Towards the protein-protein interactions in minimal genome: Changing the cloning compatibility of the plasmid vectors for the high-throughput protein-protein interaction assay in the minimal genome

Miroslav Gasparek[1], Keoni Gandall[2], Drew Endy[2]

Abstract— **Engineering of a functioning artificial cell has drawn an increasing attention of the synthetic biology community over the last few years. The deletion of the redundant genes from the genomes of the smallest naturally occurring organisms in the effort to find the set of genes essential for life led to the construction of the synthetic organism JCVI-syn3.0, which contains only the essential genes necessary for the autonomous life of the organism [1]. However, the function of 149 out of the 473 genes present in the minimal genome remains only generically understood or completely unknown. In this work, we describe our effort to contribute to the identification of the essential unknowns through preliminary work towards the protein-protein interactions (PPI) assay in the genome of the minimal synthetic organism JCVI-syn3.0 using the high-throughput double-barcode sequencing system combined with the dihydrofolate reductase protein-fragment complementation assay in Saccharomyces cerevisiae as described by Schlecht et al. [3]. We detail the experimental approach used to transform the Gateway cloning compatible bait and prey yeast plasmid vectors into the Golden Gate cloning compatible plasmids through the insertion of the BsaI recognition sites into the plasmid backbones by the PCR amplification. This modification of the plasmids is crucial for the high-throughput seamless assembly of the plasmid vectors and JCVI-syn3.0 inserts. We examined the validity of our experimental approach by the diagnostic digest of the modified plasmid vectors by BsaI and the Sanger sequencing results. We also report the cloning of a JCVI-syn3.0 gene rpoA (DNA-directed RNA polymerase subunit alpha) into the bait plasmid vector. We also discuss the challenges and obstacles encountered during the experimental process, and the lessons drawn from these challenges.**

I. INTRODUCTION

Building of a synthetic cell is a monumental task. However, the successful construction of a functioning cell would be a tremendous contribution to our understanding of the and on the nature and would have a major practical and philosophical implications. The creation of the minimal cell, a cell which contains only the genes essential for its survival, has been a goal of the scientists since 1930s. They believed that by studying the simplified structure of a minimal cell would enable them to gain insight into the essence of more complex living systems. As John Glass et al. stated, ... the minimal cell is the hydrogen atom of biology [2].

[1]Dept. of Bioengienering, Imperial College London, SW7 2A7, London, UK miroslav.gasparek15@imperial.ac.uk

[2]Bioengineering Department, Shriram Center, 443 Via Ortega, Stanford CA94305, USA

Although the construction of JCVI-syn3.0 by Hutchinson et al. [1], a reproducing cell with the genome smaller than in any other autonomously occurring living organism in 2016 was a tremendous success it has not provided a definitive answers about the nature of the simplest forms of life. At the time of the publication of the work by JCVI, 149 out of 473 genes (with 438 coding for the proteins and 35 coding for the DNA) of the minimum genome was either of unknown or only generically known function [1]. The limited understanding of the significant portion of the minimal genome can represent a significant obstacle on the path to de novo, bottom-up design of a synthetic cell. Attempts to engineer the living synthetic cells using the genes with unknown function is akin to construction of a new, delicate electric circuit from the components taken from another circuit, without the knowledge of whether they are active or passive, and if they are resistors, capacitors, or inductors. The significance of identification of the function of the essential unknown genes, or the essential unknowns, as they are sometimes referred to in the synthetic biology community jargon, goes beyond the construction of the artificial cells - the undiscovered biomolecular pathways could represent the novel antibiotic targets, addressing the antibiotic crisis [5], and the causes of disease states that have not been investigated before [4].

The recent study of the minimal genome of JCVI-syn3.0 included the attempts to identify the function of the unknown genes by considering their hypothetical assignment to the essential functions that each cell must perform by Danchin and Fang [6] yielding predictions for 78 genes, in silico functional annotation combining the results from the different complementary approaches enabling assignment of the function to 94 of the 149 products of the unknown genes by Antczak et al. [7], and the machine learning-based approach based on the secondary structure element alignment, which annotated at least 136 proteins out of 149 unknown gene products by Yang et al. [8]. However, none of the above approaches have focused on the experimental investigation of the minimal genome and there is currently a need for the data enlightening the interaction of the essential gene among themselves. Consequently, the knowledge of the complete protein interactome of JCVI-syn3.0 could provide priceless dataset that could be further analyzed by the in silico techniques, such as those described above. A complete PPI assay of JCVI-syn3.0 would add a significant

piece to the puzzle of our knowledge about the minimal genome. Therefore, the main goal of our work was to contribute to experimental realization of the PPI assay in JCVI-syn3.0 - a task that, to our knowledge, has not been accomplished.

Protein-protein interactions have served as a tool for the identification of the function of the unknown proteins by the analysis of the interaction with partners of known function for more than three decades. The two-hybrid screening were placed in 1989 by Finch and Song [9] and have undergone the significant development since then. In our work, we aimed to use the Protein-Protein interaction Sequencing (PPiSeq) developed by Schlecht et al. [3]. This is a scalable and robust method that combines Protein-fragment Complementation Assay (PCA), genomic double-barcoding technology, time-course barcode sequencing of the competing cell pools and an analytical framework enabling the estimation of the fitness of a stream from the lineage trajectories [3]. The estimation of the fitness is very attractive feature of this approach, as it extends the information extracted from the interactome beyond the binary data about whether two proteins interacted or not. Another useful feature of this approach is the possibility of perturbing the interaction between two proteins by changing of the environmental conditions [3]. The PCA method of determination of the PPI is based on the covalent linking of the proteins of interest, bait and prey proteins (in this case, products of the genes of JCVI-syn3.0) to the fragments of a third reporter protein, in this case, to the fragments of the murine Dihydrofolate Reductase (DHFR). If the bait and prey proteins interact, the fragments of a third protein are brought close enough to reconstitute a functional protein. In this case, the DHFR provides a resistance to anti-folate drug methotrexate (MTX), providing a very sensitive assay [10]. The genes producing the protein of interest need to be cloned into the bait and prey plasmids. The plasmids with PCA fragments are then inserted into the haploid yeast strains mated with the barcode acceptors strains with barcodes (SHA345-barcode + prey strain, and SHA349-barcode + bait strain) [3]. The diploid strains are then selected by the antibiotic markers and are inserted into the sporulation media. Spores are then inserted into the selection media to pick the correct haploids containing both a barcode and a split DHFR construct [3]. These haploids are then mated and the double barcode signatures and PCA constructs that are now in each cell are subsequently used to measure the fitness of the strain and hence the PPI scores [3]. The process is outlined in the Figure 1 adopted from [3].

The PPiSeq method is described in further details in [3]. Levy and his colleagues have developed a high-throughput framework, accompanied by the software that is able to evaluate the fitness of the final strains from the measurements and hence determine the PPI strength. Hence our objective was to use the bait and prey plasmid kindly

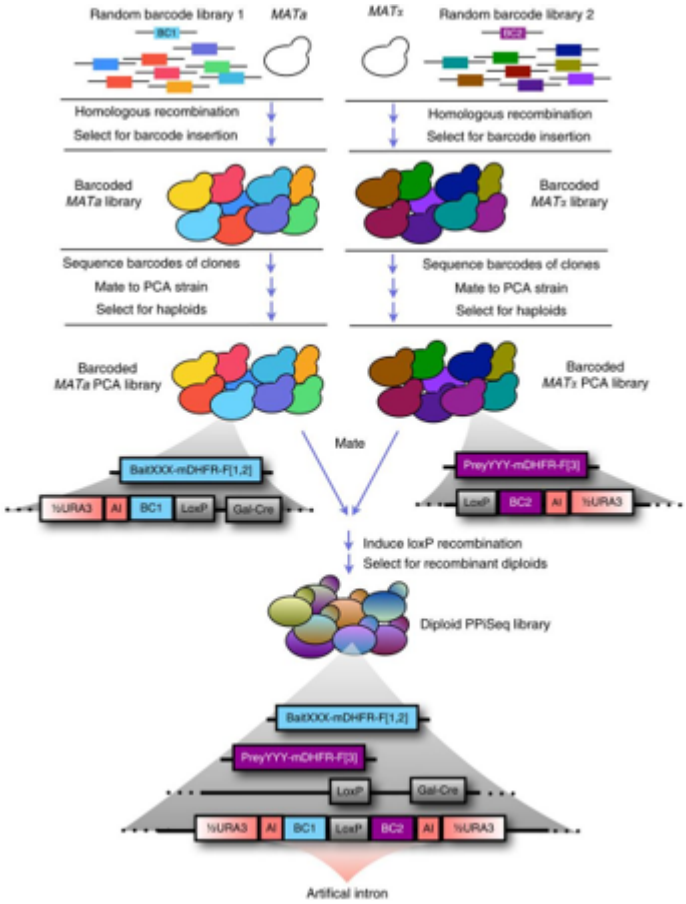

Fig. 1: The schematics of the construction of the PPiSeq library, adopted from [3].

provided by the Levy lab and clone all the JCVI-syn3.0 genes available in the Endy Lab into the bait and prey vectors and to pass them to the Levy lab for the further analysis. However, the obstacle on our way to quickly clone the JCVI-syn3.0 genes into bait and prey plasmids was the fact that the JCVI-syn3.0 genes available to the lab were synthesized as Golden Gate compatible, while the bait and prey plasmids were Gateway cloning compatible.

