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Development of gene silencing with dCas13d in *Streptomyces* spp.

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201911571

Submitted in accordance with the requirements for the degree of Master of Science in Programme of Study MRes Molecular and Cellular Biology

The University of Leeds

School of Molecular and Cellular Biology

December 2025

Word Count: 12515

I confirm that the work submitted is my own and that appropriate credit has been given where reference has been made to the work of others.

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Dr. Ryan Seipke, for affording me this opportunity and welcoming me into his lab group. I am deeply thankful for his insight and encouragement, his reliable presence and approachability, and for creating an environment where there was never a moment of awkward silence.

I also want to share my thanks with everyone in the Astbury office for fostering a warm atmosphere every day and for providing me with support or advice whenever I needed it. In particular, I want to thank Trent McLean-Ash, who always made time to offer me help in and out of the lab, organised all of the office outings, kept my windowsill green with plants and saved me from a year of horrible lunches.

Finally, I want to thank my family for never losing faith in me and making me want to push myself to become the best scientist I can be, as to make them proud.

Abstract

After the end of the antibiotic “Golden Age”, the discovery of antimicrobial natural products with unique mechanisms of actions has drastically decreased while at the same time antimicrobial resistance has been on the rise. Advancements in next-generation sequencing has unlocked hundreds of thousands of microbial genomes, offering the opportunities to study some of the most prolific antibiotic-producing taxa such as *Streptomyces* spp. in greater detail. To fully capitalise on this potential, robust functional genomics tools are essential for developing our understanding of these powerful organisms.

Existing functional genomics tools for *Streptomyces* spp. are effective but are not without flaws. Recently, dCas13d, a programmable, RNA-targeting Cas protein has emerged as a promising candidate for genetic study with the ability to knock down an individual gene at the translational level, showing a reduced impact on other genes present within an operon. This work lays the foundation for establishing dCas13d in *Streptomyces albidoflavus* J1074 by constructing the first integrative, customisable and codon optimised dCas13d plasmid and confirming its activity against an artificial gene. Although further study is needed, it is possible that dCas13d may become a key component of the *Streptomyces* spp. functional genomics toolkit in the future.

Contents

Abbreviations

µg	Microgram
µL	Microlitre
µM	Micromolar
µm	Micrometre
AMR	Antimicrobial resistance
aacC4	Apramycin resistance gene
asRNA	Antisense RNA
aTc	Anhydrotetracycline
bp	Base pairs
BSA	Bovine serum albumin
℃	Degrees Celcius
carbR	Carbenicillin resistance gene
CFU	Colony forming units
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
CRISPRi-art	CRISPR Interference through Antisense RNA-Targeting
CRISPRi-seq	CRISPR interference – sequencing
crRNA	CRISPR RNA
dCas13d	Nuclease-deficient Cas13d
dCas9	Nuclease-deficient Cas9
DNA	Deoxyribonucleic acid
DR	Direct repeat
dsRNA	Double-stranded RNA
E. coli	*Escherichia coli*
e.g.	For example
*Es*Cas13d	*Eubacterium siraeum* Cas13d
FDA	Food and Drug Administration
*Fpe*Cas13c	*Fusobacterium perfoetens* Cas13c
g	Gram
G/C	Guanine/cytosine
gDNA	Genomic DNA
gusA	β-glucuronidase encoding gene
HEK293FT	Human Embryonic Kidney 293 Fast-growing T-antigen-expressing
HEPN	Higher eukaryotes and prokaryotes nucleotide-binding domain
hph	Hygromycin resistance gene
i.e.	In other words
IDT	Integrated DNA Technologies
ISP-2	International Streptomyces Project-2
neo	Kanamycin resistance gene
Kb	Kilobases
kDa	Kilodaltons
L	Litre
LBA	Luria Broth Agar
*Lwa*Cas13a	*Leptotrichia wadei* Cas13a

M	Molar
Mb	Megabases
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
NCBI	National Centre for Biotechnology Information
NEB	New England Biolabs
ng	Nanogram
NGS	Next-Generation Sequencing
nM	Nanomolar
NP	Natural product
nt	Nucleotide
NUC	Nuclease lobe
PAM	Protospacer adjacent motif
PBS	Phosphate-Buffered Saline
*Pbu*Cas13b	*Prevotella buccae* Cas13b
PCR	Polymerase chain reaction
PFS	Protospacer flanking sequence
pre-crRNA	Precursor CRISPR RNA
*Psp*Cas13b	*Prevotella sp.* Cas13b
RBS	Ribosome binding site
REC	Recognition lobe
*Rfx*Cas13d	*Ruminococcus flavefaciens* XPD3002 Cas13d
RNA	Ribonucleic acid
RNAi	RNA interference
S. albidoflavus	*Streptomyces albidoflavus*
S. coelicolor	*Streptomyces coelicolor*
S. pyogenes	*Streptococcus pyogenes*
sec	Second
SFM	Soy Flour Mannitol
sgRNA	Single guide RNA
smBGC	Secondary metabolite biosynthetic gene cluster
*sp*Cas9	*Streptococcus pyogenes* Cas9
ssRNA	Single-stranded RNA
TBS	Tris-buffered saline
TBST	Tris-buffered saline + Tween20
tcp830p	tcp830 promoter
Tn-seq	Transposon insertion sequencing
tracrRNA	Trans-activating CRISPR RNA
USD	United States Dollar
UV	Ultraviolet

1. Introduction

1.1 *Streptomyces* spp. Characteristics

1.1.1 *Streptomyces* spp. Morphology

"*Streptomyces*" is a genus within the Actinomycetota order with over 700 species validated in publication (1). Gram-positive, primarily soil-dwelling organisms, *Streptomyces* species are unique among bacteria for their complex multicellular fungal-like growth cycle, shown in Figure 1. Initially, in suitable growth conditions, a dormant spore will germinate and branch out by tip extension to establish vegetative mycelium on a surface or digging into in solid media. Subsequently, in response to environmental stresses such as food depletion, using nutrients recycled from the programmed cell death of vegetative mycelium, aerial hyphae coated in a hydrophobic sheath grow vertically out of the surface of the colony into the air (2). The apical sporogenic cells of aerial hyphae later undergo cell division into chains of weakly-bound metabolically dormant, unigenomic exospores which act as a lightweight, robust vessel for genetic information. Resistant to heat, desiccation and mechanical disruption, the spores compensate for the mycelium's immobility by establishing new colonies elsewhere after dispersion by abiotic factors (i.e. wind and rain) or even by biotic factors such as insects (3, 4). Once the colony has a well-established vegetative mycelium where only the peripheral regions are growing, and while aerial hyphae and spores are developing, in response to stress stimuli, non-growing mycelial regions shift from synthesising primary metabolites to producing a range of secondary metabolites that may assist survival (5).

