if the CDS is on the negative strand, paste the Reverse complement);
- use the preset; select “Acinetobacter baumannii ATCC 19606 (ASM1139851v1)” or “Klebsiella pneumoniae str. SGH10 (CP025080)”, “CRISPR/Cas9” and “repression”; settle Target specific region of gene on “Coding region”; filter for Minimum required GC [%] content has to be between min: 30 and max: 70.
- search for a guide coded by the negative strand (-), which should have the lowest Self-complementarity and number of off-targets (with 0, 1, 2 or 3 mis-matches), and the highest Efficiency.

Cas-Designer settings:
- paste the Target sequence (if the CDS is on the negative strand, paste the Reverse complement);
- use the preset; select “Acinetobacter baumannii (ASM151871v1)” or “Klebsiella pneumoniae str. ATCC 43816 (ASM1607173v1)”;
- search for a guide coded by the negative strand (-) with the lowest number of off-targets (with 0, 1 or 2 mis-matches).

CRISPOR settings:
- paste the Target sequence (if the CDS is on the negative strand, paste the Reverse complement);
- use the preset; select “Acinetobacter baumannii ATCC 19606” or “Klebsiella pneumoniae ATCC 43816 (ASM1607173v1)”;
- search for a guide in the reverse Strand (“rev”) with the lowest number of off-targets (with 0, 1, 2, 3 or 4 mis-matches), especially if next to PAM.

The reported sequences of any off-targets in all the tools are aligned with the genomes of the bacteria to verify that it is not the guide itself instead of an actual off-target, then the position and binding strength with respect to the off-gene are evaluated.

3. RESULTS AND DISCUSSION

3.1 dCas9 standardized expression cassette

The iGEM Registry contains genetic parts that include dCas9 sequences; however they are marked as "Not in stock" or "Sample is complicated". So it was decided to utilize the plasmid J116-dCas9_3k3 (composed of the backbone pSB3K3 and the insert BBa_J107201, Bellato et al., 2022), which was already available in stock and theoretically exploitable for the purposes of the project; it already had an origin of replication for *Escherichia coli*, the kanamycin resistence gene and in particular an expression cassette for d(Sp)Cas9 flanked by Prefix and Suffix (Figure 6). The sequence of the dCas9 was obtained from the plasmid pdCas9 (addgene code: 44249). The cassette used cannot be considered as a Part for iGEM due to an EcoRI site in position 1339 5'-end of the dCas9 coding sequence.