The Gateway cloning is based on the site-specific recombination of the phage lambda system [11] and relies on two reactions. BP reaction takes place between attB sites of the gene and the attP sites of the donor vector. LR reaction takes place between the attL sites of the entry clone containing the gene of interest, and the attR sites of the destination vector, which contains the negative selection marker ccdB [11]. The reaction creates the expression clone. While the Gateway cloning has advantages such as fast cloning, multiple fragment cloning, and high efficiency because of the ccdB selection marker [11]. Plasmids from the Levy lab came as the prepared destination vectors with attR sites. However, the Gateway cloning requires increased preparation time, is more expensive, and also leaves the scars because of the recombination of the attB sites [11].

In addition to that, the modification of all the Golden Gate compatible JCVI-syn3.0 genes would represent a redundant work, compared to the modification of two bait and prey plasmids.

Consequently, to use the Levy lab pipeline, we decided to modify the bait and prey plasmids to be compatible with the Golden Gate assembly, also referred to as Modular Cloning, or MoClo, presented by Weber et. al. [12]. This assembly method relies on the TypeIIS restriction enzymes (BsaI or BpiI/BbsI) that cut outside of their recognition sites. The cutting outside of the recognition sites creates the four base long flanking overhangs which, if properly designed, can provide a scarless assembly, with no risk of the scars altering the function of the final assembled product, as the recognition sites are removed from the reaction [12]. This cloning method allows for a single-tube assembly of the multiple DNA fragments in a directional order through a simple and fast reaction, which involves only several reactants. However, the major drawback of the Golden Gate assembly is the requirement that the TypeIIS restriction enzymes are not present within the fragments that are to be assembled - otherwise, the fragment will be cut at the undesired site. A way to overcome this problem is to introduce a silent point mutation through PCR amplification at the unwanted BsaI recognition site [13].

In this work, we describe the experimental approach used to transform the Gateway compatible bait and prey plasmids used for the PPiSeq assay into Golden Gate compatible plasmids through the introduction of the BsaI sites into the appropriate loci, and the removal of the undesired sites in the plasmids through introduction of the silent mutation by the PCR amplification. In the Methods section, we describe step-by-step approach that we followed to obtain the Golden Gate compatible plasmids, as well as the challenges and obstacles encountered during our experiments. In the Results section, we present the outcomes; we analyze the Sanger sequencing of the modified plasmids and their regions containing the introduced recognition sites and the removal of the undesirable BsaI recognition sites. We also show the results of the diagnostic cutting of the final assembled plasmids by BsaI enzyme. Furthermore, we show the results of the successful Golden Gate cloning of the rpoA JCVI gene (MMSYN1 0645), which encodes the alpha subunit DNA-dependent RNA polymerase (RNAP) [14] into bait plasmid. Finally, in the Discussion, we discuss the outcome of our effort and the learning outcomes achieved. Our work yields the experimental approach that can be used to clone the JCVI-syn3.0 genes into bait and prey plasmids used for the PPiSeq assay in a high-throughput and cost effective manner, and overcomes the most difficult steps on our way towards the acquisition of the complete PPI assay of the minimal genome.

II. METHODS

A. Analysis of the bait and prey plasmids and the design of the primers

The original bait (bdestorf1) and prey (bdestorf2) plasmids received from Levy lab are shown in the Figure 2 with the annotated features and the BsaI recognition sites as originally present in the plasmid backbones. In the Gateway cloning design, the toxic ccdB gene (performing the role of the negative control) located between the attR1 and attR2 sites is replaced by the gene insert during the LR reaction. Therefore, two modifications of the original plasmids were required:

a) The BsaI recognition sites for the Golden Gate cloning needed to be introduced in the proximity of the Gateway recombination sites, so that the ccdB gene can be replaced by the Golden Gate compatible JCVI-syn3.0 during the Golden Gate assembly to allow the transformation of the plasmids into the non-ccdB resistant cells.
b) The BsaI sites outside of the attR1 and attR2 recombination sites needed to be removed to avoid the cleavage of the plasmids by the restriction enzymes during the Modular Cloning.

To make the desired modifications of the plasmid, we designed the primers containing the BsaI recognition sites at the appropriate positions within the attR1 and attR2 recombination sites. We also designed the primers that introduced the silent mutations in the undesired BsaI sites (at positions 1881 and 6713 in bdestorf1, position 1122 in bdestorf2) to remove these sites. The Figure 3 displays the positions of the designed primers and Figure 4 displays the results of the simulation of the PCR amplification of the created plasmid fragments and their subsequent assembly, the result that we aimed to accomplish experimentally. The design of the primers was conducted using the SnapGene software. The PCR primers containing the BsaI recognition sites or the silent mutations removing these sites from undesired locations are listed in the Table I. The primers were designed according to the basic design rules, such as the GC content close to 40% and 60% of the primer and C or G base at the 3'-end. Also, the dimerization analysis, self-dimerization analysis and hairpin formation analysis were conducted using online IDT Oligo Analyzer. The primers were ordered from the IDT Technologies.

B. Bait and prey plasmids transformation and outgrowth

After the purified bait and prey plasmids were received from the Levy lab, they were first transformed into the ccdB resistant cells (One Shot ccdB Survival 2 strain by Invitrogen, catalog number: A10460), and the culture was plated on the LB agar medium and left in the 37C incubator overnight. After 16 hours of the incubation, the plate with bait colonies and the plate with prey colonies were stored at 4C for any subsequent manipulations.

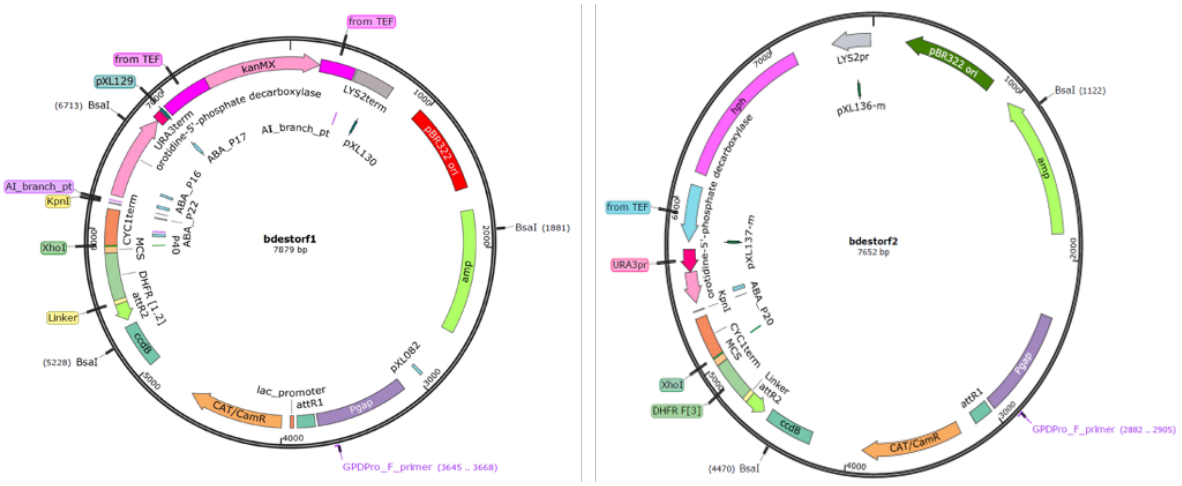

Fig. 2: The original annotated bait plasmid bdestorf1 (left) and prey plasmid bdestorf2 (right) used for the PPiSeq as received from the Levy lab. The position of the BsaI recognition sites is indicated, as well as the position of the target locus of insertion of the JCVI genes between the attR1 and attR2 sites, where the ccdB gene resides prior to the cloning reaction. The other important features include the split URA3 marker for the barcode-loxP-barcode recombination on a single chromosome [3], selection PCA marker domains DHFR-F[1,2] in the bait plasmid and DHFR domain DHFR-F[3] in the prey plasmid, and the antibiotic resistance genes, such as hph (Hygromycin res.), amp (Ampicilin/Carbenicilin resistance), CAT/CamR (Chloramphenicol resistance).

Primer number	Primer name	Primer sequence
Primer sequences for both ORFs		
1	best_amp_for	5-CAGTGCTGCAATGATACCACGAGAaCCACGCTCACCGGCTCCAG
2	bestorf_amp_rev	5-TGATAAATCTGGAGCCGGTGAGCGTGGtTCTCGTGGTATCATTGCAGCACTGGGG
3	bestorf_insert_for	5-GTACAAAAAAGCAGGCTAATGAGAGACCattaggcaccccaggctttacac
4	bestorf_insert_rev	5-caccgcctcctgatccgccaccgTGAGACCcagactggctgtgtataagggagcc
5	bestorf_vec_for	5-aggctcccttatacacagccagtctgGGTCTCAcggtggcggatcaggagg
Primer sequences for bait plasmid bdestorf1		
6	bestorf_vec1_rev	5-cctggggtgcctaatGGTCTCTCATTAGCCTGCTTTTTTGTACAAACTTGtGGGGGCTAG
7	bestorf1_DH_for	5-gtggatgatgtggtTtctacaggatctgacattattattgttggaag
8	bestorf1_DH_rev	5-tcttccaacaataataatgtcagatcctgtagaAaccacatcatccacggttctatactg
Primer sequences for prey plasmid bdestorf2		
9	bestorf_vec2_rev	5-atGGTCTCTCATTAGCCTGCTTTTTTGTACAAACTTGtTAGCCCACGTGCCTCAGGtcg

TABLE I: The primer sequences used for the insertion of Type IIS restriction enzymes recognition sites into attR1 and attR2 regions and for the removal of the recognition site from the other regions of the plasmids. GC content of all the primers is between 38% and 61%. The optimal annealing temperature T_m is between 58C (bestorf_vec_for) and 72C (bestorf_amp_rev).