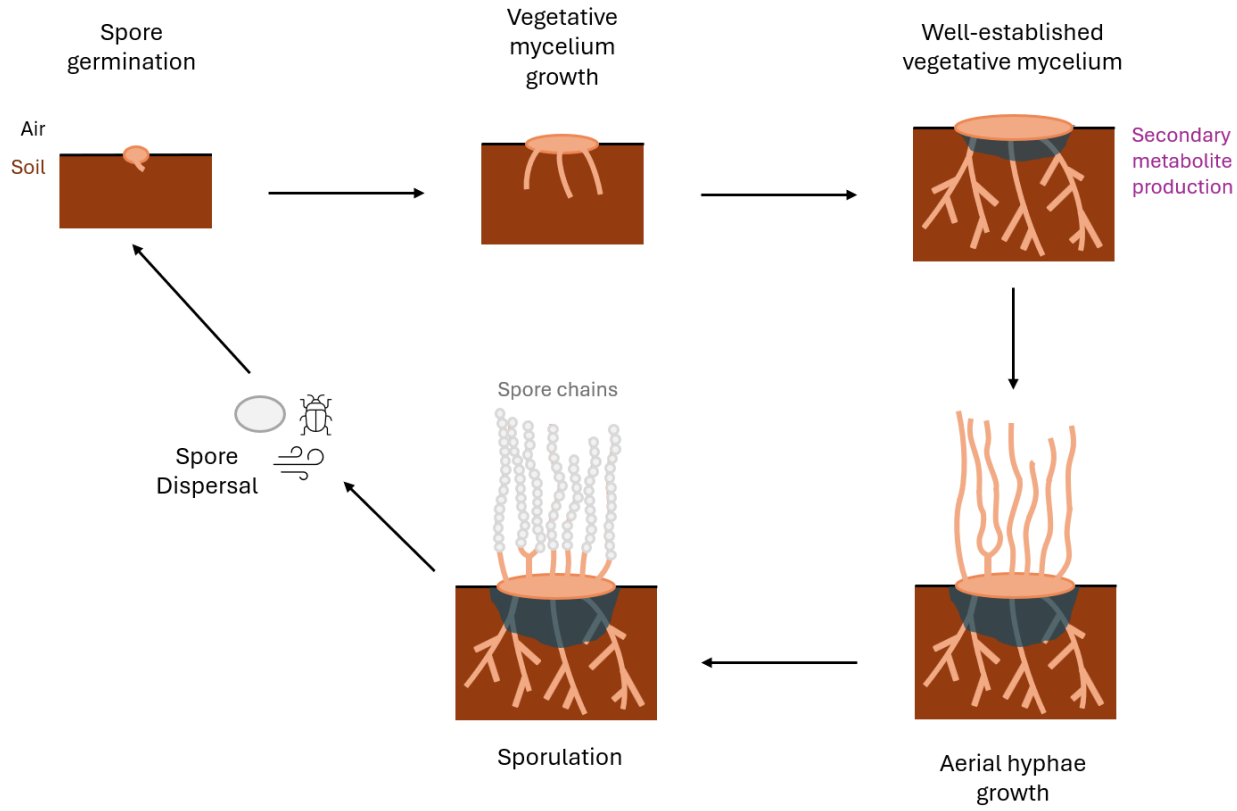


Figure 1. The *Streptomyces* life cycle. Starting with spore germination in a favourable environment, vegetative hyphae growth establishes the colony where then, in response to environmental stimuli, aerial hyphae grow and aerial sporogenic cells may undergo cell division into spore chains (sporulation) and eventual dispersion. Elements of the figure were adapted from (6).

1.1.2 *Streptomyces* spp. Secondary Metabolites and Antibiotics

The true adaptive advantage of streptomycetes lies in their repertoire of bioactive compounds encoded in secondary metabolite biosynthetic gene clusters (smBGCs) - an interconnected collection of biosynthetic genes, regulatory genes and resistance genes all functioning toward metabolite synthesis. The genomes of *Streptomyces* spp. are linear, ~70% G/C rich and range between 5.9 and 12.7 metabases (Mb) in length, with a number of smBGCs present per genome that varies considerably (7-9). The secondary metabolites produced can provide survival benefits to the host, including pigmentation to provide UV-shielding, metal sequestering to acquire essential metal ions during scarcities or bioactive compounds, including antibiotics, to help outperform competing microorganisms. The bioactive compounds produced by *Streptomyces* spp. are very diverse and can exhibit a range of effects including antimicrobial, antiviral, antitumour, insecticidal, herbicidal, immunosuppressive and more (7, 10).

Streptomyces species were first identified as a source of antibiotics in the 1940's during World War II when demand was extremely high and since then, particularly during the antibiotic "Golden Age" that took place between the late 1940's and 1960's, *Streptomyces* spp. represent one of the most prolific and historically valuable antibiotic producers – the discovery of different classes of antibiotics derived from *Streptomyces* spp. can be visualised in Figure 2. Actinomycetes produce two thirds of all microbial antibiotics, with 80% of those compounds being derived from *Streptomyces* species (7). Furthermore, filamentous actinomycetes (including *Streptomyces* spp.) make 64% of the known natural product (NP) antibiotic classes which, even if not clinically deployed, can provide a framework for structural modifications or synthetic antibiotic design to produce new effective antimicrobials (11). From the early 1960s, the discovery of novel bioactive compounds declined sharply as the most readily accessible actinomycetes had already been interrogated and screening only kept rediscovering the same known compounds. The last novel class of clinically approved natural product antibiotic, daptomycin, was discovered in *Streptomyces roseosporus* in 1987 and approved by the FDA in 2003 (11).

Typically, smBGCs are only expressed in response to very specific biotic or abiotic stimuli (i.e. under stress or extreme constraints) and thusly, under standard laboratory growth conditions, while useful gene clusters maybe be present, the majority of *Streptomyces* spp. smBGCs are silent. Nevertheless, *Streptomyces* stands atop any other genus in terms of its capacity to produce antibiotics and that makes it a tremendously valuable field of study (12).

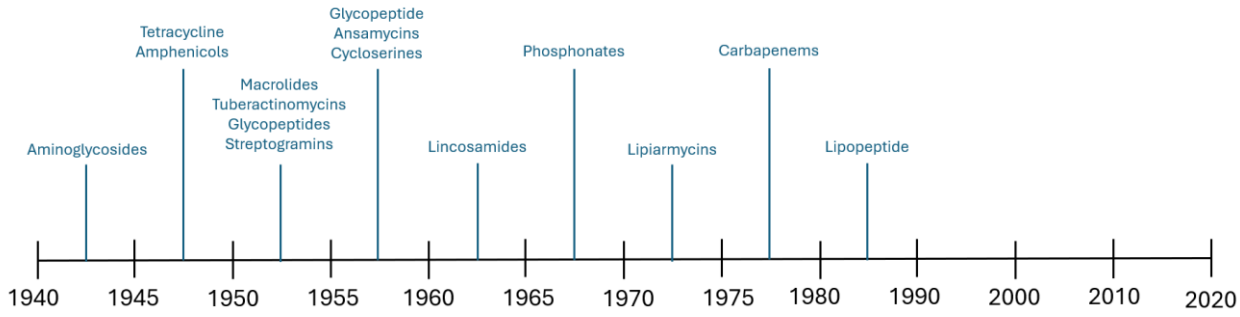


Figure 2. A timeline of the reported discovery of clinically used antibiotic classes derived from actinomycetes, discoveries are grouped into 5-year intervals. Adapted from (11).

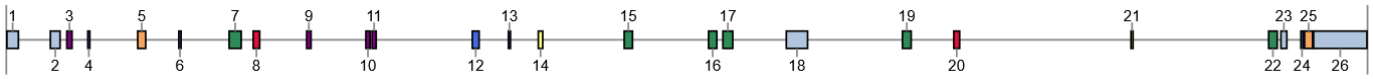
1.2 The Rise of Antimicrobial Resistance

Presently, the appearance of multi-drug-resistant pathogenic bacteria far outpaces the rate of discovery or development of new antibiotics, which poses a severe public health risk for the near future, with bacterial antimicrobial resistance (AMR) directly responsible for 1.14 million global deaths in 2021 (13). AMR is particularly the result of overuse and incorrect prescription of specific antibiotics to the point where their efficacy is substantially reduced. Resistance can be acquired by bacteria by the uptake of resistance-conferring DNA in horizontal gene transfer or by random mutation that occurs roughly every 10^{10} nucleotides. The four main mechanisms by which the bacteria can exhibit resistance are by limiting uptake of the antibiotic, inactivating or degrading the antibiotic, modifying the internal target site or by activating efflux (14). The continual evolution of bacterial β-lactamases exemplifies this process, where a single point mutation can modify the enzyme's structure such that it can degrade a newly introduced β-lactam antibiotic, producing a resistant "escape mutant" that will be selected for and pass on its valuable mutation (15).

As it stands, newly approved antibiotics are protected and used cautiously for only the most dire cases, and instead older antibiotics are deployed for most treatments; this combined with the fact that antibiotics are typically used for short one-off cycles only serves to push pharmaceutical investors further away (16). With that said, the introduction of daptomycin (Cubicin) onto the market in 2003 has led to what is a market value of 3.66 billion USD in 2025, so the reward for discovering an effective compound is still present. Without major intervention, AMR is estimated to cause 1.91 million deaths directly and 8.22 million deaths linked to AMR in 2050, not only this but it is estimated that antibiotic resistant infections cost the United States and China tens of billions of dollars in health care costs and in lost productivity every year (13, 17). With antibiotics still playing an irreplaceable part in how infectious diseases are tackled, there is a demand for new antibiotics with novel modes of action and with 92 new antibacterial natural products reported from 39 *Streptomyces* spp. between 2015 and 2020, it remains the most promising natural source of antibiotics that must be explored (18).

1.3 Characterising Genomes

A variety of new species have been found in soils from previously unexplored regions such as deserts, mountains, polar regions, marine environments or even engaging in symbiosis with animals, plants and fungi, so there is a wealth of new genomes to be searched (7, 18). The decreased cost and widespread availability of next-generation sequencing (NGS) means that the number of publicly available genomes sequences has surged; whereas there were only 588 *Streptomyces* spp. genomes (draft and complete status combined) on the NCBI database in 2015, the number has exponentially increased to 12,826 genomes as of December 2025. By virtue of the large number of high-quality, complete *Streptomyces* spp. genomes available, *in silico* genome mining techniques have arisen that can rapidly make rule-based predictions and offer insights into evolutionary developments, secondary metabolism and smBGCs, morphological development and stress responses. antiSMASH is a widely used web-based genome mining tool for smBGCs that in the latest version (version 8 as of December 2025) contains detection rules for 101 different cluster types with a low false positive rate, an example of antiSMASH analysis is shown in Figure 3 (19). Genome mining of 213 *Streptomyces* species with antiSMASH analysis identified an average of 31 smBGCs per soil species, but in order to efficiently investigate *Streptomyces* spp. smBGCs we must first understand the organism (20).