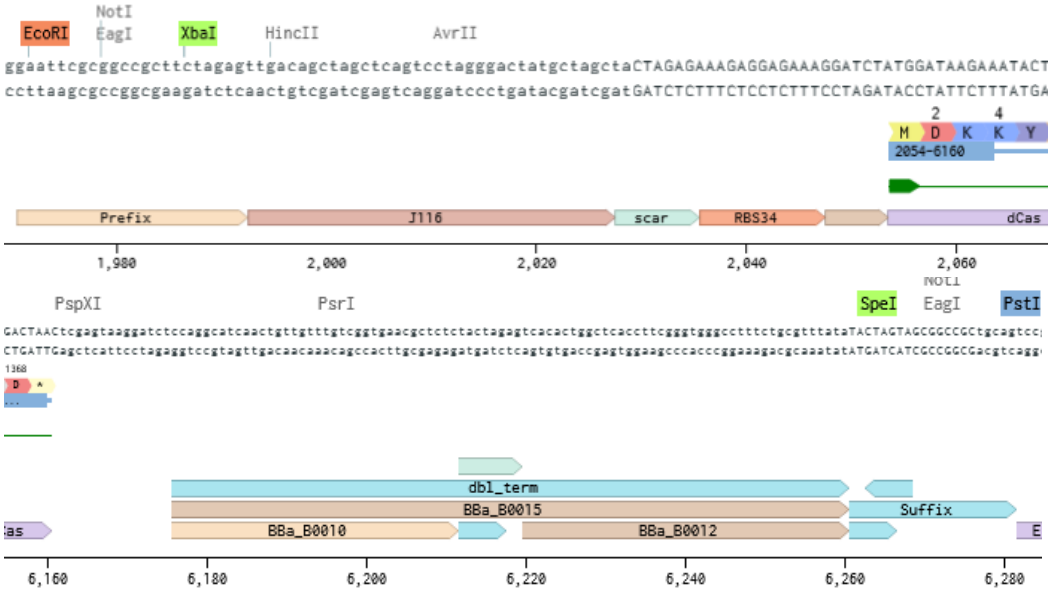
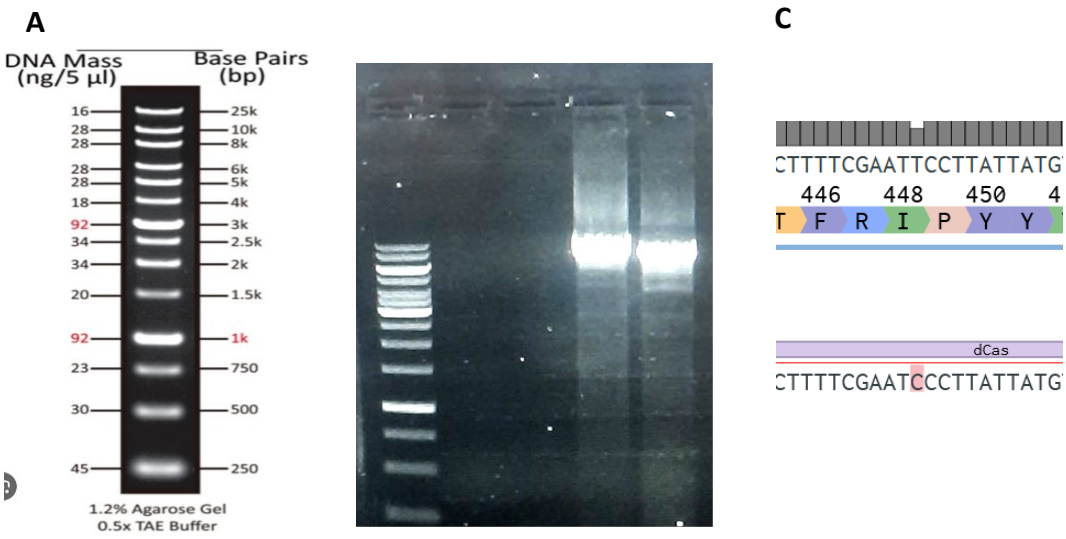


FIGURE 6| dCas9 expression cassette between Prefix and Suffix of plasmid J116-dCas9_3k3. In order, there are: the Promoter *J23116* (BBa_J23116), a scar (from a previous cloning), the RBS BBa_B0034 (an iGEM part), the coding sequence of d(Sp)Cas9 (4,107 kb long), the Double Terminator BBa_B0015 (composed by the *rrnBT1* terminator BBa_B0010, in this case only a part of it was cloned, and the TE from coliphageT7 BBa_B0012).

To obtain a cassette compatible with the iGEM standards, a PCR mutagenesis was carried out to eliminate the EcoRI site while maintaining the codon usage in *E. coli*. The last thymine of the ATT codon, encoding an isoleucine, has been replaced by a cytosine. The protocol performed was the Quick Protocol for Q5® Site-Directed Mutagenesis Kit (with any optimizations reported in the materials and methods section of this document). The starting concentration of the miniprepped Template DNA was 43.6 ng/µl, and $\frac{260}{280}$ was equal to 2.00, while $\frac{260}{230}$ was equal to 2.62; so, the plasmid DNA was diluted 1:10 in nuclease free water and then added to the reaction mix.

