pDel: A One-Plasmid, One-Step System for Gene Knockout in *Escherichia coli*

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Introduction

Gene knockout is a widely used method in all fields of biological research as it is crucial for understanding gene function. Permanently disrupting expression of a specific gene allows researchers to study the effects of that gene in a controlled way. Gene knockouts are applied in several different organisms, including mice, bacteria, and yeast. The gene knockout method has been used for many years now, and knockout approaches have continuously been improving. Homologous recombination is an intrinsic mechanism that cells use to fix DNA mutations (Sun et al., 2020). Several knockout methods rely on this mechanism – for example, the red recombination system initiates homologous recombination with exogenous DNA in prokaryotic cells and the suicide vector system utilizes vectors containing homologous DNA (Zhang et al., 2000, Wang et al., 2019). Alternatively double stranded breaks, or DSBs, can be introduced to the target site. The cell has a different intrinsic repair system – non-homologous end joining or NHEJ – for DSBs. While homologous recombination may be fairly accurate in correcting mutations, NHEJ is significantly error-prone and often results in insertions and deletions (indels) at DSB sites. These indels would leave a gene non-functional, or "knocked out" (Li et al., 2023). Several knockout strategies have been developed based on initiating DSBs. ZFNs and TALENs, which are based on nucleases with genome editing capabilities, are most notable, although they are rarely adapted for prokaryotic use (Zhang et al., 2023). Most recently, the advent of CRISPR-Cas9 technologies for targeted DNA engineering has been groundbreaking for gene editing. CRISPR allows gene editing to be much more efficient than it ever has been and is undoubtedly the new standard. However, for gene knockout specifically, CRISPR-Cas9 often has to be utilized alongside the Red system, also referred to as recombineering, or requires multiple plasmids to obtain successful mutants (Pyne et al., 2015).

Despite all the advancements in gene knockout methods, the development of even more efficient and accurate tools is still necessary to continue to drive research forward. We want to build a robust, streamlined gene knockout system, starting in *E. coli*, that only requires one plasmid and one step. In the lab, we hope that researchers will be able to quickly and easily adapt our system to knockout any gene they want to study. In order to achieve this goal, we combine an inducible expression system with CRISPR-Cas12a. CRISPR-Cas12a has several advantages over CRISPR-Cas9. Instead of producing blunt ends, Cas12a generates staggered cuts. Cas12a also does not require a tracrRNA and can process its own crRNA (Zetsche et al., 2015). As a result, an entire autonomous CRISPR-Cas12a system can be encoded onto a single plasmid. Additionally, Cas12a exhibits significantly fewer off-target effects than Cas9 (Modrzejewski et al., 2020). We aim for our system to be self-clearing as well, meaning it removes itself from the cell once the knockout has been performed. We are starting the development of pDel in *E. coli*, an important and universal model organism. pDel is based on pFREE, a "a universal plasmid-curing system" that utilizes CRISPR-Cas9 to clear plasmids in gram-negative bacteria. pFREE is also a one-step system and has "self-curing" capabilities – pDel's proof of concept strove to replicate pFREE's plasmid clearance model (Lauritsen et al., 2017).

The knockout system, which we have chosen to name pDel, is a plasmid construct with multiple components. First, the inducible expression system we chose consists of the pTet promoter controlling expression of the enzymes encoded on pDel and the tetR protein, which acts as a repressor. The inducer for this system is anhydrotetracycline, or ATC. When the system is in its uninduced state, tetR is bound to pTet, preventing transcription of Cas12a. When induced, ATC binds to tetR, releasing it from pTet, allowing for expression to occur. ATC is a common inducer for *E. coli* and can easily be added to bacterial liquid cultures for overnight induction. This pTet-tetR system was adapted from pFREE. pDel has a p15a origin of replication, a common *E. coli* ORI. The CRISPR-Cas12a system on pDel consists of the Cas12a gene and our engineered crRNA. The crRNA contains a conserved spacer, a repeat sequence, the 20 base pair protospacer, the same repeat, and a conserved tail. It is an individual cassette, meaning transcription of the crRNA is controlled by a constitutive promoter, J23119. There is also a terminator at the end of the crRNA, rrnB T1, that prevents the rest of the plasmid from being constitutively expressed. The protospacer is the key to pDel's adaptability; users can be guided to design the protospacer with their specific sequence of interest and PAM. pDel will