Region	Type	From	To	Similarity Confidence	Most similar known cluster	
Region 1	T1PKS , NRPS	3,011	61,711			
Region 2	NRPS , T1PKS	224,752	274,162	High	SGR PTMs/SGR PTM Compound b/SGR PTM Compound c/SGR PTM Compound d	NRPS:Type I+PKS
Region 3	terpene	308,626	335,190	High	hopene	terpene
Region 4	RiPP-like	415,688	425,903			
Region 5	PKS-like	669,953	711,002	Medium	paulomycin	other:other
Region 6	RiPP-like	879,946	891,274			
Region 7	NRPS	1,136,409	1,198,114	High	cyclofaulknamycin	PKS
Region 8	NI-siderophore	1,259,256	1,292,288			
Region 9	terpene	1,531,863	1,554,148	High	geosmin	terpene:Sesquiterpene
Region 10	terpene-precursor	1,833,901	1,854,944			
Region 11	terpene	1,865,338	1,886,312	Low	julichrome Q3-3/julichrome Q3-5	PKS
Region 12	azole-containing-RiPP , RRE-containing	2,376,785	2,411,468			
Region 13	RiPP-like	2,560,811	2,571,323			
Region 14	lanthipeptide-class-iii	2,713,361	2,735,940	High	SAL-2242	ribosomal:RiPP:Lanthipeptide
Region 15	NRPS-like	3,151,596	3,194,010	Medium	pamamycin-607/pamamycin-621A/pamamycin-635G/pamamycin-663A/homopamamycin-677A/pamamycin-649B	PKS:Type II
Region 16	NRPS-like	3,581,613	3,624,027	Medium	pamamycin-607/pamamycin-621A/pamamycin-635G/pamamycin-663A/homopamamycin-677A/pamamycin-649B	PKS:Type II
Region 17	NRPS	3,654,983	3,705,272			
Region 18	NRPS , azole-containing-RiPP	3,978,361	4,087,014	High	surugamide A/surugamide D	NRPS:Type I
Region 19	NRPS	4,570,659	4,615,053	Low	dudomycin A	NRPS:Type I
Region 20	NI-siderophore	4,832,632	4,862,452	High	desferrioxamin B	other:other
Region 21	ectoine	5,736,509	5,746,907	High	ectoine	other:other
Region 22	NRPS-like	6,438,546	6,482,376	High	minimycin/indigoidine	NRPS:Type I+saccharide
Region 23	terpene , RiPP-like	6,501,239	6,531,291	Medium	isorenieratene	terpene
Region 24	RiPP-like	6,603,064	6,614,431	Medium	streptamidine	ribosomal:unmodified
Region 25	T3PKS	6,621,671	6,662,768	Low	valinomycin/montanastatin	saccharide:hybrid/tailoring+NRPS:Type I
Region 26	T1PKS , terpene-precursor , NRPS-like , NRPS , lanthipeptide-class-ii	6,667,720	6,939,942	High	candicidin	NRPS:Type I+PKS

Figure 3. Displaying the antiSMASH results using the genome of *Streptomyces albidoflavus* J1074 NZ_CP059254.1. The antiSMASH version 8 software predicts regions where biosynthetic gene clusters are likely to be present with varying levels of confidence, which in this case was 26 regions. While the majority of regions above are only predicted to contain one BGC, large regions such as region 26 are predicted to contain up to five BGCs. (19).

1.4 Functional Genomics Tools

Functional genomics examines the entire genome of an organism to understand how genes are expressed, how they interact, and their larger function within the biological system. High-throughput approaches combine genome-wide genetic manipulation strategies with large-scale screening to provide insights at a magnitude far beyond traditional single-gene knockout studies. The most straightforward way to narrow down a gene's function is by removing its activity and monitoring the phenotype frequency in a population as well as the cells morphology, motility and host interactions all while varying growth conditions or stresses. Gene fitness is the simplest point of evaluation; knocking out an essential gene is lethal to the cells and therefore no mutant cells will survive whereas silencing a non-essential gene will not be lethal. While *Streptomyces* spp. are a cornerstone of natural antibiotic discovery, it's complex genome architecture, slow growth and low transformation efficiency means that it is much more challenging to adapt genetic approaches for than it is for simpler bacteria such as *E. coli*. With that said, there have been various tools adapted to *Streptomyces* spp. that can produce good results.

1.4.1 Traditional *Streptomyces* spp. Functional Genomics Strategies

Streptomyces spp. functional genomic tools include low-throughput methods such as individual gene knockouts using allelic exchange mutagenesis; traditionally implemented using a λ-Red cosmid modification system or Cas9 double stranded break-mediated genome editing (21, 22).

The λ-Red phage recombination system drastically increases homologous recombination. Starting out in *E.coli* with a cosmid that contains a large ~40 kb section of the *Streptomyces* spp. genome, the λ Red proteins mediate recombination between a section of the cosmid and a PCR product containing terminal 39 nt homology arms (21). The sequence encoded in the PCR

product will replace part the original cosmid sequence defined by the homology arms and typically encodes a selection marker. Following selection for the strains that have undergone homologous recombination, the cosmid is then conjugated from a specialised *E. coli* strain into the *Streptomyces* strain. Inside the cells, the long regions of sequence identity encoded on the cosmid promote efficient integration by homologous recombination, where the region edited into the cosmid by PCR will now be present in the host genome and can be selected for (21). This classical technique has been utilised to disrupt hundreds of genes within *S. coelicolor* for study; however, relative to more modern tools it is much more time-consuming and not scalable (23).

The pCRISPomyces CRISPR-Cas9 editing technique for *Streptomyces* spp. involves using a plasmid that contains a selectable marker, a gene encoding a Cas9 protein, a single-guide RNA (sgRNA) that targets a specific location on the genomic loci, and an editing template sequence containing two 1 kb homology arms corresponding to the upstream and downstream region of the target region (22). The sgRNA will bind onto its target where Cas9 will implement a double stranded break which allows for homologous recombination of the editing template into the host genome to disrupt the gene. After selection and editing, a temperature-sensitive *rep* region of the plasmid allows it to be disposed of, leaving an edited host without the plasmid (22).

These lower-throughput approaches are powerful and precise for investigating on a smaller scale, able to knockout a single gene with high efficiency and specificity by disrupting the coding region, removing part of the coding region or possibly causing a frame shift. However, bacterial genomes encode thousands of genes, *Streptomyces albidoflavus* J1074 for example contains 5832 genes across its 6.8 Mb genome, in the face of this many genes to investigate, these low-throughput techniques simply cannot compete with more streamlined high-throughput techniques available today (24). Currently, there are only two truly viable high-throughput functional genomic techniques used for genome-level investigations in *Streptomyces* spp.: transposon-insertion sequencing (Tn-seq) and CRISPRi-sequencing (CRISPRi-seq) (25, 26).

1.4.2 Transposon-insertion Sequencing

Transposon-insertion sequencing (Tn-Seq) is a widely employed, high-throughput mutagenesis technique that uses a transposase to mediate the insertion of a transposable DNA element (transposon) at a random point across the genome, summarised in Figure 4. There are no specific design requirements, and this approach relies on the randomness of insertions to essentially cover the whole genome. Tn5 transposase derivatives are favoured for use in *Streptomyces* spp. because of their weaker insertion preferences conferring wide coverage across the G/C rich genome, the transposase is encoded separately from the transposon so that once it undertakes the random insertion, the insert sequence remains stably integrated and cannot be mobilised again (27). The transposable sequences contain a selective marker to select for cells containing an insertion mutation and also contain inverted complement terminal repeats that are used to later identify the affected region. Tn-seq can generate a diverse mutant library containing tens of thousands of strains each containing a single insertion at a random points across the genome, meaning the fitness of thousands of genes can be tested at once in a pooled experiment (28). Isolating genomic DNA and digesting it with restriction enzymes specific to cut sites engineered into the transposon terminal repeats followed by adapter binding and PCR amplification leaves a small stretch of native genomic DNA that can be used to pinpoint the location of the insertion in the genome using Illumina next-generation sequencing (28). By taking initial population sequencing measurements and comparing the frequency of each mutation to a population that has been grown for a set amount of time, it makes it possible to see which mutations have a dropped out of the population (essential genes) and which non-essential genes have not.