Additionally, during the annealing step of the 25 PCR cycles, a temperature gradient has been tested since the 65°C temperature (suggested by NEB Tm Calculator™) did not lead to any amplification of the plasmid J116-dCas9_3k3 (as verified by gel electrophoresis analysis). As seen in figure 7.A, only the temperatures of 60°C and 58°C led to amplification, and it was decided to use the sample from the former in the subsequent KLD reaction because it had a better quality.
This step was successful: after transformation, *E. coli* colonies grew in both plates with antibiotic kanamycin (Figure 7.B); the sequencing of the products obtained from the MiniPrep of some colonies revealed one plasmid, named J116-dCas9_m_3k3, positive for the mutagenesis (Figure 7.C).



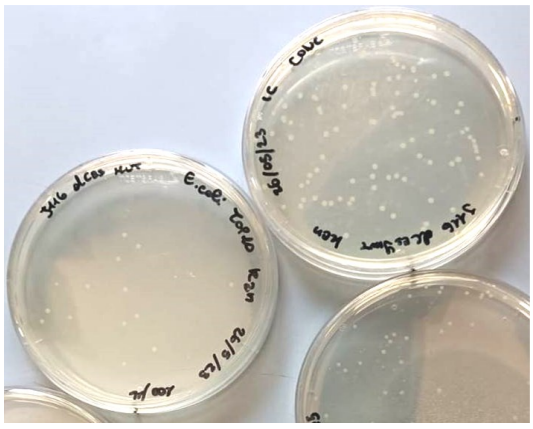


FIGURE 7| PCR mutagenesis results. A) Gel electrophoresis screening, from left to right: the 1kb GeneRuler, the blank, the PCR sample at 63°C (no amplification), at 60°C (amplification with few aspecific bands), at 58°C (amplification with nonspecific bands below the expected one at about 7 kb); B) The colonies grown on the LB+kanamycin plates, (left) 100 µl of *E.coli* TOP10 transformed with the product of the KLD reaction were plated; (right) the result of the concentration of the bacterial suction from the first one. C) Alignment between the original sequence of dCas9 around nucleotide 1339 5'-end and the sequencing of the colony 1C (C replaces T).

3.2 Cloning of dCas9

Usually, a CRIPSR/Cas9 or CRISPRi system consists either of a single plasmid carrying both the expression cassettes of the dead-nuclease (dCas9) and of the guide, or two plasmids each carrying one cassette of the two. The latter option offers some advantages, such as creating smaller, and therefore easier to handle plasmids, that could carry also the sequence encoding for a repression factor, for example the *lacI* gene, of an inducible promoter; smaller plasmids can be an important factor to consider when working with non-model organisms (i.e. *A. baumannii*, *K. pneumoniae* and *P. aeruginosa*) as in the case of the project PASTA.

The design of the experiment theoretically foresees the use of two different resistance genes for the two constructs; the iGEM competition rules allow the use of the ampicillin, kanamycin and chloramphenicol resistance genes, however, the selected bacterial strains resulted resistant to two antibiotics each, and specifically *A. baumannii* and *K. penumonie* are sensitive only to kanamycin. This led to the choice of a monoplasmid system.

Two plasmids were used in different studies relating to genome editing throught Cas9 in the two bacteria treated in this thesis, opening up the possibility of optimizing times thorugh the exploitation of a single construct. This plasmids, defined pSGKP-km (used in *K. pnumoniae*) and pSGAb-km (used in *A. baumannii* and derived from the previous one), added resistance to kanamycin and the expression of an sgRNA under the constitutive Promoter *J23119*.

Then, pSGAb-km (addgene code: 121999) has been engineered to make it compatible with the iGEM BioBrick RFC Standard [10] and to insert the Prefix and the Suffix (Asia Picchi, 2023), and later into this resulting plasmid, named pSGAb-km_iGEM, an expression cassette for the RFP was cloned to obtain pSGAb-I13507_iGEM; it consists of the Promoter *J23119* (BBa_J23119), the RBS BBa_B0034 and the complete Double Terminator BBa_B0015. The construct was electroporated in *A. baumannii* and *K. pneumoniae*, which led to the expression of the fluorescent protein, and allowed to draw the following conclusions:

-the constitutive promoter *J23119* allows the expression in the specific ATCC electroporated strains, as already known from various studies (precisely, it was applied to other strains of *K. pneumoniae*);

-the RBS and the Double Terminator tested work in both strains, confirming what was found for *A. baumannii* in the study by Bai et al., (2021) (while no information about these parts was found in the literature for any strain of *K. pnuemoniae*).