have two crRNAs – one for the gene being knocked out and another for self-clearance. The self-clearance crRNA will always be targeting its own p15a ORI. By disrupting its own origin, pDel will stop replicating and ultimately be cleared from the cell. This self-clearance concept is also adapted from pFREE. The expression levels of pDel's various components will be tuned such that gene knockout occurs before self-clearance a large majority of the time. pDel has ampicillin and carbenicillin antibiotic resistance as a form of selection. We are uncertain about the exact repair mechanisms that take place in *E. coli* following CRISPR-Cas12a generating a DSB in the genome. Either way, the exonuclease and ligase hypothetically work to ensure the two ends from the break are re-annealed and the rest of the genome remains intact.

Materials and Methods

For the pDel system, our basic cloning protocol consists of PCR to isolate our plasmid backbone and gene of interest and insert restriction sites, gel electrophoresis to confirm correct amplification of DNA, cleanup of DNA to isolate the PCR products, Golden Gate assembly to insert the sequence of interest into a plasmid utilizing restriction sites, transformation into *E. coli* and allowing colonies to grow on plates, picking colonies that successfully expressed the desired phenotype, performing miniprep to isolate the plasmid DNA, and finally sequencing to verify the contents of the plasmid DNA.

Our pDel1 consisted of a p15a ORI, the Bla ampicillin resistance gene, the GFP reporter gene, and the TetR cassette consisting of the TetR repressor gene and the pTet promoter. pDel1 was transformed into cells and picked into +/- ATC cultures to display fluorescence. We designed and ordered a gBlock for the gRNA sequence in 2 fragments for pDel2 and inserted it into the pDel1 via 3 fragment assembly. The gRNA in pDel2 included a 20 bp protospacer, promoter, repeats, terminator, and two fragments with BsaI sites for assembly. The gRNA would identify a TTTN PAM target for the ColE1 origin. In pDel3, we inserted the Cas12a gene in place of GFP, but observed leaky expression of Cas12a. pDel5 was created by reinserting the gRNA into pDel1 after the GFP gene to prevent its interference with GFP or Cas12a expression. pDel7 was created as an analog of pDel3 by replacing the GFP gene in pDel5 with Cas12a. In order to tune the Cas12a expression further, we decided to use a ribosome-binding site (RBS) library for pDel7. Using PCR with an oligonucleotide primer containing three degenerate bases at a key position in the RBS, we experimentally generated a library of pDel7 clones with unique RBS sites and different expression levels, out of which we could select for high inducibility of Cas12a activity (along with sufficient cell density).

To assay pDel7, we tested its clearance of reporter plasmid p20n31, which constitutively expresses GFP and contains the ColE1 origin that pDel7 is targeting. Our plasmid clearance assay protocol for pDel7 consisted of transforming mach1 E. coli cells with the reporter plasmid

p20n31, making competent cells (mach1 + p20n31), and transforming pDel7 into the mach1 + p20n31 cells with ATC. We grew these cells in a 96 well plate with ATC for induction and analyzed fluorescence and cell density with the Tecan plate reader. After selecting desired clones, we miniprepped and sequenced them. We also transformed the purified plasmid of these clones into cells with p20n31 and grew them with ATC to confirm their clearance activity.

pDel8 was created by replacing the p15a origin of pDel7 with the ColE1 origin. To test for self-clearance, we transformed pDel8 into cells and grew on Carb +/- plates after ATC induction, expecting cell death on Carb. Our initial assay included a step where cells were grown in ATC and media without Carb before plating on +/- Carb. Our new assay maintained Carb selection pressure at all times by growing cells in Carb containing media and finally plating with and without ATC, expecting cell death on ATC.

pDel10 was created by replacing the crRNA protospacer in pDel7 with a new protospacer sequence targeting the UPP gene in E. coli. This poses a challenge because there are repeats flanking the protospacer in the crRNA sequence. To isolate the protospacer from the repeats, we performed one round of PCR that split the pDel7 backbone into two halves. We performed a second round of PCR on the products of the first round to add BsaI sites for assembly and replace the old protospacer sequence with a new sequence. The final pDel10 plasmid was assembled via 3 fragment assembly. We later added a gel purification step after the first round of PCR to purify the products and ensure the template of the first round was not interfering with the second round. We used the same assay protocol for pDel10 as pDel8 (growing in Carb to maintain selection pressure and plating on +/- ATC).