C. PCR amplification of the bait and prey plasmid fragments

The PCR amplification of the plasmid fragments was conducted according to the protocol by New England Biolabs [15]. Prior to the PCR amplification, the primers were diluted to 10uM concentration. The 7 PCR reactions were assembled in the separate tubes, as the 4 fragments of the bait plasmid (bestorf1) were to be amplified, while 3 fragments of the prey plasmid (bestorf2) were to be amplified. Each of the 50μL reactions were assembled on ice as follows (in the order of the materials used).

- Nuclease free water (ddH2O) - 19μL
- Thermo Scientific Phusion Hi-Fi PCR Master Mix with HF buffer (25μL)
- Forward primer (2.5μL)
- Reverse primer (2.5μL)
- Plasmid DNA - 1μL (based on the guidelines provided by NEB)

The combinations of the forward and reverse primers used in each tube are in the Table II. The thermocycling of the PCR was done in a single thermocycler under the conditions described in the Table III. Neither negative nor positive control reactions were realized and the implications of this are further elaborated in the discussion.

D. Verification of the correct PCR amplification and the Purification of the DNA of the fragments

To verify the validity of the PCR amplification results, the agarose gel electrophoresis was performed and the expected sizes of the fragments were compared to the observed ones. 10μL samples of the PCR reactions were mixed with 2μL of the NEB 6x DNA Purple Gel Loading Dye. 10μL of the mixture was then loaded into the wells in the gel, which was prepared using the 1% solution of agarose in the TBE buffer, mixed with 5μL of the Invitrogen SYBR Safe DNA Gel Stain after the pouring.

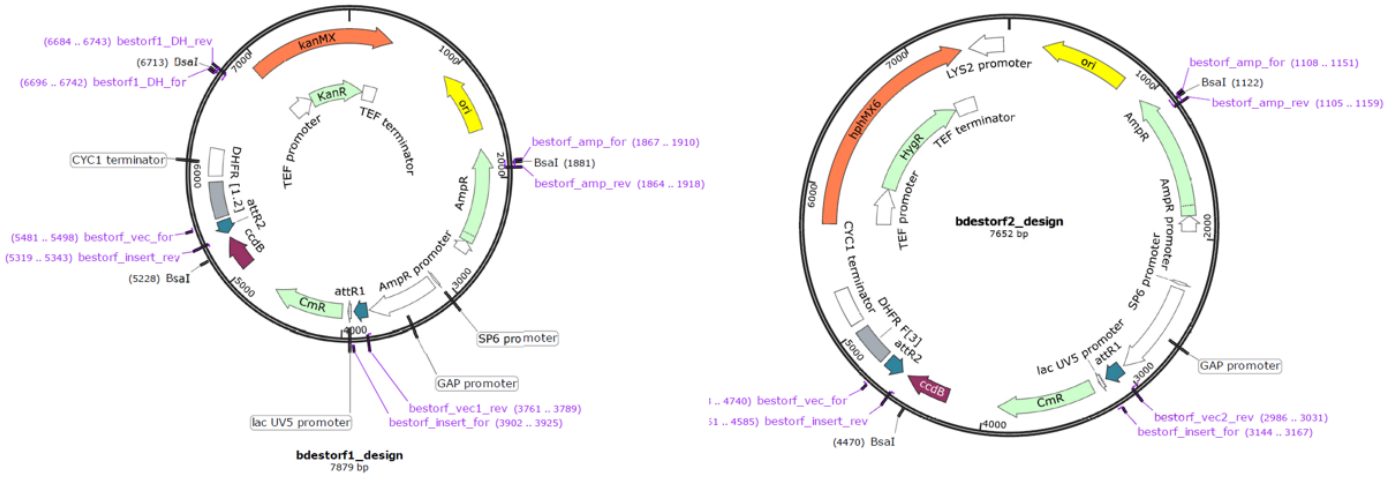

Fig. 3: The bait plasmid bdestorf1 (left) and prey plasmid bdestorf2 (right) indicating the positions of the primers designed to add the BsaI recongition sites at the appropriate positions within the attR1 and attR2 recombination sites and to introduce the silent mutations in the undesired BsaI sites (at positions 1881 and 6713 in bdestorf1, position 1122 in bdestorf2) to remove these sites.

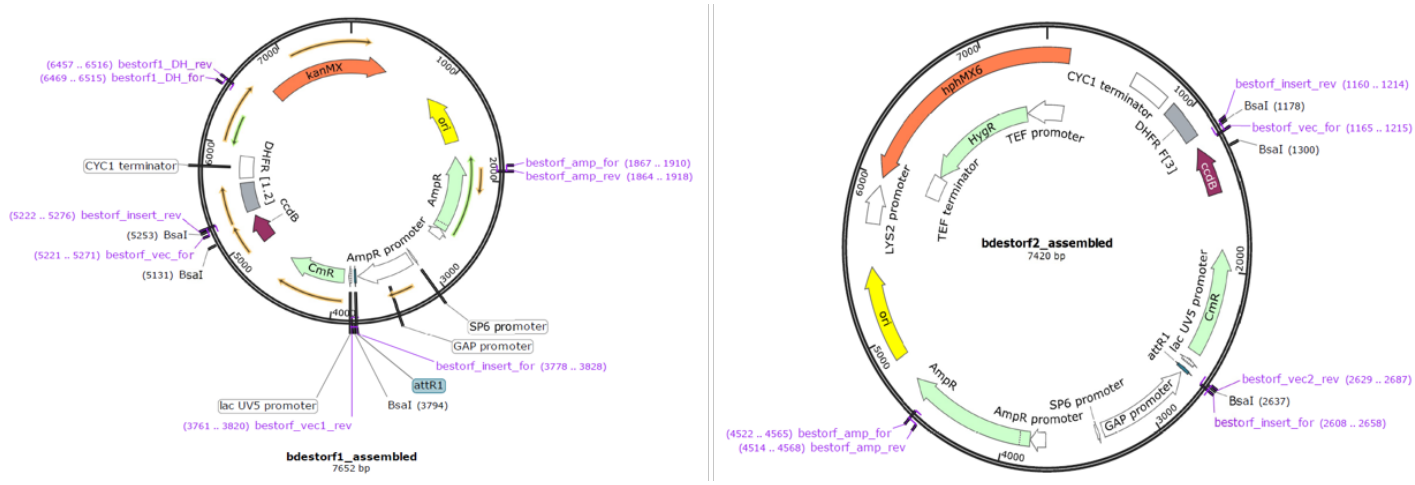

Fig. 4: The simulated results of the PCR amplification and the assembly of the bait plasmid bdestorf1 (left) and prey plasmid bdestorf2 (right) indicating the final desired Golden Gate compatible plasmid ready for the Modular Cloning of the JCVI-syn3.0 genes. BsaI recognition sites are present at the appropriate positions within the attR1 and attR2 recombination sites and the unwanted BsaI sites (at positions 1881 and 6713 in bdestorf1, position 1122 in bdestorf2) are removed.

Tube	Plasmid	Primers in the tube (forward and reverse)	Primer combination	The expected amplified fragment length [bp]
1	bdestorf1	bestorf_amp_for + bestorf_vec1_rev	1+6	1954
2	bdestorf1	bestorf_insert_for + bestorf_insert_rev	3+4	1499
3	bdestorf1	bestorf_vec_for + bestorf1_DH_rev	5+8	1296
4	bdestorf1	bestorf1_DH_for + bestorf_amp_rev	7+2	3102
5	bdestorf2	bestorf_amp_for + bestorf_vec2_rev	1+9	1937
6	bdestorf2	bestorf_insert_for + bestorf_insert_rev	3+4	1499
7	bdestorf2	bestorf_vec_for + bestorf_amp_rev	5+2	4122

TABLE II: The combinations of the forward and reverse primers for the PCR amplification of the bait (bdestorf1) and prey (bdestorf2) plasmid fragments and the expected sizes of the fragments as inferred from the SnapGene design.

Step	Temperature [C]	Time
Initial denaturation	98	30 s
30 cycles	98	10 s
	61	30 s
	72	2 min
Final extension	72	10 min
Hold	4	Forever

TABLE III: Thermocycling conditions for the PCR amplification of the bait and prey plasmid fragments.

During the first trial of the gel electrophoresis, the molecular-weight size marker was not used and hence the size of the PCR fragments was estimated only using the the relative position of each loaded sample. In the subsequent experiments, the NEB 2-log DNA ladder was used for the more precise estimation of the size of each fragment and the comparison with the predicted sizes of the fragments as listed in the Table 2. Figure 5 displays the pictures of the four gel electrophoresis conducted for the four repeats of the PCR amplification that were performed. It can be seen that the efficiency of the PCR was greatest for the first set of PCR reactions, despite the fact that the experimental procedure was the same in all cases. While the fragments that were observed on the gel are approximately matching the expected sizes in the Table 2, some bands were missing on occasion. The possible reasons for this are disputed in the Discussion section.

After having confirmed that the PCR reactions yield all seven fragments of the approximately appropriate size. To purify the fragments and to degrade the template DNA, 0.8μL of DpnI restriction endonuclease and 0.8μL of the 10X NEB CutSmart buffer were added the remaining 40μL of each of the PCR product. The samples with DpnI and CutSmart were incubated at 37C for 30 minutes and then the enzyme was deactivated by the incubation at 80C for 20 minutes following the Gibson Assembly Master Mix Instructional Manual [16]. Subsequently, the fragment DNA was purified using the Macherey-Nagel NucleoSpin PCR clean-up manual [17]. Each PCR fragment sample of size of 40μL was mixed with NTI buffer (40μL of PCR sample with 80μL of NTI buffer). Then the NT3 buffer (already prepared with Ethanol) was added to the 100μL of mixture of NTI buffer with PCR fragment sample to wash the Spin column (the washing was repeated twice as recommended in the protocol for better results) and then Elution buffer was added to the cleaned up filtering column (20μL of EB into each sample), to each sample at the at the time. After the buffer was added to all samples, the samples were centrifuged at 11 000 rpm for 60 s and the flow-through was kept for further processing. The DNA concentration of the purified fragments was subsequently measured by nano-dropping and after ensuring the appropriate sample quality (good 260/280 absorbance ratio) was stored for the further processing. Because of the failures in the latter steps of the experimental work, the above purification process was repeated three times overall.