Tn-seq has already been applied to *S. coelicolor* where after over 50,000 insertions across the 7800 gene genome, 348 mutated genes were implicated in affecting the biosynthesis of the pigmented antibiotics undecylprodigiosin and streptorubin B (25). Transposon-insertion sequencing offers a large-scale, genome-wide, cost-effective functional genomics tool that has been validated for use in *Streptomyces* spp. However, its innate randomness means that it cannot reliably hit every gene of interest, especially smaller genes, with a study in *S. coelicolor* finding that 90% of the non-disrupted genes were smaller than 1 kb and 60% of below 500 bp (29). Additionally, its methodology of completely disrupting a genes coding or promoter region means that it cannot be applied to study the specific functions of essential genes.

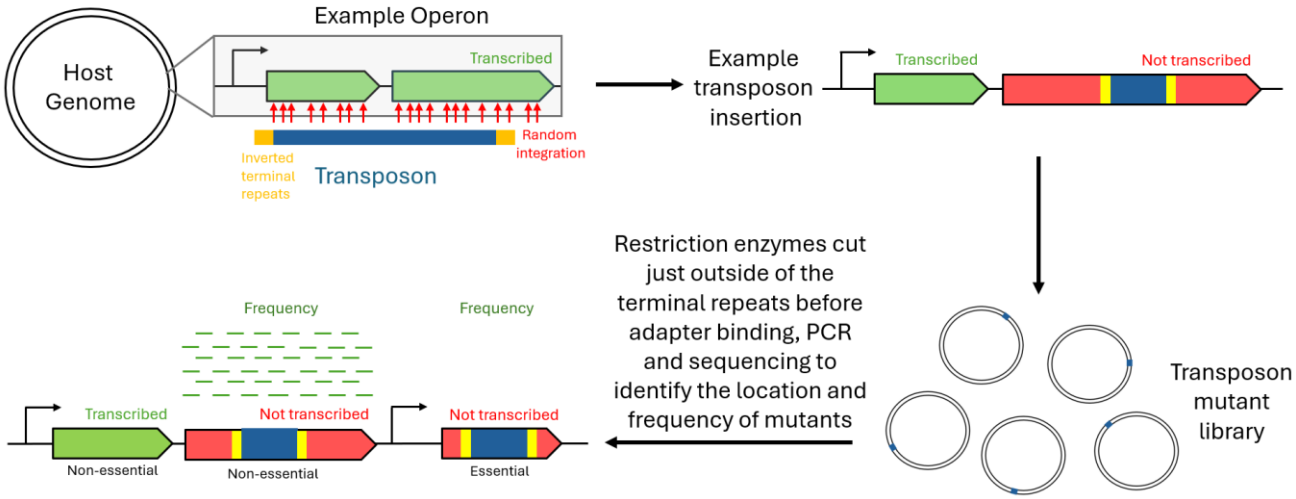


Figure 4. A simplified depiction of transposon insertion sequencing (Tn-seq). The insert will disrupt the genes coding region to prevent transcription and knock it out completely, a library of mutants can then be analysed using the type IIS restriction enzymes in the terminal repeats to cut outside of the insert where after adapter binding, PCR and sequencing, the location and frequency of each mutation can be determined to find gene fitness.

1.4.2.4 CRISPRi and CRISPRi-seq

CRISPR-Cas9 tools are the most well-characterised among the Cas families, utilising a single-guide RNA (sgRNA) containing a tail sequence to hybridise with the Cas9 nuclease and a variable spacer sequence to specifically target the upstream region of an operon. For functional genomics, a nuclease deficient Cas9 (dCas9) protein that will obstruct transcription instead of cutting is used because it has a lower growth burden and because its effects can be titrated, it permits study of essential genes. dCas9 has high efficiency targeting close to the transcription start site but knockdown will affect the entire operon, limiting its study to the operon-level (30). CRISPR interference (CRISPRi) with dCas9 uses a customisable plasmid where new sgRNAs can quickly be cloned in using restriction sites to target seemingly any region within the genome provided the target sequence has a protospacer adjacent motif (PAM) (31). CRISPRi has been proven to be an effective tool in *Streptomyces* spp. and is much more streamlined than the previously mentioned low-throughput functional genomics techniques for smaller-scale studies but it can also be expanded to large-scale tests (26).

A simplified roadmap of CRISPRi-seq is shown in Figure 5. CRISPRi-seq utilises a large sgRNA pool, typically containing more than one sgRNAs per operon designed using sequences provided from whole genome sequencing to target operons across the genome. The collection of sgRNAs is cloned en masse into dCas9 plasmids (one sgRNA per plasmid) which are initially transformed into *E. coli* before conjugation into *Streptomyces* spp., yielding a pooled library of cells all containing dCas9 plasmids with different sgRNAs targeting different operons. CRISPRi-seq uses an inducible promoter to control expression of dCas9, meaning it is possible to establish a pooled strain library in an uninduced environment so there will be no impact and then later add an inducer such as Isopropyl β-D-1-thiogalactopyranoside (IPTG) or anhydrotetracycline (aTc) to start seeing effects. Not only this but varying the level of inducer to control the level of repression opens up the potential to study essential genes by partially knocking them down instead of total knockout which would be lethal. Whereas individually processing the strains containing a unique sgRNA can permit more precise study into morphological changes, a pooled strain library allows for convenient phenotypic screening of gene fitness across countless operons at the same time (26). sgRNA frequency is analysed by growing the pooled *Streptomyces* spp. CRISPRi library in the absence and presence of inducer and then extracting genomic DNA from each sample where a region of the integrated plasmid sequence containing the sgRNA is cut out with a restriction digest for amplicon sequencing (26). Analysis of CRISPRi-seq results is much simpler than for Tn-seq as the sgRNA is a simple indicator for what is being silenced but also, the process of analysing this same region for every strain is much more straightforward.

While CRISPRi-seq has been confidently established in unicellular bacteria, it has only recently been adapted to the more genetically complex, G/C rich *Streptomyces* genus, starting with *S. albidoflavus* J1074 (26). CRISPRi-seq offers a transplantable large-scale pooled-population approach to efficiently knockdown either a small subset of genes or a broad selection of genes across an entire genome in an inducible, targetable, tuneable, reproducible manner.

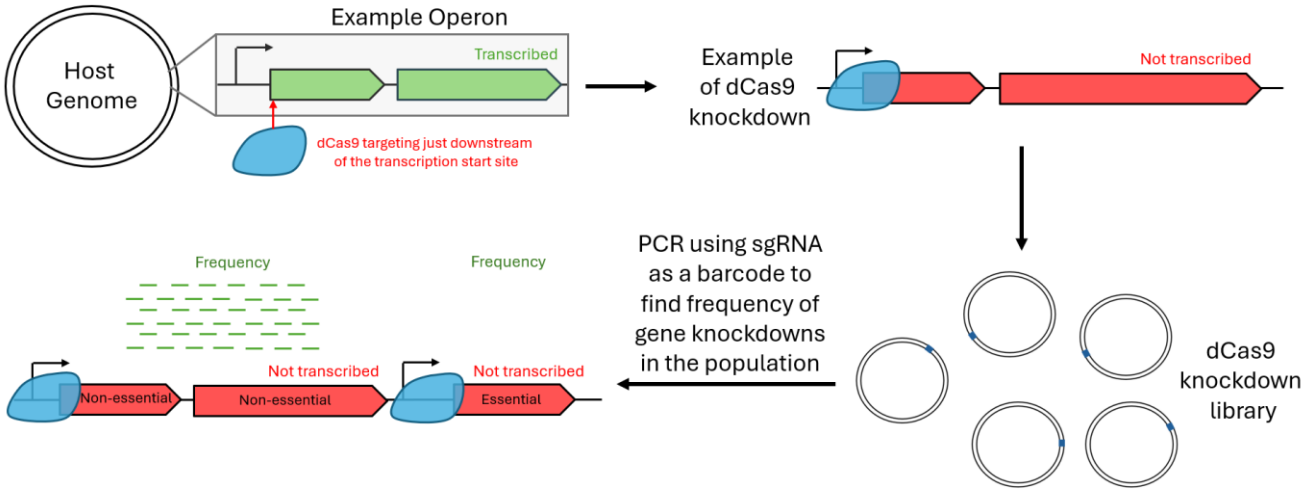


Figure 5. A simplified depiction of CRISPRi-seq targeting a simple operon for knockdown. A pool of single guide RNAs determine the selection of genes that will be targeted and knocked down in the population – with one knockdown per cell that will knockdown all downstream genes in the operon, limiting the technique to operon-level studies. The same sgRNA acts as a barcode to determine the gene knockdowns present in the population and their frequency.