Results

The pDel1 cloning protocol formed the backbone for all of our future, more complex developments of pDel. It covers the basics and confirms that our antibiotic resistance and GFP gene work according to the inducible TetR system, allowing us to use this plasmid as a template for future pDel plasmids.

Figure 1: ATC-inducible expression confirmed. GFP expression screenings conducted via pDel1 transformations in (A) pDel1A, (B) pDel1B, wherein “A” and “B” in transformation names imply distinct colonies. The cells fluorescing with GFP expression contain ATC, which indicates successful TetR system operation. Then, pDel1 was refined to include gRNA targeting the ColE1 origin for the system to be able to operate with CRISPR-Cas12a, resulting in pDel2. The gRNA was inserted before the Tet promoter.

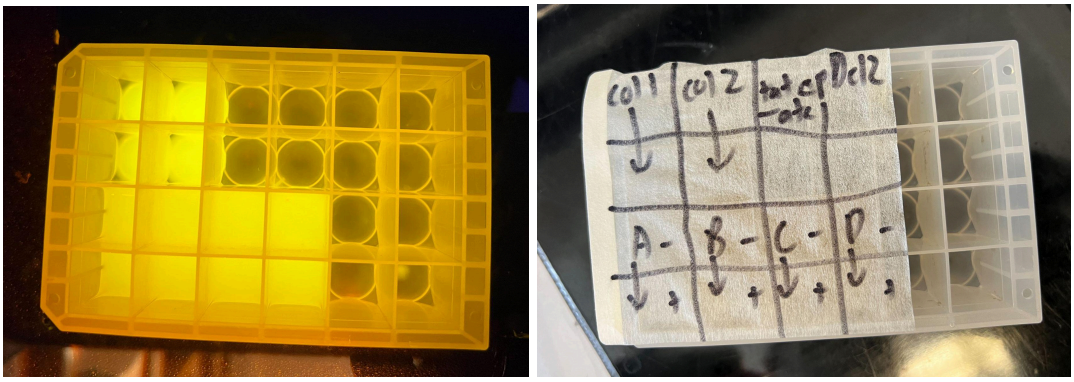

Figure 2: Six colonies from pDel2 transformation picked and cultured with (+) and without (-) ATC confirms ATC-inducible expression. Fluorescent cells expressing GFP contain ATC, suggesting successful TetR system operation.

We initially expected white colonies in our results for pDel3 due to the presence of ATC, which activates Cas12a expression, causing cleavage of the ColE1 origin, clearing p20n31 (reporter plasmid containing GFP), resulting in a lack of GFP, consequently leading to white colonies.

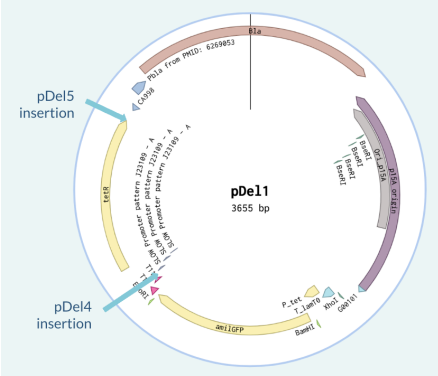

Figure 3: To the left is a plasmid map of pDel1 showing the new insertions of our gRNA for the new pDel5 and pDel4 plasmids.