E. Gibson assembly of the modified plasmids

Gibson assembly of the bait (bestorf1) and prey (bestorf2) fragments was realized according to the protocol [16]. The Gibson assembly was repeated several times, with the difference in the total amount of the DNA for each of the construct. Initially, the DNA volume of bestorf1 fragments giving 0.2 pmols of DNA per each fragments was used (assembly of the fragments 1, 2, 3 onto a bestorf1 backbone, which was the fragment 4), and the DNA volume of bestorf2 fragments giving 0.15 pmols of DNA per each fragment was used (assembly of the fragments 6, 7 onto a bestorf2 backbone, which was the fragment 5). However, in the subsequent repeats of the Gibson assembly, the target DNA volume for bestorf1 plasmid was 0.1 pmols of the plasmid DNA and 0.06 pmols of the plasmid DNA for bestorf2. As the subsequent transformation (as described later) of the bestorf1 plasmid was successful, while the transformation of the plasmid bestorf2 was not succesful, we repeated the Gibson assembly with the 0.1 pmol of the fragment DNA per fragment for bestorf2 as well and this led to the desired result. We calculated the conversion factor between the target amount of pmols and the volume of the DNA needed for each fragment, based on its base-pair length and the nano-dropped concentration. Then the 20μL Gibson assembly reactions were assembled in two tubes (one for bait plasmid and the other for the prey plasmid) as follows:

- Nuclease-free water (ddH2O): to fill to 20μL reaction volume.
- DNA fragments (four fragments for the bestorf1, three fragments for the bestorf2): volume as calculated based on the base-pair length of the fragment and amount (pmol) used.
- Gibson Assembly Master Mix (2X): 10μL.

The samples were subsequently incubated in the thermocycler at 50C for 45 min and then left at 10C until they were further used in the next steps. In the experiments, no positive control was used.

F. Bacterial transformation of the plasmids and the challenges encountered.

The transformation of the Gibson-assembled plasmids was the most difficult and time consuming part of the experimental protocol. At first, we attempted to transform the Gibson-assembled bait and prey plasmids (further referred to as bestorf1 and bestorf2, or simply as plasmids) into the Invitrogen One Shot Top10 chemically competent E. Coli cells (further referred to as Top10 cells). The chemically competent Top10 cells were taken out of the -80C freezer and thawed on ice for approximately 30 min. The reaction was assembled in two tubes, and 2.5μL of each of the Gibson assembled plasmids was added to a single aliquot of the competent cells and mixed by a pipette. Cells with the plasmid DNA was left on ice for 30 min. Then the cells with the plasmid DNA mixture were heat-shocked for 30 sec. at 42C and transferred on ice for another 5 min. Cells were then plated on the Carbenicilin plates and left

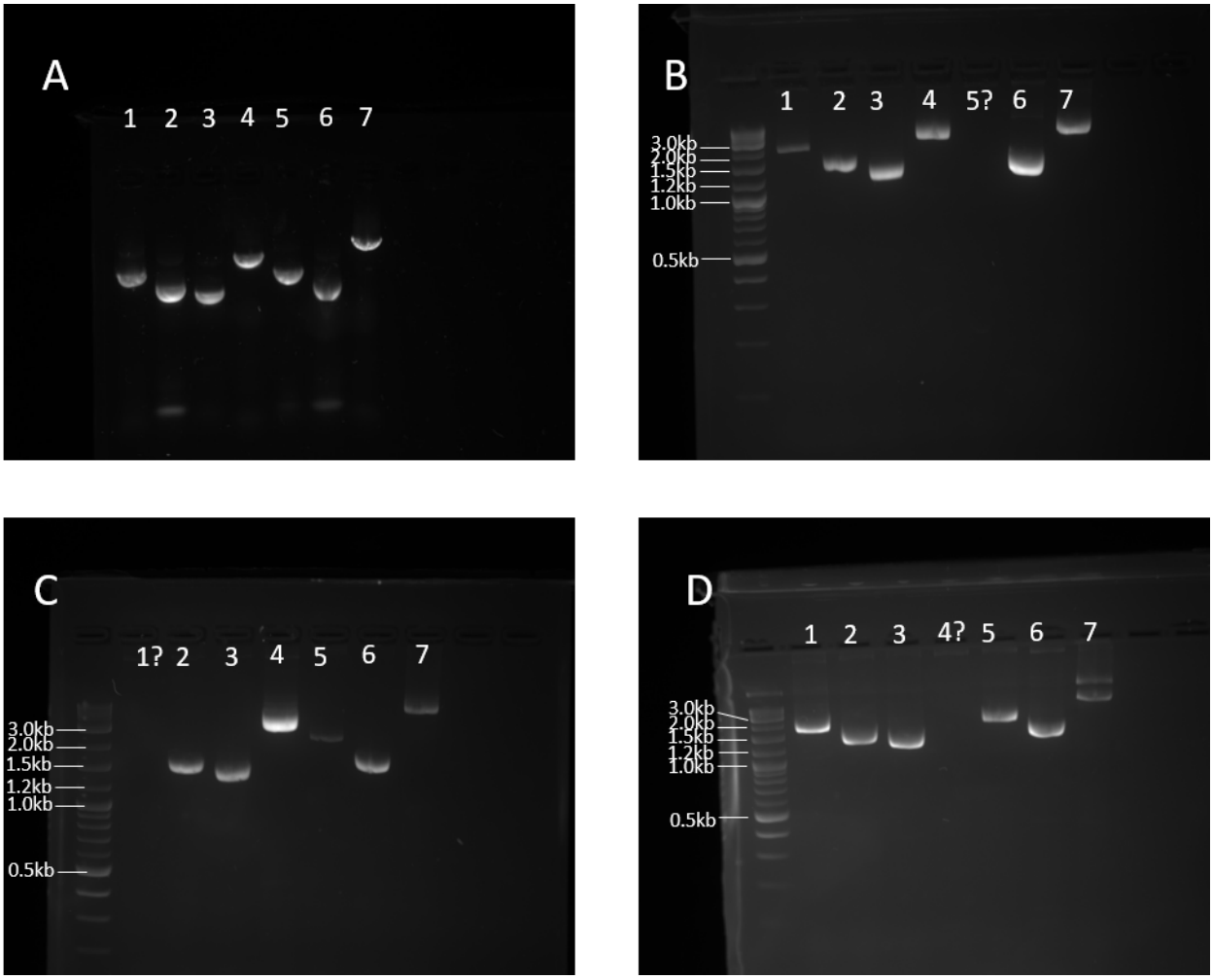

Fig. 5: (A-D) Images of the agarose gel loaded with the DNA ladder and the amplified fragments of the modified bait (bdestorf1) and prey (bdestorf2) plasmids. Samples numbered 1-4 correspond to the bait plasmid fragments, while the samples 5-7 correspond to the prey plasmid fragments. The question mark designates a missing band.

in the 37C incubator. However, no colonies were observed at the plates. This transformation was unsuccessful, as the successful transformants carried the ccdB gene, which is lethal for E. Coli, while the unsuccessful transformations led to the deaths of the Top10 cells due to the absence of any antibiotic resistance.

Preparation of the electrocompetent cells

After the first attempt, the Invitrogen One Shot ccdB Survival 2T1R Competent cells were used for the subsequent transformations. These cells were chemically competent as of the time of the delivery, however, due to the cells being delivered during the weekend of the Build-a-Cell meeting, the cells were exposed to the high temperature and lost their competency. We attempted to chemically transform the plasmids into non-competent ccdB resistant cells, but we were not successful due to the low efficiency of the uptake of the plasmid by the cells. To achieve the highest transformation efficiency possible, we attempted to make the ccdB resistant cells electrocompetent. The pre-culture of Top10 cells was grown in 2YT overnight from an individual colony and then diluted 1:100 in 500mL SOB-Mg. The centrifuge was cooled down to 4C. The cells were grown for approx. 2.5 hours, until the OD600 was within the range of 0.4-0.6 (in this case, the final measured OD600 was 0.56). The cells were subsequently immediately chilled in ice water bath for 10 min, while the culture was constantly swirled to cool the cells evenly. The cells were subsequently transferred from the flask to the test tubes and then centrifuged at 4C, 2500rcf for 10 min. The supernatant was discarded (The following steps were conducted in the cold room at 4C). Cells were resuspended in a chilled 15% sterile glycerol (25 mL/tube). Subsequently, the cells were spinned down at 4C, 2500rcf for 10 min. The supernatant was discarded and the cells were placed on ice immediately. Cells were then again re-suspended in chilled 15% sterile glycerol (25 mL/tube) and spinned-down in the same way as before. As much of the supernatant as possible was discarded without disturbing the pellet by inverting the bottle and then aspirating the remaining liquids by the pipette

tip. The cells were then re-suspended in the 15% residual glycerol and transferred to a chilled tube. Cells were then diluted 1:100 with ddH2O (5μL of cells with 1mL of the water) and the OD600 of the diluted solution was measured. The target value of the OD600 was between 0.85 and 1.0. If the OD600 was under, cells were spinned-down for 5 min at 2500rcf, at 4C and enough supernatant to achieve the desired OD600 was removed. If the OD600 was above the given interval, more sterile 15% glycerol was added to the solution. Finally, the cells were aliquoted into 50μL sterile eppendorf tubes and stored in the -80C freezer.