To compare the two frontrunning *Streptomyces* spp. functional genomics tools, Tn-seq and CRISPRi-seq, in terms of building the systems, CRISPRi-seq is much more complex as each sgRNA in the pool requires individual design to target each operon whereas Tn-seq relies on random insertion, and therefore does not require extensive genomic knowledge but must be freshly built for each test and requires characterisation of large numbers of insertions to be confident about 'transposition cold spots' in the genome. In regard to the coverage of each approach, CRISPRi-seq can seemingly target any operon provided it contains a protospacer adjacent motif (PAM) in the early sections of its coding region, meanwhile the random nature of Tn-seq means that it cannot reliably hit every gene of interest, especially the shorter genes with a relatively smaller hitbox. CRISPRi-seq knocks down all downstream genes in the target operon and thus is limited to study gene function at the operon level; in contrast Tn-seq disrupts single genes by insertion which allows gene-level study. The tuneable nature of CRISPRi-seq permits study of essential genes by not completely knocking out their function, whereas this is not an option with Tn-seq that disrupts a gene entirely. All that to say that neither approach is perfect and there is still room for new tools to enrich the *Streptomyces* functional genomics toolkit.

Early studies into the RNA-targeting dCas13d indicate that it can efficiently target and knock down individual genes at the translational level with a reduced impact on adjacent genes in the operon compared to CRISPRi-seq while maintaining the targeting and titratable nature of the CRISPRi system. Therefore, it has great potential as a functional genomic prospect that could be used as a complementary strategy with established approaches such as CRISPRi-seq to overcome its limitation of only knocking down at the operon level.

1.5 CRISPR-Cas13

1.5.1 Background of Cas Proteins

Since its discovery in *E. coli* in 1987, CRISPR-Cas has been established as a widely-used versatile genetic tool for use in eukaryotes, bacteria and archaea with different Cas classes boasting different modes of action. Clustered regularly interspaced clustered short palindromic repeats (CRISPR) are 20-50 bp, conserved sequences located on a CRISPR locus, found in 40% of bacteria and 90% of archaea, with various unique viral DNA fragments known as spacers encoded between each direct repeat (32). A CRISPR-associated (Cas) gene is located upstream of the CRISPR array, encoding a diverse nuclease that can be made up of multiple subunits (Class 1) or just one protein (Class 2). There are 6 types and 33 subtypes of Cas proteins, classified based on their structure, target and mechanism of action, with the most commonly used model being the Class 2 type II CRISPR-Cas9. Cas9, directed by a sgRNA, targets DNA at any location in the genome provided it has a protospacer adjacent motif (PAM) present – in the case of the most popular *S. pyogenes* spCas9 this a 3 nt 5' -NGG-3' sequence on the opposite strand to the one being targeted (33). In 2015, a new type of Cas protein was computationally discovered by scanning of a genomic database for loci containing the conserved Cas1 and Cas2 genes; Cas13 is a relatively small Class 2 type VI Cas protein and the only type to exclusively target RNA (34). Since then, many subtypes of Cas13 have been discovered, the best characterised types are Cas13a, Cas13b and Cas13d, ranging from ~930 to ~1250 amino acids in length, of those Cas13d has is the most studied subtype (35, 36).

The native function of all CRISPR-Cas in bacteria is as a form of adaptive immune defence against foreign nucleic acids that is passed on to future generations as the mobile genetic elements added to the CRISPR library from each past infection are stably maintained in the locus. New protospacers are first captured and processed by a Cas1-Cas2 complex and integrated between

the leader sequence and the first repeat of the CRISPR array by nucleophilic attack before a new repeat is formed upstream, meaning that the spacers are encoded in inverse chronological order (seen in Figure 6)(37). Transcription of the CRISPR cassette yields a long precursor CRISPR RNA sequence (pre-crRNA) that is processed into individual mature crRNAs made up of the spacer and a direct repeat (DR) section. The crRNA:Cas effector complex uses the spacer as a template to search for the target sequence and once complementarity is found, it will cut the DNA or RNA using a nuclease mechanism specific to the Cas protein, removing the foreign nucleic acid and providing immunity (Figure 6).

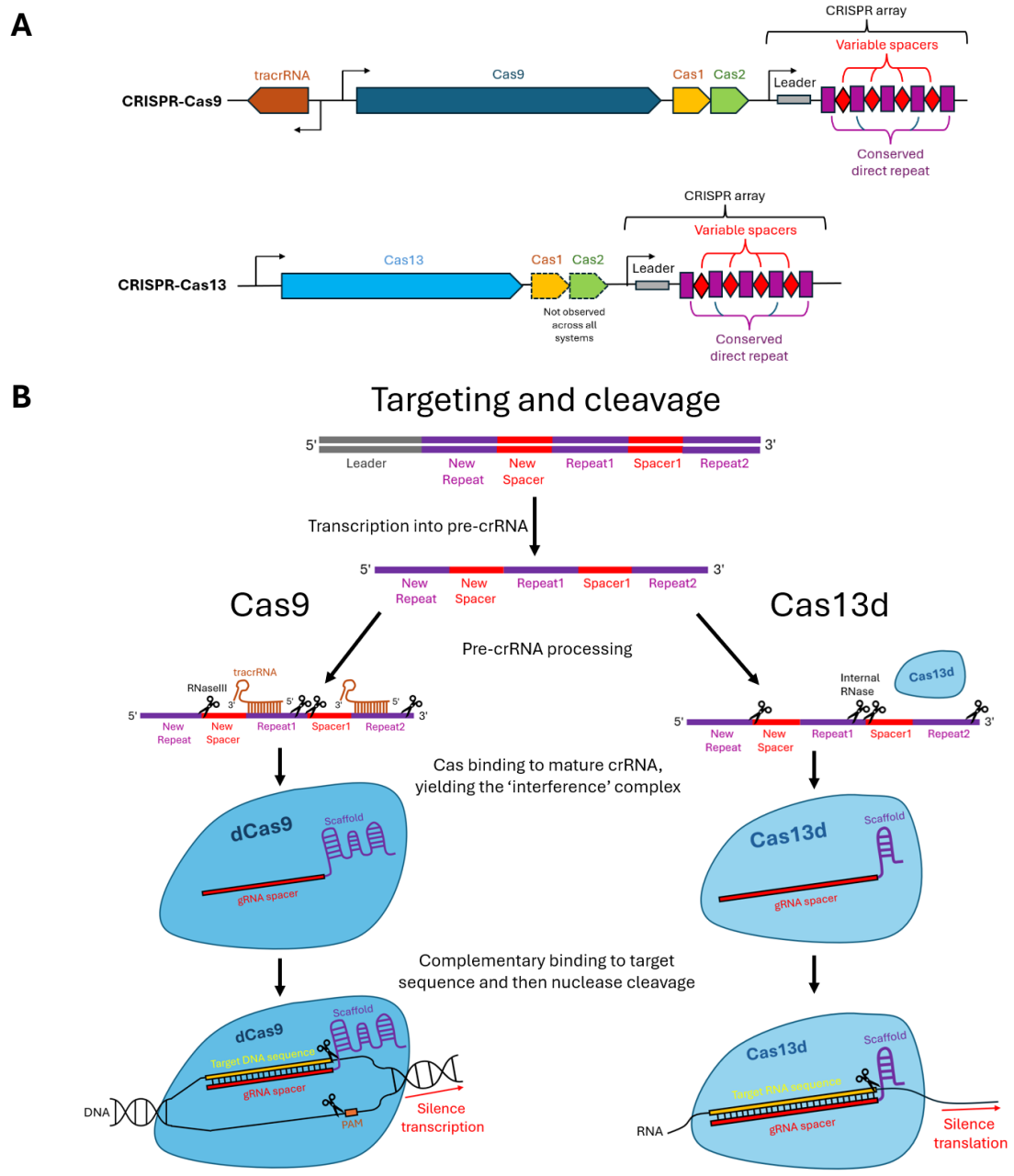


Figure 6. A simplified diagram of Cas9 and Cas13d functionality. A. The general coding operons for CRISPR-Cas9 and CRISPR-Cas13 containing the CRISPR-array of conserved direct repeats and variable spacers corresponding to a target foreign nucleic acid. The tracrRNA is not required for Cas13 and the presence of Cas1 and Cas2 varies between each variant of Cas13. **B.** A figure representation of Cas9 and Cas13d targeting and cleavage of DNA and RNA respectively. Cas9 acquires a mature spacer using tracrRNA as a scaffold and targets the template strand of DNA to supress downstream transcription while dCas13d can bind onto RNA to silence translation without an accessories.