After these evidences, it was decided to clone the standardized expression cassette of dCas9 inside J116-dCas9_m_3k3 into the pSGAb-I13507_iGEM, thanks to the Standard Assembly creating the pSGAb-dCas9_m (Figure 8). Both plasmids were digested with EcoRI and PstI enzymes to make a directional cloning. The starting miniprepped template DNAs of pSGAb-I13507_iGEM and J116-dCas9_m_3k3 were around 88.4 ng/μl ($\frac{260}{280}$ equal to 1.95, $\frac{260}{230}$ equal to 2.23) and 56,6 ng/μl ($\frac{260}{280}$ equal to 2.00, $\frac{260}{230}$ equal to 1.98) respectively. Then, almost 1 μg and 800 ng of the respective miniprepped DNAs were digested. After the gel

extraction of the bands shown in Figure 8.B (point 2.2.1), the ligation ratios performed were 3:1, 2:1 and 1.1. After the transformation, the pick of some of the grown colonies and the minipreps, a digestion with the restriction enzymes EcoRI and PstI was performed using around 500 ng of the DNAs. The following gel screening (point 2.2.2, Figure 8.C) shows (from left to right) that a sample from the 3:1 ratio plate (the first run) and two from the 1:1 ratio plate (the last ones) seem to be positive for the insertion of the dCas9 because of the presence of the corresponding band. While two colonies from the 2:1 plate are negative.

A

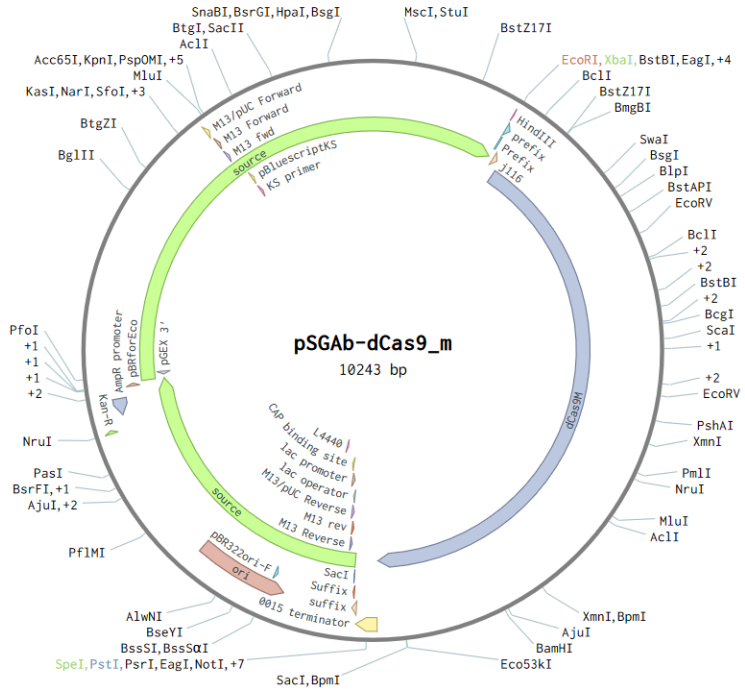
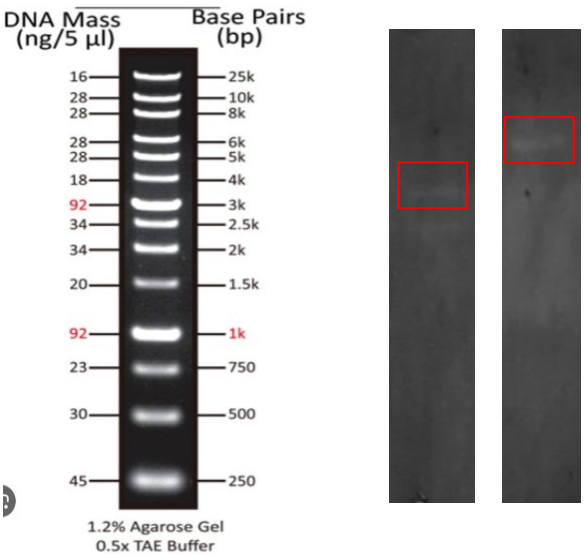
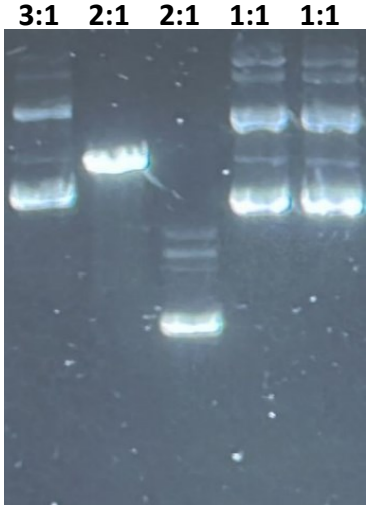