It was found during the experimentation that 500 mL of the initial medium can yield 3-6 tubes of the electrocompetent cells, what makes the process very inefficient. After the preparation of the electrocompetent cells, we attempted to transform the plasmids into electrocompetent cells. The electrocompetent cells were thawed on ice and 5μL of the Gibson-assembled bait and prey plasmids and the positive control RFP plasmid (with very high transformation efficiency) were added to the thawed electrocompetent cells. We expected the control plasmids to be transformed, which would validate the process of the electroporation and the successful preparation of the electrocompetent cells. The mixtures of cells and plasmids were transferred into a electroporation couvette and the electric pulse for E. Coli: 1mm, 1.8kV, capacitance 25 uF and resistance 200 Ohm was applied. This approach was repeated during all three electroporation attempts.

In the first attempt to prepare the electrocompetent cells, the medium 2YT was used instead of SOB-Mg. This led to the unsuccessful electroporation of the plasmids into the cells and to the electrical discharge (arcing) of all the samples, probably due to the higher salinity content of the medium. In the second attempt, the appropriate medium (SOB-Mg) was used and no arcing was observed for any of the samples. However, we were not able to observe the growth of the bait and prey plasmid-electroporated cells on the LB agar,despite the significant growth of the RFP control, as shown in the Figure 6. This enabled us to conclude that the Gibson Assembly performed earlier did not work properly and that it must be repeated. The third attempt to achieve the transformation of the plasmids through the electroporation did again result into arcing of all the samples.

Preparation of the chemically competent cells

After three unsuccessful trials, we decided to abandon the electroporation and to prepare the chemically competent ccdB resistant cells to attempt to achieve the bacterial transformation of the bait and prey plasmids. To prepare the chemically competent cells, we used the Mix Go E. Coli Transformation Kit Buffer Set and the corresponding protocol [18]. The 0.5 mL of the fresh E. Coli culture was used to inoculate 50mL of the ZymoBroth SOB medium in a 250 mL culture flask. The OD600 of the shaked culture with cells was regularly measured until it reached the value of 0.49, when the culture was placed on ice and swirled for 10 minutes. The cells were subsequently centrifuged down at 2500g at 4C and were pelleted. The supernatant was completely removed and the cells were resuspended in 4mL of 1X Wash Buffer. The cells were then centrifuged at 2500g at 4C, pelleted, and the supernatant was completely removed. The cells were resuspended in 4mL of 1X Competent Buffer and transferred to the test tubes. Approx 20 test tubes containing 200μL of the cell suspension were then stored in the -80C freezer.

Subsequently, the plasmids were transformed into the chemically competent cells using the Addgene protocol [19]. Four DNA samples were chemically transformed: RFP plasmid (positive control), non-modified purified bestorf2 plasmid (positive control), assembled bait plasmid bestorf1, assembled prey plasmid bestorf2. The chemically competent ccdB resistant cells were thawed on ice in 50μL aliquots (four tubes). The agar plates with Carbenicilin resistance were preheated at 37C to increase the efficiency of the growth of the transformants. 2.5μL of DNA of each plasmid (two controls, bait, and prey) were added to the competent cells and mixed by the gentle flicking. The mixtures of the competent cells and DNA were incubated on ice for approx. 30 min. The transformation tubes were then heat-shocked in 42C water bath for 45 s. The tubes were then left on ice for another 2 min. 500μL of SOC media (without antibiotic) was added to the bacteria and grown in 37C rotating incubator for approx. 50 min. 50μL of the cultures were put on the pre-warmed agar plates with Carbenicilin and incubated overnight. Important improvements with respect to the previous protocols were the pre-heating of the Carbenicilin agar plates to 37deG in the incubator prior to transformation, gentle flicking of the transformation tubes instead of vigorous suspension, and the growth in the SOC medium. The results of the transformation are in the Figure 7. After approx. 20 hours, there were numerous observable colonies at the RFP plasmid plate and original non-modified plasmid, as well as cca 8. observable colonies at the plate with the assembled bait plasmid bestorf1. Unfortunately, no colonies were observed on the prey plasmid plate.

The Gibson assembly of the prey plasmid bestorf2 was repeated with 1.0 pmols of the fragment DNA per fragment were used (as outlined in the section about the Gibson Assembly). The bacterial transformation of the plasmid with chemically competent cells was repeated. In this case, the different amounts of the plasmid (2.5μL, 2.0μL, 1.0μL) were added to the chemically competent ccdB resistant cells to test the hypothesis that the excessive concentration of cells was preventing the cells from the sufficient growth. Non-modified prey plasmid bestorf2 was used as the positive control. In this case, the growth was observed for all of the tranformants, as shown in the Figure 8.

As a negative control, we decided to transform the ccdB resistant competent cells with the fragment 5, fragment 6, and fragment 7, i. e. the PCR amplified and purified fragments of the prey plasmid, and to culture them on a Carbenicilin plate. We expected that due to the linearity of these DNA sequences, no colonies will be observed.

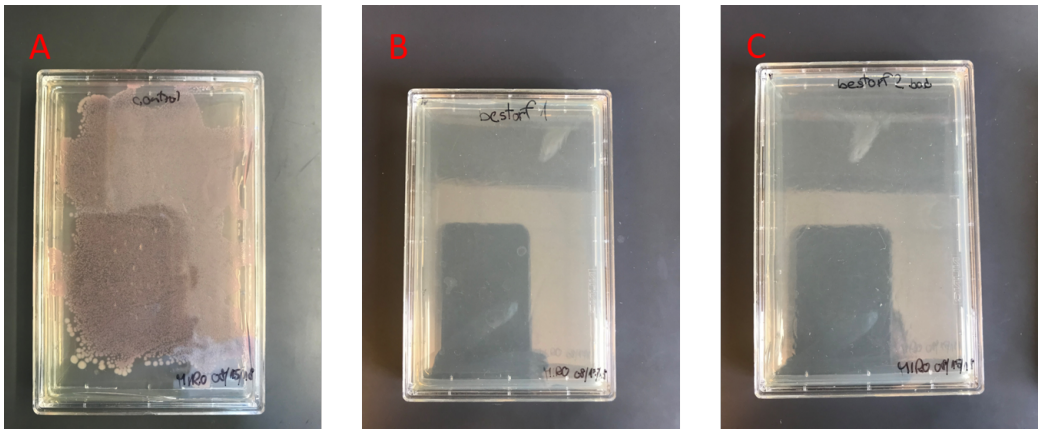

Fig. 6: The results of the transformation by electroporation. (A) Multiple colonies observed on the plate with the RFP plasmid (positive control). (B) No colonies observed on the Gibson-assembled modified bait plasmid (bestorf1). (C) No colonies observed on the Gibson-assembled modified prey plasmid (bestorf2).

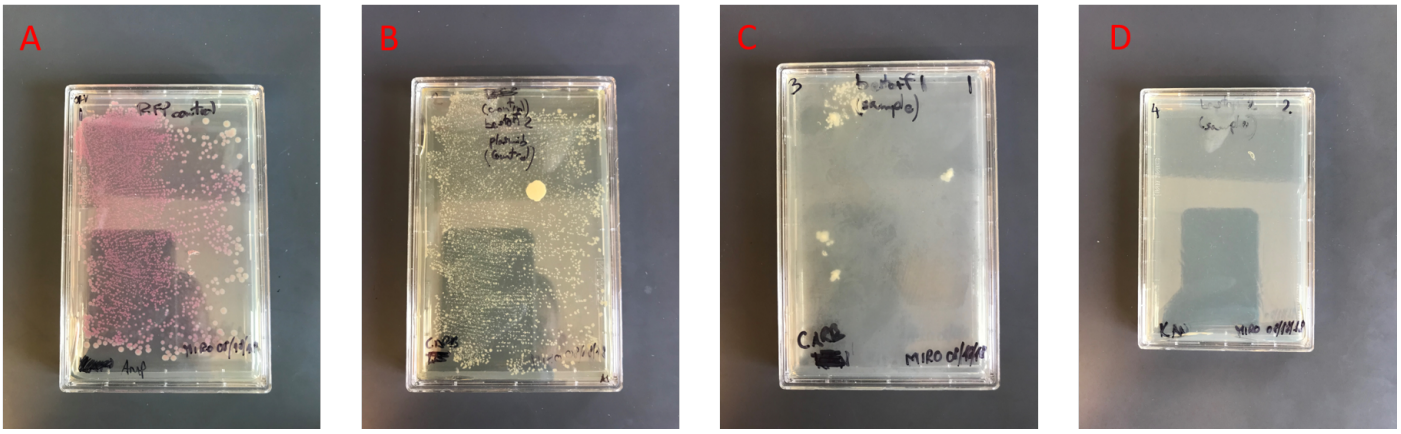

Fig. 7: The results of the transformation of the chemically competent bacteria with RFP plasmid positive control (A), non-modified bestorf2 prey plasmid (B), modified bestorf1 bait plasmid (C) , modified prey plasmid bestorf2 (D). Successful transformation is observed for all plasmids but for the modified bestorf2 plasmid.

However, transformation with fragment 6 (which contains the Chloramphenicol resistance gene and ccdB toxin) and fragment 7 (which contains Hygromycin resistance marker) eventually enabled the growth of the colonies, as shown in the Figure 9.