Instead, we found green colonies before and after ATC. After careful consideration, we realized that this result was due to leaky expression of the plasmid, since our promoter, J23119, is extremely strong and our terminator was not strong enough to fully halt the expression of Cas12a. In pDel3, our gRNA is

located directly upstream of pTAT + Cas12a, which causes our leaky expression. So, to develop new plasmids with refined pDel3 function, we inserted our gRNA after the GFP gene to fix the leaky expression issues (Figure 3). pDel5 resulted from an insertion of the gRNA downstream of the TetR cassette to prevent any interference with Cas12a expression.

pDel7 is an altered version of pDel5 in which the GFP gene is replaced by the CRISPR Cas12a gene with a protospacer, so that the plasmid is able to target the ColE1 origin of a reporter plasmid. Figure 5 displays the results of the screen for a library of 96 clones generated by our pDel7 RBS library. After selection of the desired pDel7 clones, we confirmed their phenotype further. Notably, a significant contrast in fluorescence emerged between cells cultivated with and without ATC in two clones as compared to a control (refer to Figure 6). This finding substantiates the effective cleavage of the crRNA target sequence using CRISPR-Cas12a, affirming pDel7's efficacy in eliminating plasmids.

pDel7 RBS Library

0.2949	0.2753	0.2587	0.2585	0.2632	0.2936	0.2794	0.2865	0.2811	0.2798	0.3013	0.2754
0.3311	0.2925	0.2791	0.3189	0.3166	0.2912	0.2888	0.3177	0.2865	0.3000	0.3049	0.3037
0.3014	0.2612	0.2827	0.3262	0.2740	0.2840	0.2662	0.3035	0.2820	0.2861	0.2528	0.2763
0.2697	0.2781	0.2738	0.2998	0.2903	0.2655	0.2939	0.2885	0.2897	0.2827	0.2789	0.2803
0.2748	0.2784	0.2798	0.2897	0.2795	0.2974	0.3247	0.3061	0.3095	0.3188	0.2861	0.3119
0.3178	0.3028	0.3089	0.3406	0.3213	0.3044	0.3151	0.3421	0.3359	0.3363	0.3396	0.2532
0.2881	0.2804	0.2910	0.2755	0.2852	0.2912	0.2883	0.3079	0.2905	0.3000	0.3017	0.2260
0.2818	0.3017	0.2785	0.3177	0.3001	0.3043	0.3075	0.3208	0.3184	0.3297	0.3371	0.3131

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	4199	44	83	131	71	2047	4333	46	97	890	4111	145
B	99	4170	55	94	4605	52	71	4460	98	4275	4153	4014
C	3741	62	4215	4012	287	4149	85	74	4299	70	46	80
D	97	4362	43	4054	4173	40	50	4354	4404	4292	48	60
E	4475	4556	49	4121	4096	2349	4520	4770	409	4951	3853	3339
F	4866	4563	4734	4415	4318	4458	**50**	4932	**54**	4862	3595	898
G	93	76	4404	56	3489	2758	4516	4418	642	115	3711	967
H	3984	5041	4485	4973	**49**	4496	4806	4916	1042	3294	4952	**52**

Figure 5: pDel7 RBS Library (a) Optical density reads of 96 colony screen with ATC induction (measuring cell growth). Colonies above average OD have red background. (b) GFP fluorescence data of 96 colony screen with ATC induction – darker shades of red correspond to lower values. Reporter plasmid, p20N31, expresses GFP; GFP expression is expected to be reduced upon p20N31 clearance with pDel7. Therefore, ideal candidates have reads with low GFP fluorescence, but above average OD. Through this method, F7, F9, H5, H12 (bolded) were chosen as candidates.