Figure 8| A) pSGAb-dCas9_m plasmid; the Prefix and the Suffix are reconstituted, in order to maintain the possibility of making other cloning operations using the BioBrick RFC Standard[10].
B) Gel electroforesis after the plasmid digestions; the extraceted bands are (left) dCas9_m, (right) pSGAb-I13521_iGEM (indicated in red boxes) .
C) Gel screening of the miniprepped DNAs from the grown colonies.

B



C



3.3 sgRNA expression cassette and target genes

As described earlier in the sgRNA design section of this document, a sgRNA consist of a variable part (that defines the binding site on the target DNA) and the constant part of the scaffold.
The sgRNAs of the mentioned pSGAb-km and pSGKP-km are composed by 83 nt long scaffold sequence (developed by Qi et al., 2013; Figure 4.A and 5.2 Supplemental Material). Therefore, the same scaffold can be exploited in different bacterial strains, so it was decided to include this costant part in the final sequence of the RNA guides. To gain greater efficiency, an additional Terminator *rrnBT1* was copied downstream the scaffold, and finally, the *J23119* promoter was inserted exactly upstream of the 20 nt sequence. Thus, the defined expression cassette is synthesized, flanked by the Prefix and the Suffix, as well as by 6 random bases or more on each side (Figure 9). The Prefix and Suffix will allow the insertion of the construct into pSGAb-dCas9_m, downstream the dCas9_m cassette.

FIGURE 9| Standard expression cassette synthesized for every sgRNA used in the project; the 20 nt of the guide are specific for the target gene.

It was decided to use 20 nt long spacers as it's the length with the best trade-off between efficiency and low off-target score (Cui et al., 2018); their design was created by running the target gene sequences in the CHOPCHOP, Cas-Designer, CRISPOR and Benchling tools. The analysis of the results and the relative most important parameters was made on the basis of the considerations set out in 1.4, searching for two appropriate guides for each gene. The steps carried out were the same for all the genes of the different strains. Therefore, in this document, only the chosen sequences of the guides binding to the ampicillin resistance genes in *A. baumannii* ATCC 19606 and *K. pneumoniae* ATCC 13883 are reported. They are *ampC* and *blaSHV-1*: *ampC*, also known as *blaADC*, respectively. The first encodes a class C beta-lactamase, while the latter one belongs to the class A. The guides can be used individually in the silencing test or together to increase efficiency. The chosen spacer sequences (named as "sg" followed by a number stating for their binding posistion after the TSS) are shown in the Table 2 in correlation with all the parameters that were considered (2.3 section of this document); the FASTA sequences are reported in the 5.3 section of this document.