G. Diagnostic cutting of the plasmids by BsaI

After we were able to transform the plasmids into the ccdB resistant cells, we performed the diagnostic restriction digest of the assembled bait and prey plasmids by BsaI to verify the success of the assembly. To conduct the restriction digest, we picked two colonies of the bait plasmid and four samples of the prey plasmid from the plates shown in the Figure 8 and Figure 9, and incubated them overnight in the 2YT growth medium. The plasmid DNA was purified using the QIAprep Spin MiniPrep Protocol [20]. Subsequently, the following reaction was assembled on ice: 1μL of BsaI enzyme, 1μL of NEB 10x CutSmart X buffer, and 10μL of the purified DNA. The reaction was incubated for 60 min at 37C and then heat-deactivated at 65C for 20 min. Subsequently, the gel electrophoresis of the 10μL of samples was performed as described in the gel electrophoresis section above, with NEB 1 kb Plus DNA Ladder used, instead of NEB 2-log DNA Ladder. We also simulated the cutting of the assembled plasmids with BsaI in the SnapGene, as well as the cutting of the original plasmids provided by the Levy lab. Unfortunately, the diagnostic cutting of the original plasmids was not performed because of the insufficient time.

H. Modular cloning of the rpoA into bait and prey plasmids and transformation into Top10 cells

To prove the validity of our approach, we decided to clone one of the JCVI-syn3.0 genes, rpoA into the assembled bait and prey plasmids using the Golden Gate assembly. The Golden Gate cloning was performed on two samples of the bait plasmid and two samples of the prey plasmid, following the Modular Cloning protocol. Briefly, the following reaction was assembled on ice in a PCR tube in a 10μL reaction for each of the four samples:

- ddH2O to 10μL
- Plasmid vector (bait or prey) DNA (40 fmol)
- Gene insert rpoA (MMSYN1 0645) into the bait plasmid vector. (40 fmol)
- 1 μL 10X CutSmart buffer
- 1 μL ATP
- 0.5 T4 Ligase
- 0.5 μL BsaI

The reaction was subsequently placed into the thermocycler for 60 min at 37C. After thermocycling, the samples were transformed into the Invitrogen One Shot Top10 chemically competent E. Coli cells using the approach for the chemical transformation outlined above and in [19]. The transformed cells were distributed over a Kanamycin agar plate and incubated for 16h in the 37C incubator.

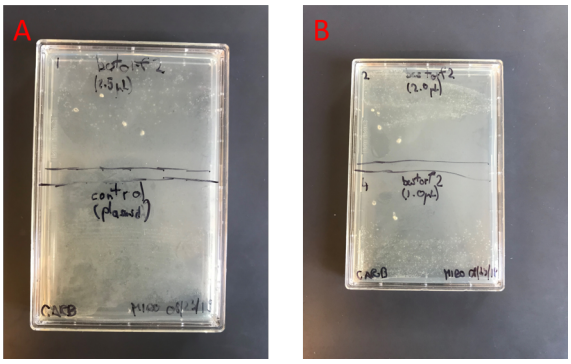

Fig. 8: (A) The colonies of the bacteria control plasmid bestorf2 (non-modified) and the colonies of the bacteria with the modified bestorf2 plasmid, when 2.5μL was added to the 50μL of the ccdb resistant competent cells. (B) The colonies of the bacteria with the modified bestorf2 plasmid, when 2.0μL was added to the 50μL of the ccdb resistant competent cells, and 1.0μL of the plasmid was added to the 50μL of the ccdB resistant competent cells.

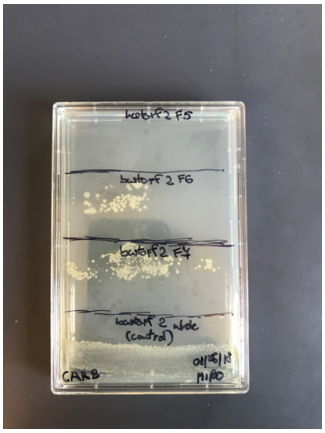

Fig. 9: The unexpected growth of the colonies on the negative control (the bestorf2 prey plasmid fragments) in the first three sections of the plate. The assembled plasmid grows colonies as expected.

I. DNA sequencing of the modified bait and prey plasmid samples

As the final step of the verification of our approach, we sent a sample of the bait plasmid and two samples of the prey plasmid for the Sanger sequencing. The sequences for the analysis were selected so that the successful insertions of the BsaI recognition sites in the attR1 recombination regions can be confirmed. For both plasmids, the presence of BsaI site in the attR1 recognition site was sequenced twice, and the non-modified region of the plasmids was sequenced once to provide a check on the sequencing quality. Sequencing samples were prepared according to the Sequencing Sample Preparation instructions provided by Elim Biopharm, submitting 500 ng of the plasmid DNA with the primers.

III. RESULTS

A. The diagnostic restriction digest by BsaI enzyme suggests the successful modification of bait and prey plasmids into Golden Gate compatible plasmids

We expected that the BsaI enzyme added to the DNA will cut at introduced BsaI sites, and it will not cut at the BsaI sites that were to be removed from the plasmids (at positions 1881 and 6713 in original bdestorf1, and at position 1122 in the original bdestorf2). The SnapGene simulation of the diagnostic digest is in the Figure 10A. The simulation suggested that the cutting should result into following fragments:

- Modified bait plasmid: 1. 6193bp, 2. 1337bp, 3. 122bp.
- Non-modified bait plasmid: 1. 3347bp, 3047bp, 1485bp.
- Modified prey plasmid: 1. 5961bp, 2. 1337bp, 3. 122bp.
- Non-modified prey plasmid: 1. 4304bp, 2. 3348bp.

From these results, we expected a clear distinction between the largest fragments of the modified and non-modified versions of the plasmids. The experimental results in the Figure 10B show the results of cutting two samples of the modified bait plasmid bestorf1 (BO1_GG2, BO1_GG1) and four samples of the modified prey plasmid bestorf2 (BO2_GG1, BO2_GG2, BO2_GG4a, BO2_GG4b). The largest fragments have the sizes of approx. 6kb, clearly distinguishable from 3.3kb, 3.0kb, or 4.3kb bands that would suggest the unsuccessful modification. Also, the careful examination of the samples BO1_GG1, BO2_GG1, BO2_GG4a shows the band in between 1.5kb and 1.2kb. This band is not expected at all for he prey plasmid samples BO2_GG1, BO2_GG4a, and while the band could in principle correspond to the expected 1485bp band in the non-modified bait plasmid bestorf1, it is unlikely that cutting of the plasmid would give the expected result for the largest fragment in the case of the Golden Gate compatibility modification failure. The 122bp bands are not observed for any of the plasmids, what can most likely be ascribed to their small size and hence very difficult observation.

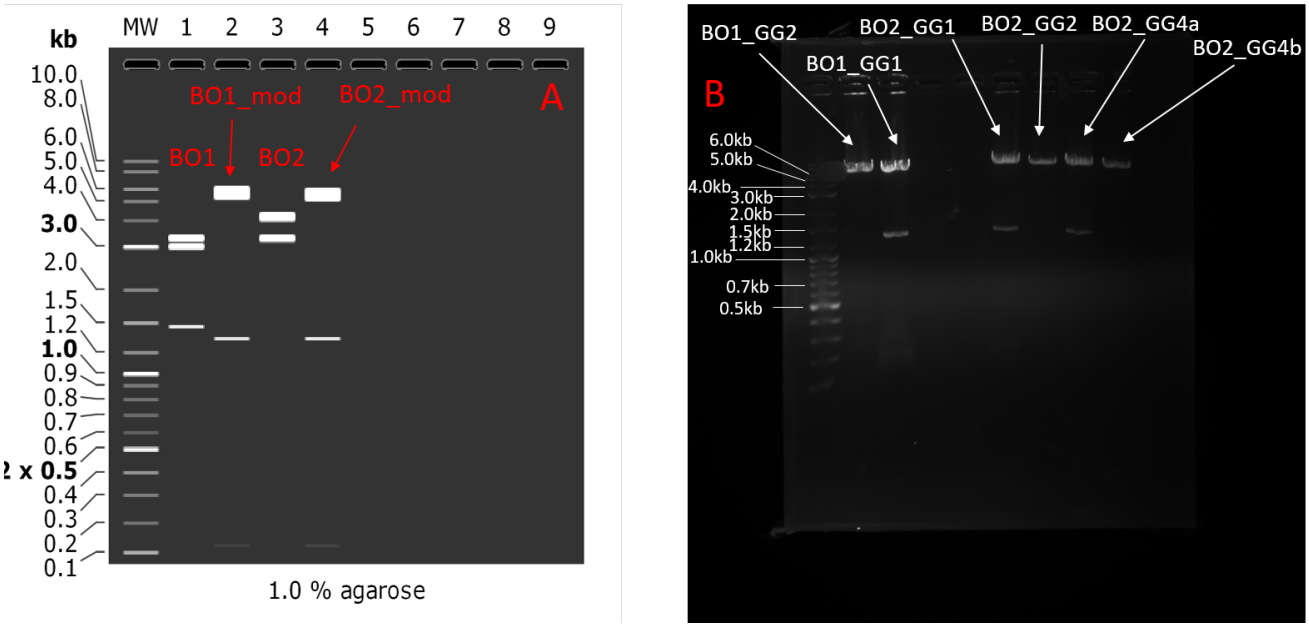

Fig. 10: (A) SnapGene gel simulation where the plasmids are cut by BsaI enzyme. Lane 1: BO1, original, non-modified bait plasmid bestorf1. Lane 2: BO1_mod, modified, Golden Gate compatible bait plasmid bestorf1. Lane 3: BO2, original, non-modified prey plasmid bestorf2. Lane 4: BO2_mod, modified, Golden Gate compatible prey plasmid bestorf2. The differences between the non-modified and modified plasmids are clearly seen. (B) The image of the gel electrophoresis of the modified plasmid samples. All the samples exhibit the band at approximately 6 kb, which is expected for the successfully modified plasmid (expected 6193bp for the modified bestorf1, and 5961bp for the modified bestorf2). Samples BO1_GG1, BO2_GG1, BO2_GG4a show the band that seems to correspond to the expected 1337bp band of the simulated cutting. The 122bp band expected for the both modified plasmids is not observed for any of the samples. This might be due to the small size of the fragment and suboptimal exposure conditions.