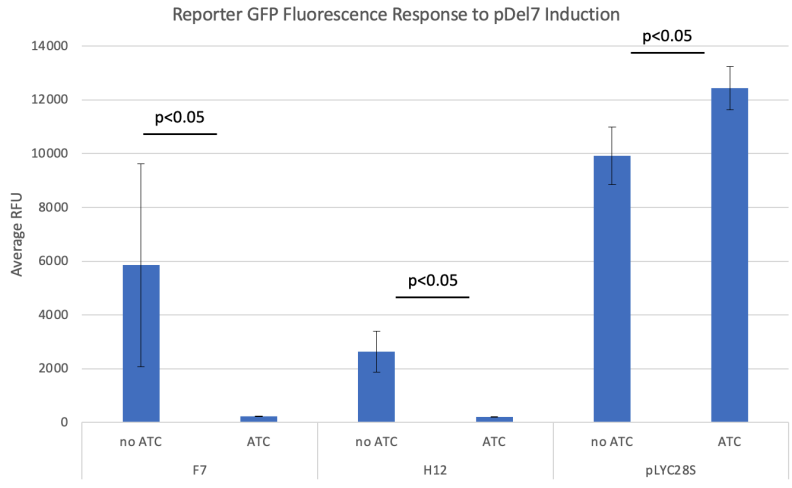

Figure 6: pDel7 clones F7 and H12 show significant reduction in fluorescence with ATC while the control pLYC28S does not.

pDel8 follows a similar mechanism, however with an inserted ColE1 origin instead of the original p15a origin. This allows for the pDel8 system to clear its own origin of replication. To further validate this capability, quadruplicate colonies were cultured overnight with ATC, subsequently diluted by a factor of 10^-6, and then plated both with and without ampicillin in parallel following the introduction of pDel8 into Mach1 cells. The plates lacking ampicillin exhibited an average of around 100 colonies, while no colonies were observed on the ampicillin plates. This outcome proves pDel8's ability to eliminate itself from cells without the need for the removal of antibiotic selection (see figure 7).

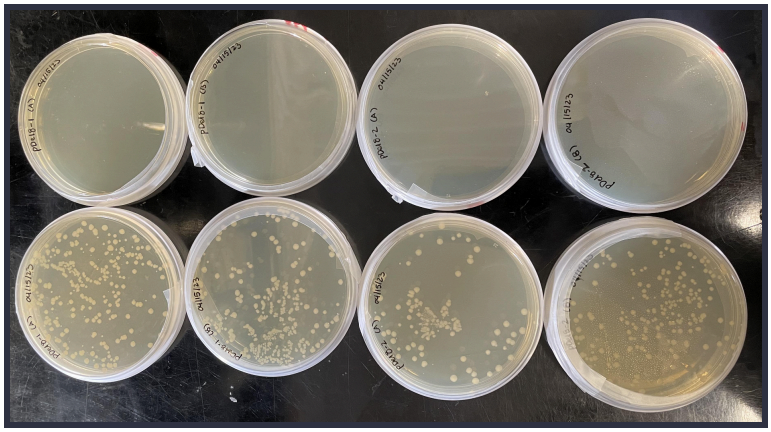

Figure 7: pDel8 plated on LB + Carb without ATC (bottom row) and with ATC (top row)

To fine tune pDel8, we created a RBS library to select the best version of pDel8 (which would be renamed to pDel9). However, our results did not at all represent what we expected as none of the pDel9 colonies cleared (see Figure G).

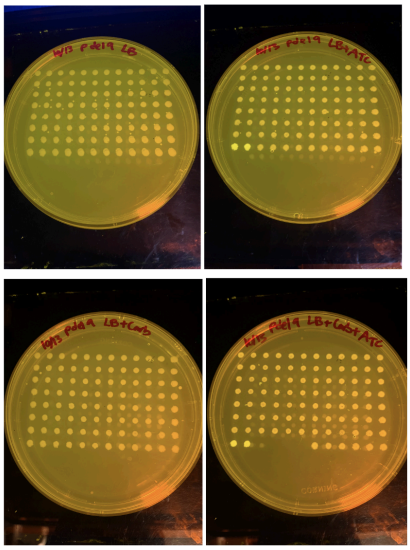

Figure 8: 4 plates with different LB, antibiotic and ATC environments. We expected pDel9 colonies to clear after ATC induction on Carb only which was achieved by pDel 8, the four missing colonies in the bottom right plate. The two glowing colonies show pDel5 which was our control, expressing GFP as expected.