1.5.2 Cas13d Structure

Structurally, Cas13 proteins range from ~775 to ~1250 amino acids (aa) in average length per subtype and showcase a compact bilobed architecture divided into a recognition lobe (REC), composed of an N-terminal domain (NTD) and helical-1 domain, and then also a nuclease lobe (NUC), made up of a helical-2 domain and two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains, HEPN1 and HEPN2; a helical-3 domain is present in larger proteins such as Cas13a but missing from the smaller ~930 aa Cas13d subtype - structures are shown in Figure 7 (38). Interestingly, while Cas9 requires additional factors such as RNaseIII to process the pre-crRNA and a tracrRNA to act as a scaffold to adopt the crRNA, Cas13 can simply bind onto the stem loop handle of the direct repeat (DR) and hold the pre-crRNA between the two lobes in a positively charged channel where a dedicated nuclease cleaves at the DR hairpin junction to generate a mature crRNA (shown in Figure 6B)(35). With the mature crRNA or an artificial sgRNA (made up of a variable spacer and the relevant direct repeat), the crRNA:Cas protein forms a surveillance complex which seeks out its target sequence for cutting as seen in 6B. In the case of Cas13d, the variable spacer is organised such that part of it is held internally and another section protrudes externally, but, like other sgRNAs, it will only accept a specific target sequence with full complementarity to the spacer. Each HEPN domain contains an R-X_4-H RNase motif, and after formation of the spacer:target dsRNA helix, a conformational shift brings the two motifs into close enough proximity to form a composite RNase active site that will cleave the target RNA (39).

Many Cas proteins including type 1, 2 and 5 require recognition of a protospacer adjacent motif (PAM), a 2-6 bp conserved motif that must be present on the 3' or 5' end of the target sequence for the Cas protein to recognise it, and while some Cas13 proteins, such as certain Cas13a and Cas13b subtypes show preference for a single base protospacer flanking site (PFS), Cas13d shows no such restrictions and can therefore target any sequence with no limitations (40, 41). In regard to the spacer itself, its optimum length varies between Cas variants, with *Sp*Cas9 favouring a 20 nt spacer and *Rfx*Cas13d showing the highest knockdown efficiency with a 28 nt spacer (30, 42). Each Cas protein also has a defined 'seed' region within the spacer that demands strict complementarity to the target and is very sensitive to mismatches, in the case of *Sp*Cas9, the seed region spans the ~8-10 nucleotides adjacent to the PAM and *Rfx*Cas13d's seed region spans nucleotides 15-21 (counting from the 5' end of the 28 nt spacer)(38). A WYL domain-containing accessory protein has been found in some loci of some Cas13d variants including *Es*Cas13d where it can enhance ssRNA cleavage by an unknown mechanism, however it is not an essential component for experiments involving *Rfx*Cas13d (42, 43).

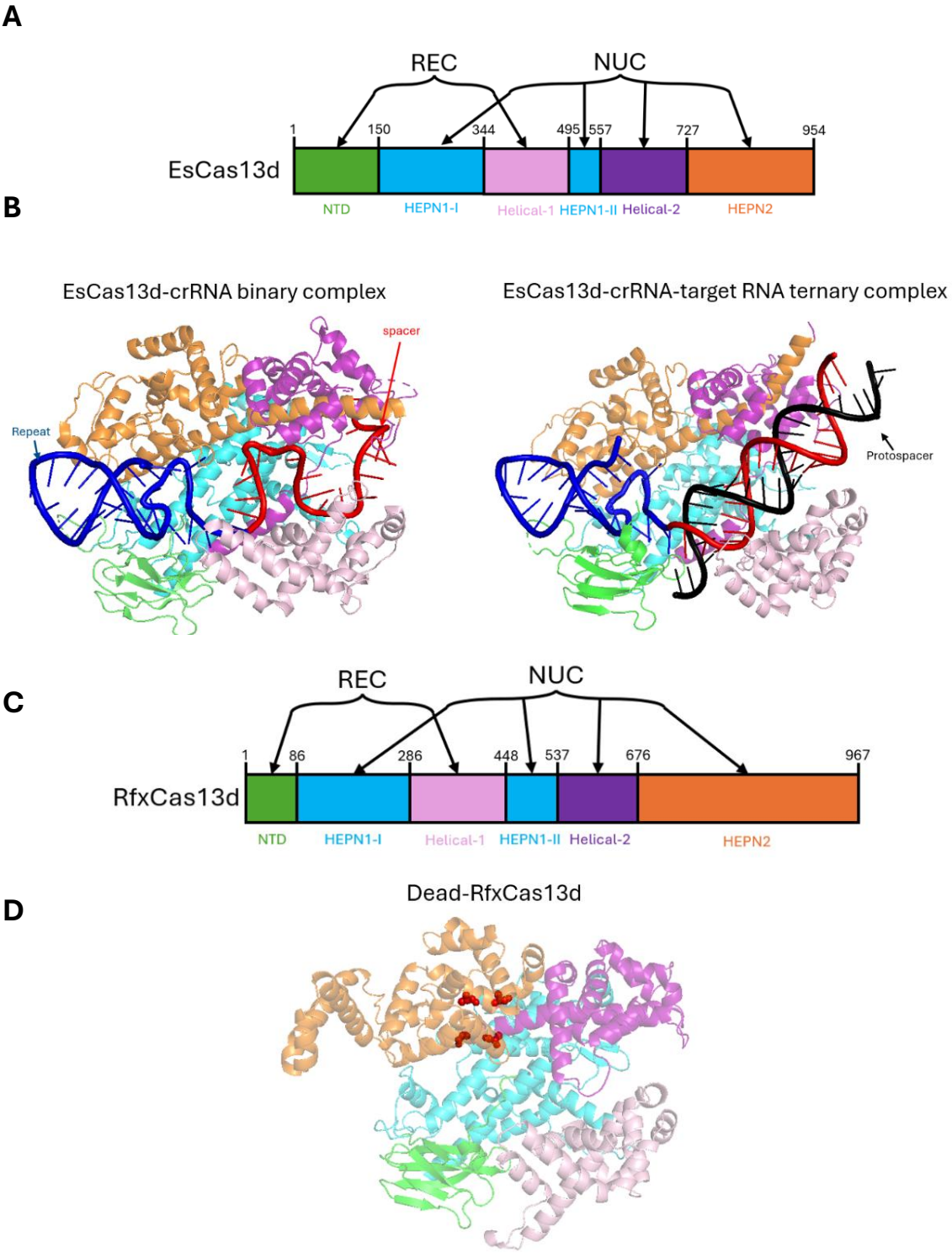


Figure 7. The domain organisation and protein structures of Cas13d variants. A. Domain organisation of the 954 amino acid *Eubacterium siraeum* Cas13d (*Es*Cas13d), inspired by (44). **B.** A solved cartoon representation of EsCas13d-crRNA binary complex (PDB code: 6E9E) and *Es*Cas13d-crRNA-target RNA ternary complex (PDB code: 6E9F) made using electron microscopy structures with resolutions of 3.4 Å and 3.3 Å respectively (44). **C.** Domain organisation of 967 amino acid *Ruminococcus flavefaciens* Cas13d (*Rfx*Cas13d), adapted from (45). **D.** Structure of dead-*Rfx*Cas13d with the altered catalytic residues highlighted in red, generated by AlphaFold2 but not solved in the PDB (46).

It is also worth mentioning that Cas13 proteins have been linked to collateral cleavage in bacterial, mammalian and plant systems. Upon shifting into a cleavage-competent state and cutting the bound target ssRNA *in cis*, the surface exposed RNase can also non-specifically cleave other encountered RNA in *trans*, which in its natural setting would be an inherently useful property to degrade not just target phage DNA but also host DNA in a 'suicide' response to infection (47, 48). While collateral cleavage sounds detrimental to the use of Cas13d, for applications in gene-knockdown, using a nuclease-deactivated 'dead' Cas13 (dCas13) with the key catalytic residues altered shows a significantly reduced growth burden and removes collateral activity as an issue (42). As seen in Figure 7D, for *Rfx*Cas13d these mutations are R238A, H243A, R858A, H863A; mutations that do not impact overall structure of the Cas protein, maintaining its ability to acquire a guide RNA and seek out the target sequence, but removing its nuclease activity. Instead dCas13d will locate its RNA target and remain tightly bound to block any of the translation machinery from continuing downstream, silencing a gene.

1.5.3 Genetic Application of Cas13d

Early studies comparing the Cas13 subtypes found *Rfx*Cas13d to be the most promising subtype, one study in Drosophila using fluorescent protein assays showed that *Rfx*Cas13d had an 82% knockdown efficiency compared to 35-40% for *Lwa*Cas13a, 45-51% for *Psp*Cas13b and 25% for *Fpe*Cas13c (36). Adding onto this, an *in planta* study also found that *Rfx*Cas13d (along with the less established Cas13x and Cas13y) showed significantly higher levels of silencing than *Lwa*Cas13a and *Pbu*Cas13b (49). Taking a closer look at Cas13d specifically, a study in HEK293FT cells confirmed that *Rfx*Cas13d outperformed the other subtypes at knocking down a fluorescent protein (50). With *Rfx*Cas13d established as the frontrunner of RNA directed Cas13-based translational knockdown, recent application in *E. coli* proved the effectiveness of dCas13d in a simple unicellular bacteria which supports the potential application of dCas13d in the more complex multicellular *Streptomyces* spp. (42).