B. Successful Golden Gate cloning of the rpoA gene into bait plasmid

Our key objective was to obtain a pair of Golden Gate compatible bait and prey plasmids that would allow the Modular Cloning of the JCVI-syn3.0 genes into these plasmids in an efficient and simple reaction. Following the Modular Cloning protocol outlined in the subsection 8 of the Methods section , we attempted to clone the JCVI-syn3.0 gene rpoA (MMSYN1 0645) into the bait plasmid samples (BO1_GG1, BO1_GG2) and into the two prey plasmid samples (BO2_GG1, BO2_GG4a) for which the diagnostic digest by BsaI indicated the successful modification and assembly. The Modular Cloning reaction was followed by the chemical transformation of the plasmids into chemically competent Top10 cells on the LB agar plate with Kanamycin antibiotic. As the Top10 competent cells do not have any antibiotic resistance, they cannot survive unless the transformation of the plasmids into the cells is successful. Furthermore, ccdB gene is toxic for these cells and hence they cannot survive if the ccdB gene between the BsaI sites is not replaced by the non-lethal JCVI-syn3.0 gene rpoA. The bait plasmid bestorf1 contains the kanMX gene which confers the Kanamycin antibiotic resistance and hence we expected to see the colonies formation on the kanMX plate. Prey plasmid bestorf2 does not contain the Kanamycin-resistance gene and hence we expected that no colonies will be observed. Therefore, the prey plasmid fulfilled the role of the negative control. Unfortunately, the experiment could not be repeated with a different antibiotic selection marker (i. e. Hygromycin or Chloramphenicol) due to unavailability of the appropriate plates and the shortage of time. After the incubation, three large colonies of the bait plasmid were observed on the Kanamycin plate, confirming the successful transformation of the rpoA gene into the bait plasmid, as displayed in the Figure 11.

C. Sanger Sequencing of the attR1 recombination sites of bait and prey plasmids indicates successful introduction of the BsaI sites

The portions of the bait plasmid sample BO1_GG1, and the prey plasmids samples BO2_GG1, BO2_GG4a were sequenced by the Elim Biopharm, using the Sanger sequencing method. The portions of the plasmids that were sequenced included the attR1 recombination sites of the original plasmids. The aim was to confirm the presence of the BsaI recognition site within the sequenced region and hence confirming the successful plasmid assembly. To analyze the sequence, we used the SnapGene software and its utility of sequence alignment to the reference sequences. The locations of the sequenced regions for both bait plasmid sample and prey plasmid samples are in the Figure 12.

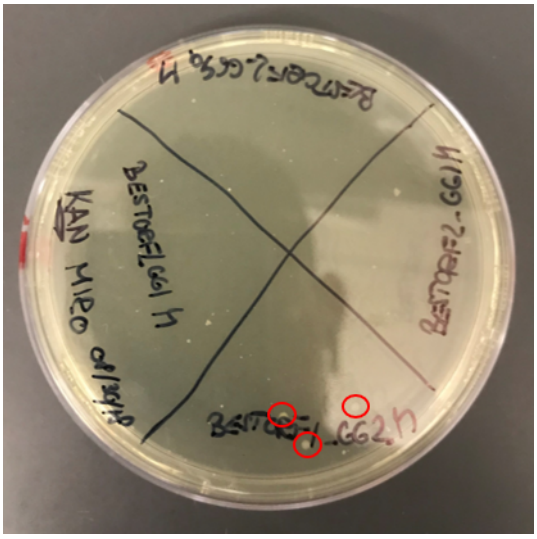

Fig. 11: The Kanamycin LB agar plate with the cell cultures with transformed bait plasmid samples (BO1_GG1, BO1_GG2) and transformed prey plasmid samples (BO2_GG1, BO2_GG4a) with a cloned rpoA gene of JCVI-syn3.0. No colonies were observed for the samples BO1_GG2, BO2_GG1, and BO2_GG4a. Cell colonies can be observed for the modified bait plasmid sample BO1_GG1 (marked by red circles), confirming the successful cloning of a JCVI-syn3.0 gene into the bait plasmid.

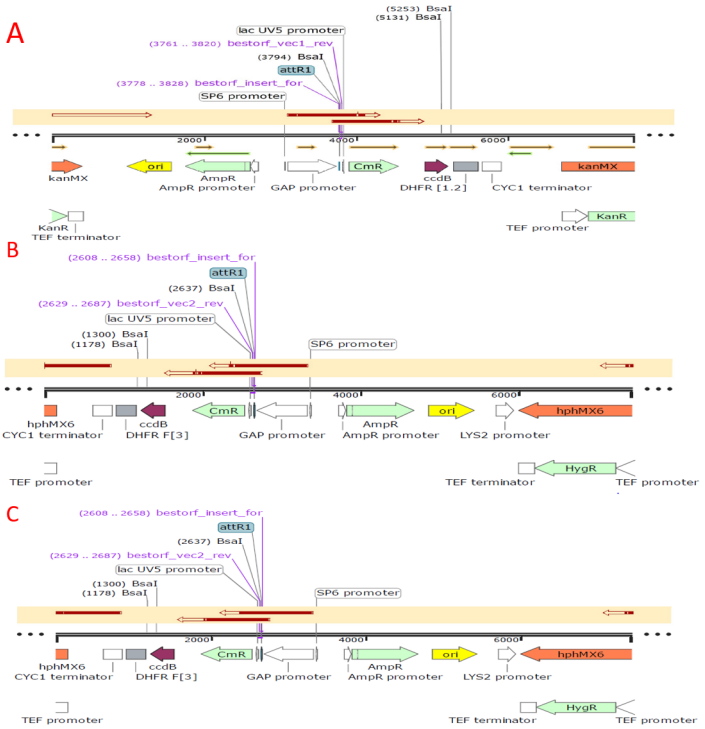

Fig. 12: (A) Two Sanger sequencings of the attR1 site of bait plasmid sample (BO1_GG1) indicates the successful insertion of the BsaI recognition sites is marked by the full red arrows. Another, failed sequencing of the region between kanMX gene and ori origin of replication marked by the empty red arrow sequencing reaction. The primer likely did not anneal to the template. (B) Two Sanger sequencings of the attR1 site of prey plasmid sample (BO2_GG1) and another sequencing of the hphMX6 region is marked by the full red arrows. The sequenced regions suggests that the plasmids were assembled correctly. (C) Two Sanger sequencings of the attR1 site of prey plasmid sample (BO2_GG4a) and another sequencing of the hphMX6 region is marked by the full red arrows. The high quality of the sequenced regions suggests that the plasmids were assembled correctly.

The analysis of the bait sample BO1_GG1 sequencing reveals the failed sequencing in between the kanMX and ori regions. As no alignment was observed, the most probable reason for the failed sequencing is the inability of the primer to anneal to the template, as the template DNA was added to the reaction and the quality of the DNA in the other regions is good. Two sequencings of the attR1 region of the plasmid are of higher quality, with a single clear mismatch in each of the two sequences, and three undetermined (N) bases observed in one of the sequences. However, the BsaI recognition site is present in both Sanger sequences.

In the prey plasmid BO2_GG1 sample, the first attR1 sequencing contains one inserted C base at the beginning of the sequence, where the quality of the sequencing is not very accurate. Also, two N bases are observed approx. 50bp from the beginning of the sequence. The second sequencing of the attR1 region contains N 30bp from the beginning, insertion of C approx. 15bp from the beginning of the sequence, and a misplaced A 60bp from the start of the sequence. However, BsaI recognition sites are clearly present in both sequencing results. The sequencing of the hphMX6 region is without any gaps and contains a single N towards the end of the sequence, which is impossible to distinguish between G base and T base.

The last sample of the prey plasmid, BO2_GG4a also contains the BsaI recognition sites. The first sequencing of the attR1 region contains a single N base approx. 15bp from the end. The second sequencing of the attR1 region contains a single misplaced A base, 15bp from the beginning of the sequence and a deletion at the very end (8bp from the end of the sequencing). Finally, the sequencing of the hphMX6 region contains a single N approximately 100bp from the beginning and the sequence is otherwise without any fails or mismatches. Overall, 8 out of 9 Sanger sequences contain at most four mismatches and all the attR1 regions contain the BsaI recognition site. As we achieved successful Golden Gate assembly of the plasmid and rpoA gene for the plasmid in which the sequencing faile, we can conclude that the sequencing supports the successful modification of the plasmids.

IV. DISCUSSION

Our work has demonstrated the success, but also the intricacy of the experimental techniques used in the synthetic biology. Although our results indicate the success in the experimental modification of the Gateway compatible

plasmids to the Golden Gate compatible plasmids, the most important aspect of this effort was its educational value that was best delivered through the experimental failures and their subsequent analysis. Our initial goal of delivering a complete PPiSeq assay of JCVI-syn3.0 within six weeks was proven to be too ambitious, given no prior experience of the experimenter with the techniques used and the short time frame to conduct the experiments. This lack of experience led to several not-to-be-repeated errors, such as not adding the antibiotics to the media during the cultures outgrowth and subsequent need to discard the cultures, leading to several short delays. Also, we realized that many techniques such as primer design require iterations and that the single optimal solution does not exist (such as the minimization of the risk of self-dimerization of the primers and formation of the hairpins) and hence entail not only engineering aspects, but also the artistic ones. Below we discuss several challenges encountered during the experimentation and the lessons learned from them over the course of the work.