	ampC spacers		*blaSHV-1* spacers	
Parameter	sg56	sg148	sg85	sg181
PAM	AGG	TGG	AGG	CGG
GC [%] content	40	50	45	30
On-target score by Benchling	Not found	57.0	62.2	50.2
Off-target score by Benchling	Not found	49.8	98.7	50.0
Self-complementarity	0	1	0	0
Efficiency by CHOPCHOP	57.28	57.39	62.17	50.15
CHOPCHOP off-targets	0,0,0,0	1,0,0,0	0,0,0,0	0,1,0,0
Cas-Designer off-targets	1,0,0	1,0,0	0,0,0	1,0,0
CRISPOR off-targets	0,0,0,0,0	0,0,0,0,1	0,0,0,1	0,0,0,0,0

Table 2| Designed spacers and relative parameters.

All off-target sequences were aligned with the genomes of the bacteria of interest and none revealed an actual off-target, except sg56; the putative off-target aligned in an unannotated CDS region with 4 mis-matched (which weren't in the seed region) and the PAM was non-canonical (CAG). The CDS was analysed on BLAST selecting "A. baumannii ATCC 19606", with the resulting hits being two hypothetical proteins, one of which had the length equal to the number of AA (120) of the translet putative off-target (NCBI Reference Sequence: WP_000881947.1). After these considerations, the choice of the sg56 guide is in any case confirmed.

4. CONCLUSIONS

During the development of the project, the BioBrick RFC Standard [10] initially posed the challenge of engineering the DNA sequences used so that they do not contain illegal sites, as in the case of the J116-dCas9_3k3 plasmid. But beyond this step, the Standard has made the design of subsequent assemblies easy and quick; moreover the number of available parts of the registry is growing.
Regarding the PCR mutagenesis technique, despite the optimization of the temperature and of some steps of the protocol, the setup of the experiment has been simple and ultimately successful: a standardized expression cassette was obtained for dCas9 (dCas9_m), which can be inserted into plasmids exploiting the Standard.
Thus, the cassette was cloned into pSGAb-I13507_iGEM (able of replicating within the bacterial strains of interest of *A. baumannii* and *K. pneumoniae*), creating pSGAb-dCas9_m plasmid.
Once the cloning is confirmed also by sequencing, it will be possible to insert the sgRNA sequences for *ampC* and *blaSHV-1* (designed for the iGEM competition) using the same Standard.
This versatile platform will make it possible to test other guides in order to improve the silencing efficiency , or to avoid the evolution of bacteria that are resistant to the CRISPRi system.
The immediate future application of the plasmid pSGAb-dCas9_m will be the addition of an encapsidation sequence, or the expression cassettes for dCas9will be cloned into a phagemid vector (DELIVERY).

5. SUPPLEMENTAL MATERIAL

5.1 Primers for dCas9 mutagenesis

Forward primer: F_dCas9_3397_c
5' GACTTTTCGAATcCCTTATTATGTTGGTC 3'

Length	29
GC Content	37.9%
Melting Temp. T_m	56.5°C

Reverse primer: R_dCas9_3397_c
5' AAGATTTTTTCAATCTTCTCACGATTGTCTT 3'

Length	31
GC Content	29.0%
Melting Temp. T_m	56.5°C

Primer per il sequenziamento: FWD_dCas9_c
5' TGCTGATTTGTTTTTGGCAGCT 3'

Length	22
GC Content	40.9%
Melting Temp. T_m	56.3°C

5.2 FW_seq_pSGAb_Acineto

5' GCAACTGGTCTATTTTCCTC 3'

Length	20
GC Content	45.0%
Melting Temp. T_m Params	50.5°C

5.3 sgRNA scaffold (dCas9 handle + *S. pyogenes* Terminator)

5' gttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttg
aaaaagtggcaccgagtcggtgc 3'

5.4 sgRNA spacer sequences

ampC sg56:
5' TGTATTGCCCGCATAAATTG 3'
ampC sg148:
5' ATAACACCCACAGCCATACC 3'

blaSHV-1 sg85:
5' ACCACATACATCCTTGAGTG 3'
blaSHV-1 sg181:
5' CTTAGTTTAATTTGCTCAAG 3'

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