The most exciting factor surrounding the application of dCas13d is obviously its ability to knockdown gene expression at the translational level, targeting RNA. The major limitation of CRISPRi-seq is the fact that disrupting transcription of the upstream gene will also preclude expression of downstream genes in the operon – limiting it to operon-level studies. However, the presence of numerous ribosome binding sites (RBS) throughout an operon transcript means that even if translation of one gene is blocked using dCas13d, ribosomes can bind elsewhere to translate the other genes present within the operon – opening up the potential to study individual genes within an operon (42). All this while, dCas13d application maintains the other strengths of CRISPRi-seq which includes providing a targetable high efficiency platform capable of studying essential genes in a high-throughput reproducible manner.

1.6 Project Aims

The ultimate aim of this project is to adapt dCas13d translational silencing in *Streptomyces* spp. where it may complement existing strategies in the *Streptomyces* spp. functional genomics toolkit. The specific aims of the project are to: (1) Design, construct, and introduce mono- and tri-cistronic model operons into *S. albidoflavus* J1074, and (2) design, construct and introduce an inducible, integrative, customisable dCas13d system into *S. albidoflavus* J1074 and evaluate its performance in translational silencing in mono- and tri-cistronic contexts against dCas9 using phenotypic assays and Western blots.

2. Methods

2.1 Materials

2.1.1 Strains

Name	Description	Reference or source
Escherichia coli		
NEB5α	General cloning host	New England Biolabs
ET12567	Non-methylating host for transfer of DNA into *Streptomyces* spp. Cam^R	(51)
ccdB Survival 2T1^R	General cloning host for constructs containing the *ccdB* toxin gene. Contains a DNA gyrase mutation to alter CcdB binding site	Invitrogen
Streptomyces strains		
S. albidoflavus J1074	*Streptomyces albidoflavus* with the SalI restriction system knocked out to improve DNA acceptance for cloning	(52)
ΦBT1::pRFSdCas9-*aacC4*	*S. albidoflavus* J1074 derivative harbouring pRFSdCas9-*aacC4*; Apr^R	This study
ΦBT1::pRFSdCas9-targetless	*S. albidoflavus* J1074 derivative harbouring pRFSdCas9-Targetless; Apr^R	This study
ΦBT1::pRFSdCas13d-*aacC4*	*S. albidoflavus* J1074 derivative harbouring pRFSdCas13d-*aacC4*; Apr^R	This study
ΦBT1::pRFSdCas13d-targetless	*S. albidoflavus* J1074 derivative harbouring pRFSdCas13d-Targetless; Apr^R	This study
ΦBT1::pRFSdCas9-BT1	*S. albidoflavus* J1074 derivative harbouring pRFSdCas9-BT1; Apr^R	(26)
ΦC31::pKanHygGus	*S. albidoflavus* J1074 derivative harbouring pKanHygGus; $Carb^R$ Kan^R Hyg^R	This study
ΦC31::pKan	*S. albidoflavus* J1074 derivative harbouring pKan; $Carb^R$ Kan^R	This study
ΦC31::pHyg	*S. albidoflavus* J1074 derivative harbouring pHyg; $Carb^R$ Hyg^R	This study
ΦC31::pGus	*S. albidoflavus* J1074 derivative harbouring pGus; $Carb^R$	This study
ΦC31::pKanHygGus ΦBT1::pRFSdCas9-*neo*	*S. albidoflavus* J1074 derivative harbouring pKanHygGus and pRFSdCas9-*neo*; $Carb^R$ Kan^R Hyg^R Apr^R	This study
ΦC31::pKanHygGus ΦBT1::pRFSdCas9-*hph*	*S. albidoflavus* J1074 derivative harbouring pKanHygGus and pRFSdCas9-*hph*; $Carb^R$ Kan^R Hyg^R Apr^R	This study
ΦC31::pKanHygGus ΦBT1::pRFSdCas9-Targetless	*S. albidoflavus* J1074 derivative harbouring pKanHygGus and pRFSdCas9-Targetless; $Carb^R$ Kan^R Hyg^R Apr^R	This study

Table 1. A summary of the strains utilised during the study with a brief description and its source/reference

Streptomyces strains	Description	Source
ΦC31::pKanHygGus ΦBT1::pRFSdCas13d-*hph*	*S. albidoflavus* J1074 derivative harbouring pKanHygGus and pRFSdCas13d-*hph*; $Carb^R$ Kan^R Hyg^R Apr^R	This study
ΦC31::pKanHygGus ΦBT1::pRFSdCas13d-*gusA*	*S. albidoflavus* J1074 derivative harbouring pKanHygGus and pRFSdCas13d-gusA; $Carb^R$ Kan^R Hyg^R Apr^R	This study
ΦC31::pKanHygGus ΦBT1::pRFSdCas13d-Targetless	*S. albidoflavus* J1074 derivative harbouring pKanHygGus and pRFSdCas13d-Targetless; $Carb^R$ Kan^R Hyg^R Apr^R	This study
ΦC31::pKan ΦBT1::pRFSdCas9-*neo*	*S. albidoflavus* J1074 derivative harbouring pKan and pRFSdCas9-*neo*; $Carb^R$ Kan^R	This study
ΦC31::pKan ΦBT1::pRFSdCas9-Targetless	*S. albidoflavus* J1074 derivative harbouring pKan and pRFSdCas9-Targetless; $Carb^R$ Kan^R	This study
ΦC31::pKan ΦBT1::pRFSdCas13d-*neo*	*S. albidoflavus* J1074 derivative harbouring pKan and pRFSdCas13d-*neo*; $Carb^R$ Kan^R	This study
ΦC31::pKan ΦBT1::pRFSdCas13d-Targetless	*S. albidoflavus* J1074 derivative harbouring pKan and pRFSdCas13d-Targetless; $Carb^R$ Kan^R	This study
ΦC31::pHyg ΦBT1::pRFSdCas9-*hph*	*S. albidoflavus* J1074 derivative harbouring pHyg and pRFSdCas9-*hph*; $Carb^R$ Hyg^R	This study
ΦC31::pHyg ΦBT1::pRFSdCas9-Targetless	*S. albidoflavus* J1074 derivative harbouring pHyg and pRFSdCas9-Targetless; $Carb^R$ Hyg^R	This study
ΦC31::pHyg ΦBT1::pRFSdCas13d-*hph*	*S. albidoflavus* J1074 derivative harbouring pHyg and pRFSdCas13d-*hph*; $Carb^R$ Hyg^R	This study
ΦC31::pHyg ΦBT1::pRFSdCas13d-Targetless	*S. albidoflavus* J1074 derivative harbouring pHyg and pRFSdCas13d-Targetless; $Carb^R$ Hyg^R	This study
ΦC31::pGus ΦBT1::pRFSdCas9-gusA	*S. albidoflavus* J1074 derivative harbouring pGus and pRFSdCas9-gusA; $Carb^R$	This study
ΦC31::pGus ΦBT1::pRFSdCas9-Targetless	*S. albidoflavus* J1074 derivative harbouring pGus and pRFSdCas9-Targetless; $Carb^R$	This study
ΦC31::pGus ΦBT1::pRFSdCas13d-guA	*S. albidoflavus* J1074 derivative harbouring pGus and pRFSdCas13d-*gusA*; $Carb^R$	This study
ΦC31::pGus ΦBT1::pRFSdCas13d-Targetless	*S. albidoflavus* J1074 derivative harbouring pGus and pRFSdCas13d-Targetless; $Carb^R$	This study
ΦBT1::pRFSdCas9-*aacC4*	*S. albidoflavus* J1074 derivative harbouring pRFSdCas9-*aacC4*; Apr^R	(26)
ΦBT1::pRFSdCas9-BT1	*S. albidoflavus* J1074 derivative harbouring pRFSdCas9-BT1; Apr^R	(26)
ΦBT1::pRFSdCas9-targetless_1	*S. albidoflavus* J1074 derivative harbouring pRFSdCas9-targetless_1; Apr^R	(26)

Table 1 (continued). A summary of the strains utilised during the study with a brief description and its source/reference

Cam^R – chloramphenicol resistance; Carb^R – carbenicillin resistance ; Kan^R – kanamycin resistance; Apr^R – apramycin resistance; Hyg^R – hygromycin resistance.