Possible reasons for the PCR amplification failure

Why did PCR amplification failed? Failure due to the primer design is unlikely (as all of the fragments were amplified at least in one reaction), as well as the thermocycler error (other bands in the gel were fine). Therefore, most probably, the reason for the weak amplification was the low efficiency of the primer binding due to the larger difference in the optimal primer annealing temperature of the primers used to amplify the corresponding fragments compared to the annealing temperature used (61C). This exactly matches the higher annealing temperatures of the primers participating in the amplification of the fragments that are missing in any of the four gel pictures in the Figure 5: fragment 1 (primer bestorf_amp_for, optimal T_m = 71C), fragment 5 (primer bestorf_amp_for, optimal T_m = 71C), fragment 4 (primer bestorf_amp_rev, optimal T_m = 72C). As an improvement, the PCR amplification would need to be run again, with the different annealing temperature for the fragment 1, fragment 4, and fragment 5. However,the fragment 7, in which the primer bestorf_amp_rev is used, did not exhibit any problems with the assembly and this might be due to the less complex DNA sequence structure to which the primers could bind more easily.

Lessons learned from the repeating of the experimental steps

Multiple steps of the overall experimental journey were carried several times, and this enabled us to learn more about the intricacies of the experimental approach, to identify some of the systematic vs. random errors, and to move faster each time the step was repeated, while improving the practical experimental skills. PCR amplification and gel electrophoresis was conducted four times and from the distribution of the missing bands, we could identify the probable reasons for its failure (the annealing temperature that was too low for certain primers). Plasmid DNA purification step was carried three times. The Gibson assembly was repeated three times; while only two attempts were needed to get the transformation of the plasmid bestorf1 into ccdB resistant competent cells, third attempt was needed to transform the plasmid bestorf2, using the same amount of fragment DNA as the one that proved to be successful in the case of the plasmid bestorf1. The electrocompetent cells were prepared three times, and although none of the three attempts to transform the plasmids into the electrocompetent cells using electroporation was successful, each attempt gave us insight into possible causes of failure, such as different salinity content of the media used (2YT vs. SOB-Mg) leading to the arcing, or the presence of the air bubbles. All this knowledge and the general awareness of the possible obstacles will be used in the further experimental work.

Importance of thinking about the experiments prior to execution

The futile attempt to transform plasmids containing the lethal ccdB resistant gene into the Top10 cells, which also lack any antibiotic resistance bears an important lesson that it is always necessary to think about the experimental setting prior to its execution in order to save the resources and time. Due to the lethality of the environment in the case of successful and unsuccessful transformation, no useful conclusions could be drawn from this experiment. Similarly, the attempt to put the Top10 cells with transformed prey plasmid with cloned rpoA gene could not be successful, as the plasmid does not contain the Kanamycin resistance gene. Due to the lack of time, we could not repeat the experiment with a different antibiotic and thus we learned the hard way that careful thinking is important before the experiment. In this way, the prey plasmid served as a negative control.

Paying attention to details is important

Any deviation from the exact experimental protocol can have a significant effect on the experimental outcome. We found that even the subtle details, such as the use of the exact medium composition, such as in the case of the preparation of the electrocompetent cells are important - despite subtle difference between the composition of the SOB-Mg and improperly used 2YT for the bacterial outgrowth. Similarly, gentle flicking instead of resuspending of the chemically competent cells with plasmid during the chemical transformation might have been the key to successful experiment. For example, it is crucially important to avoid any impurities, such as air bubbles, during the electroporation to prevent arcing.

Apart from these experiment-specific learning outcomes, there were many other, more general skills acquired. The most important skills include the need to ask the meaningful questions (such as Why does it matter?), and the need to think about the synthetic biology more as about the art.

V. CONCLUSIONS

In this paper, we presented the experimental approach used to modify the bait and prey plasmid vectors used in the PPiSeq protein-protein interaction assay so that the plasmids are compatible with the Golden Gate assembly and allow for the high-throughput and efficient cloning of the JCVI-syn3.0 genes into these vectors. Our results were supported by the diagnostic digest of the assembled plasmids by BsaI enzyme, Sanger sequencing, and the successful Golden Gate cloning of the JCVI-syn3.0 gene rpoA into the bait plasmid. The modification of the plasmids represents an important step on our way towards obtaining a complete protein interactome of the minimum organism. We hope that this "proof-of-concept" will be followed by the cloning of all the JCVI-syn3.0 genes into PPiSeq bait and prey plasmids, their sequencing and verification, and the subsequent realization of the PPI assay of the whole proteome of JCVI-syn3.0, helping us in the identification of the unknown functions of the essential genes.

APPENDIX

10 research non-specific lessons I learned from Drew Endy:

- Before you start something, ask "why? " and "why does it matter? "
- The number one job of the PhD student is to become a better researcher - which means to ask better questions and learn when to KILL the project.
- Always communicate your lack of experience or skills vocally - you will save yourself a lot of sleepless nights.
- Personal happiness in the place where you are is important.
- Synthetic biology should be regulated as a free language.
- Synthetic biology is three-dimensional - and there is enough space for everyone.
- The power of a good metaphore.
- While experimenting, do not prepare next experiment and rush - think about the results and what they mean instead.
- Even spending every day working in the lab might not deliver desired results - take more breaks.
- Be concise and to the point when talking to Drew.

CONTRIBUTIONS

Miroslav Gasparek performed the experiments and wrote the manuscript. Keoni Gandall designed the primers and supervised Miroslav during the day-to-day work. Drew Endy suggested the project and provided the big-picture supervision and the valuable advice.

ACKNOWLEDGMENTS

I would like to thank to Professor Endy for kindly admitting me to the summer internship in his group at Stanford University. I would also like to ask to Keoni Gandall for his patience and willingness to teach me many experimental techniques and practical applications of the genetic circuit design. I would also like to thank to all the members of the Endy lab for their valuable insights, advice, and friendship that we build over the eight weeks I spent in the lab. Furthermore, I would like to thank to everyone who supported me and made this internship possible, especially Ms Danica Matakova and Mr Eduard Matak. Finally, I would like to thank to my parents for their never-fading support for my passion.

REFERENCES

[1] Hutchison, C. A., Chuang, R. Y., Noskov, V. N., Assad-Garcia, N., Deerinck, T. J., Ellisman, M. H., Venter, J. C. (2016). Design and synthesis of a minimal bacterial genome. Science.
[2] Glass, J. I., Merryman, C., Wise, K. S., Hutchison, C. A., Smith, H. O. (2017). Minimal cells-real and imagined. Cold Spring Harbor Perspectives in Biology.
[3] Schlecht, U., Liu, Z., Blundell, J. R., St Onge, R. P., Levy, S. F. (2017). A scalable double-barcode sequencing platform for characterization of dynamic protein-protein interactions. Nature Communications.
[4] Gasparek, M. (2018). Essential Unknowns: Set Theory - Interview with John I. Glass. Retrieved November 25, 2018.
[5] Ventola, C. L. (2015). The antibiotic resistance crisis. Part 1: causes and threats. PT: A Peer-Reviewed Journal for Formulary Management.
[6] Danchin, A., Fang, G. (2016). Unknown unknowns: essential genes in quest for function. Microbial Biotechnology.
[7] Antczak, M., Michaelis, M., Wass, M. (2018). Investigating the unknown functions in the minimal bacterial genome reveals many transporter proteins. BioRxiv.
[8] Yang, Z., Tsui, S. K. W. (2018). Functional Annotation of Proteins Encoded by the Minimal Bacterial Genome Based on Secondary Structure Element Alignment. Journal of Proteome Research.
[9] Fields, S., Song, O. K. (1989). A novel genetic system to detect protein-protein interactions. Nature.
[10] Remy, I., Campbell-Valois, F. X., Michnick, S. W. (2007). Detection of protein-protein interactions using a simple survival protein-fragment complementation assay based on the enzyme dihydrofolate reductase. Nature Protocols.
[11] Katzen, F. (2007). Gateway recombinational cloning: a biological operating system. Expert Opinion on Drug Discovery, 2(4), 571589.
[12] Weber, E., Engler, C., Gruetzner, R., Werner, S., Marillonnet, S. (2011). A modular cloning system for standardized assembly of multigene constructs. PLoS ONE.
[13] Engler, C., Kandzia, R., Marillonnet, S. (2008). A One Pot, One Step, Precision Cloning Method with High Throughput Capability. PLoS ONE, 3(11), e3647.
[14] Lgarashi, K., Lshihama, A. (1991). Bipartite Functional Map of the E. coli RNA Polymerase a Subunit: Involvement of the C-Terminal Region in Transcription Activation by CAMP-CRP. Cell (Vol. 65).
[15] New England Biolabs (2018). Protocol Phusion High-Fidelity PCR Master Mix with HF Buffer. Neb.com. Retrieved 27 November 2018
[16] DNA MODIFYING ENZYMES Instruction Manual Gibson Assembly Master Mix. (2017)
[17] PCR clean-up Gel extraction User manual NucleoSpin Gel and PCR Clean-up (2017).
[18] Mix Go E. Coli Transformation Kit Buffer Set Instruction Manual (2018). Zymoresearch.eu. Retrieved 27 November 2018.
[19] Addgene: Protocol - Bacterial Transformation. (2018). Addgene.org. Retrieved 27 November 2018.
[20] QIAprep Spin Miniprep Kit High-Yield Protocol - English (PDF) - QIAGEN . (2018). Qiagen.com. Retrieved 28 November 2018.