After repeating both pDel8 and pDel9 several times, we observed several inconsistencies in the phenotype displayed. We hypothesized that these inconsistencies in the phenotype pDel8 was displaying were a result of not maintaining selection pressure throughout the assay process. During our assay, we grew cells transformed with pDel8 without antibiotic overnight before inducing with ATC and then finally plating on Carb +/- plates. We theorized that pDel8 has a slow rate of clearance on its own without induction, and when growing cells without antibiotic, this could lead to the enrichment of cells that do not clear, resulting in selection for non-clearing cells at the final step of the assay. To remove any inconsistencies in phenotype, we created a new assay protocol to maintain constant selection pressure with Carb. By always growing cells transformed with pDel8 in Carb, we were able to confirm our desired phenotype.

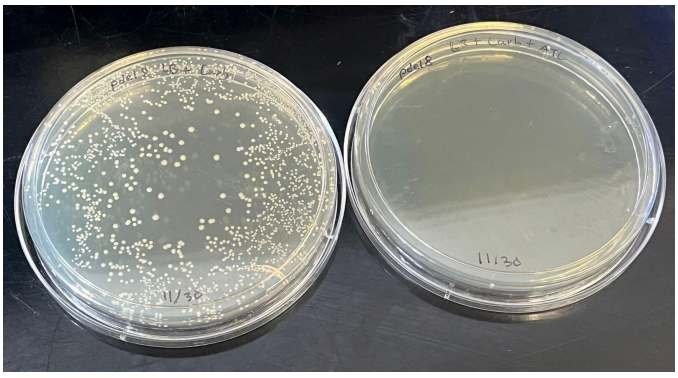

Figure 9: pDel8 plated on LB+Carb without ATC (left) and with ATC (right)

As you can see in Figure 9, cells containing pDel8 grew successfully on Carb without ATC but died on Carb when ATC was added. This indicates inducible,

reliable self-clearance with Cas12a, and with pDel8, we were able to achieve one of our primary project goals, creating a self-clearing plasmid.

After verifying pDel8, we moved onto implementing gene deletion in our system. We replaced the crRNA protospacer in pDel7 with a new protospacer targeting the UPP gene in E.Coli, a gene essential for cell growth, to prevent replication thus resulting in the death of colonies after induction. However, some adjustments had to be made to our existing cloning workflow. First, 2 rounds of PCR were required due to the direct repeats in the crRNA sequence, isolating each half of the crRNA, then replacing the old protospacer. Sequencing the result of those colonies revealed that there were insertions/changes in the sequence of the crRNA, thus we inferred that the variation in crRNA may be causing the varying clearance levels. To correct this, we added a gel purification step between the 2 PCRs to remove excess template, which may have been interfering with the 2nd PCR.

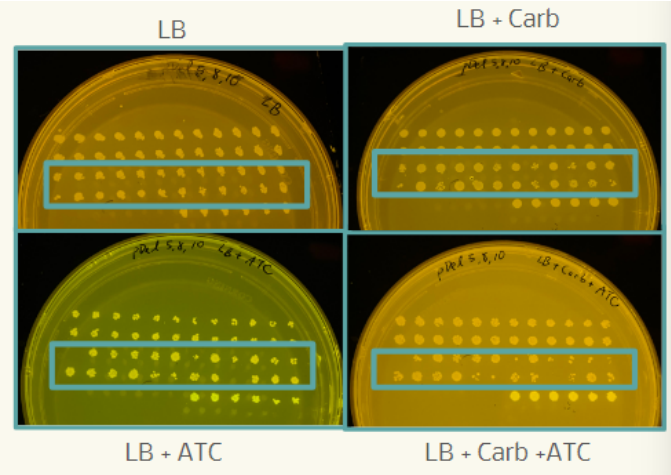

Figure 10: Boxed spots are pDel10 colonies. 2 colonies out of 24 pDel10 colonies showed complete clearance. The other colonies showed varying levels of clearance.

After making these changes, we observed death of pDel10 on plates with ATC, but growth on ATC (Figure 11), confirming that our induction system works, as there was a permanent lesion in the genomic DNA after induction.

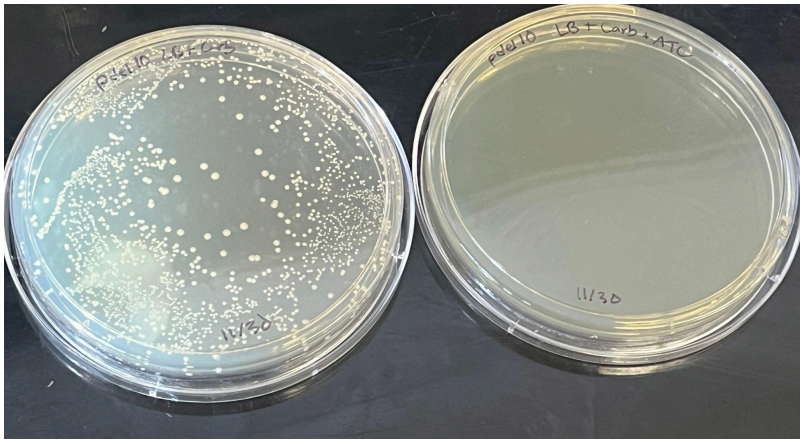

Figure 11: pDel10 on LB without ATC (left) and with ATC (right)

Conclusion

Up until recently, gene knockout methods required exogenous factors or donor templates and were time-consuming and expensive to carry out. CRISPR-Cas9 technologies have revolutionized gene editing. Our system pDel builds off of gene knockout strategies that incorporate CRISPR-Cas' ability to efficiently and accurately generate targeted breaks. In the first stage of our project, we began to build the pDel plasmid by progressively adding components to the initial backbone. We successfully demonstrated the viability of pDel's inducible expression system pTet-tetR, which is crucial for controlling exactly when gene knockout and self-clearance occurs, with pDel1 – adding ATC to bacterial cultures induced expression of GFP compared to non-induced controls. We developed the design and cloning process of our crRNA cassette with pDel2. The expression system, crRNA, and Cas12a functionalities were combined in pDel3 and repeatedly tested and modified to reach pDel7. pDel7 is most notably the product of a ribosome binding site (RBS) library. Each clone of pDel7 had a unique promoter, thus allowing us to quantitatively identify which clone displayed the most desirable phenotype. Tuning expression level in this way was vital in almost every step of our project. Our pDel7 construct produced targeted double stranded breaks in the reporter plasmid's ColE1 origin of replication after induction, subsequently clearing that plasmid from the cell. This result parallels the plasmid clearance that pFREE was able to accomplish. Observing this proof-of-concept drove the project forward into the second stage. In order to determine if self-clearance would be possible with pDel, we modified the crRNA to target pDel's own p15a ORI with pDel8. Plating pDel8 cultures induced with ATC on antibiotic selection plates (after continuously maintaining selection pressure) resulted in the growth of no colonies compared to the non-induced control, indicating effective self-targeting and clearance. pDel10 is the construct that bridges the second and final stages. pDel's ultimate goal is to create breaks as desired in the *E. coli* genome, deleting the target gene while still preserving cell viability. pDel10's crRNA was modified to target an *E. coli* gene, UPP1, that is essential for DNA replication. The same clearance assay done for pDel8 demonstrated that pDel10 successfully cut UPP1, resulting in cell clearance.

Moving forward, our team will be focusing on the final stage of our project. We will be adding a ligase and exonuclease to ensure the DSBs created by Cas12a are re-annealed and the

cell is not cleared. We will also be working to better understand the interplay between two crRNAs, one for gene knockout and the other for self-clearance, and conducting experiments that test whether both functions can be combined effectively into one step. Ultimately, we hope that pDel will be available as a construct that labs can order. We aim to lay out the cloning steps required to modify the crRNA for researchers to follow. Once the modified pDel is ready, the one step that is needed would be transformation of pDel into the cells of interest and plating those cells on antibiotic selection plates with ATC inducer. The resulting colonies can be picked into liquid culture and assayed for successful gene knockout. pDel has the potential to make gene knockouts in *E. coli*, and possibly all prokaryotic cells, much simpler, improving access and increasing productivity for gene studies conducted in all fields of biology.

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