Table 1. A summary of the strains utilised during the study with a brief description and its source/reference

2.1.2 Vectors

Vector name	Description	Reference or source
pUZ8002	Encodes all necessary conjugation machinery for mobilization of plasmids from *E. coli* to *Streptomyces*; Kan^R	(51)
pUC57 C31	pUC57 derivative harbouring an RK2 oriT, synthetic ΦC31 integrase gene and *attP* site for conjugation into *Streptomyces* spp.; Carb^R	Genscript
pKanHygGus	pUC57 C31 derivative containing the tri-cistronic reporter operon containing a 3x-3xFLAGged *neo*, *hph* and gusA gene expressed under the *aacC4* promoter. Carb^R Kan^R Hyg^R	This study
pKan	pUC57 C31 derivative containing the mono-cistronic *neo* reporter with a C-terminal 3xFLAG tag under the *aacC4* promoter. Carb^R Kan^R	This study
pHyg	pUC57 C31 derivative containing the mono-cistronic *hph* reporter with a C-terminal 3xFLAG under the *aacC4* promoter. Carb^R Hyg^R	This study
pGus	pUC57 C31 derivative containing the mono-cistronic gusA reporter with a C-terminal 3xFLAG under the *aacC4* promoter. Carb^R	This study
pRFSdCas9_	Integrative CRISPRi plasmid in which dCas9 expression is controlled by an anhydrotetracycline promoter. sgRNAs are cloned in via BsmBI golden gate cloning to replace a *cat-ccdB* counterselection cassette; integrates into the ΦC31 attB site in *Streptomyces* species; Apr^R	(26)
pRFSdCas13d	Integrative plasmid in which *Streptomyces* spp. codon-optimised dCas13d and sgRNA expression is controlled by an anhydrotetracycline-inducible promoter. sgRNAs are cloned in via BsmBI golden gate cloning to replace a *cat-ccdB* counterselection cassette; integrates into the ΦC31 attB site in *Streptomyces* species; Apr^R	This study
pRFSdCas9-*neo*	pRFSdCas9 derivative harbouring an sgRNA targeting the *neo* kanamycin resistance gene; Apr^R	This study
pRFSdCas9-*hph*	pRFSdCas9 derivative harbouring an sgRNA targeting the *hph* hygromycin resistance gene; Apr^R	This study
pRFSdCas9-gusA	pRFSdCas9 derivative harbouring an sgRNA targeting the *gusA* reporter gene; Apr^R	This study

Table 2. A summary of the vector utilised during the study with a brief description and its source/reference

Vector name	Description	Reference or source
pRFSdCas13d-*aacC4*	pRFSdCas13d derivative harbouring an sgRNA targeting the *aac(3)IV* apramycin resistance gene; Apr^R	This study
pRFSdCas13d-*neo*	pRFSdCas13d derivative harbouring an sgRNA targeting the *neo* kanamycin resistance gene; Apr^R	This study
pRFSdCas13d-*hph*	pRFSdCas9 derivative harbouring an sgRNA targeting the *hph* hygromycin resistance gene; Apr^R	This study
pRFSdCas13d-gusA	pRFSdCasI m9 derivative harbouring an sgRNA targeting the *gusA* reporter gene; Apr^R	This study
pRFSdCas13d-Targetless	pRFSdCas9 derivative harbouring a targetless sgRNA ; Apr^R	This study
$Carb^R$ – carbenicillin resistance ; Kan^R – kanamycin resistance; Apr^R – apramycin resistance; Hyg^R – hygromycin resistance.		

Table 2 (continued). A summary of the vector utilised during the study with a brief description and its source/reference

2.1.3 Oligonucleotide primers

Name	Sequence (5' - 3')	Comments
WGP1f	aaacTCTTGTTCAATCACGACATTGCACTCCA	Oligo Annealing: fwd, dCas13d *neo*-targeting sgRNA
WGP2f	aaacGAAGAGATTCTTGTGTCACCTTGTTGCC	Oligo Annealing: fwd, dCas13d *hph*-targeting sgRNA
WGP4f	aaacGCCGCAGCATATGTTTTTTCCTCCATTT	Oligo Annealing: fwd, dCas13d *gusA*-targeting sgRNA
WGP7f	atggAGGAATCATCTGAATCAATG	Oligo Annealing: fwd, dCas9 *hph*-targeting sgRNA
WGP7r	aaacCATTGATTCAGATGATTCCT	Oligo Annealing: rev, dCas9 *hph*-targeting sgRNA
WGP8f	atggGGTCGATACCGCAGTTCTCC	Oligo Annealing: fwd, dCas9 *gusA*-targeting sgRNA
WGP8r	aaacGGAGAACTGCGGTATCGACC	Oligo Annealing: rev, dCas9 *gusA*-targeting sgRNA
WGP9f	**GGCTACCGTCTCG**CAAGCAGCTTGTCTGTAAGCGG	fwd, amplification of pUC57-C31 for *neo*-3xFLAG (BsmBI)
WGP9r	**GGCTACCGTCTCC**AAAGGGCCTCGTGATAC	rev, amplification of pUC57-C31 for *neo*-3xFLAG (BsmBI)
WGP10r	**GGCTACCGTCTCC**CTTGGGCCTCGTGATAC	rev, amplification of pUC57-C31 for *hph*-3xFLAG (BsmBI)
WGP11f	**GGCTACCGTCTCG**GTCACAGCTTGTCTGTAAGCGG	fwd, amplification of pUC57-C31 for *gusA*-3xFLAG (BsmBI)
WGP11r	**GGCTACCGTCTCC**ATGTGGCCTCGTGATAC	rev, amplification of pUC57-C31 for gusA-3xFLAG (BsmBI)
WGP12f	atggGAATAGCCTCTCCACCCAAG	Oligo Annealing: fwd, dCas9 *neo*-targeting sgRNA

Table 3. A summary of all the oligomers utilised throughout the study, their sequence and a brief description

Name	Sequence (5' - 3')	Comments
WGP25	ctggTGGAGTGCAATGTCGTGATTGAACAAGA	Oligo Annealing: rev, dCas13d *neo*-targeting sgRNA
WGP26	ctggGGCAACAAGGTGACACAAGAATCTCTTC	Oligo Annealing: rev, dCas13d *hph*-targeting sgRNA
WGP27	ctggAAATGGAGGAAAAAACATATGCTGCGGC	Oligo Annealing: fwd, dCas13d *gusA*-targeting sgRNA
WGP30f	aaacGGAAGGAACCAAACAACAACAACAACAC	Oligo Annealing: fwd, dCas13d Targetless sgRNA
WGP30r	ctggGTGTTGTTGTTGTTTGGTTCCTTCC	Oligo Annealing: rev, dCas13d Targetless sgRNA
WGP31	**GGTACCGTCTCCAAAC**CAGTCACTTGTCGTCATCGT	rev, pUC57 amp starting at *neo* gene start codon (use for pKan assembly)
WGP32	**GGTACCGTCTCCGTTT**CTTGTCTGTAAGCGGATGC	fwd, pUC57 amp starting after *gusA* gene coding (use for pKan/pHyg assembly)
WGP33	**GGTACCGTCTCCTGTT**CTGATGACATCAGTCGATCATAG	rev, pUC57 amp starting on *aacC4* promoter before *neo* (use for pHyg/pGus assembly)
WGP34	**GGTACCGTCTCCAAC**AGAAGGGGCAACAAGGTGAC	fwd, pUC57 amp starting on *hph* gene start (use for pHyg assembly)
WGP35	**GGTACCGTCTCCAAAC**TTTACTTGTCGTCATCGTCCTTG	rev, pUC57 amp starting after *hph* gene coding (use for pHyg assembly)
WGP36f	CACCACCGACTATTTGCAAC	rev, sequencing primer for pRFSdCas9 or pRFSdCas13d plasmids
WGP36r	CTGATGCCGCATAGTTAAGC	fwd, sequencing primer for pRFSdCas9 or pRFSdCas13d plasmids
WGP38	**GGTACCGTCTCCAAC**AAAATGGAGGAAAAAACATATGCTG	fwd, pUC57 amp starting on *gusA* gene start (use for pGus assembly)
WGP40f	AAAGCTTTCTAGAGGATCCCC	fwd, sequencing primer for pRFSdCas13d plasmids
WGP41f	GAGAACATCATCCACCTGTTCAC	fwd, sequencing primer for pRFSdCas9 plasmids
WGP41r	GGTTCGTGTAGACTTTCCTTG	rev, sequencing primer for pRFSdCas9 plasmids
WGP43f	aaacTTGCACGACATTGCACTCCACCGCTGAT	Oligo Annealing: fwd, dCas13d *aacC4* sgRNA
WGP43r	ctggATCAGCGGTGGAGTGCAATGTCGTGCAA	Oligo Annealing: rev, dCas13d *aacC4* sgRNA
RFS876	**GGCTACCGTCTCC**GAAGAACTCGTCAAGAA	rev, amplification of tcp830/*neo* gBlock to assemble pUC57C31:tcp830/*neo*:3xFLAG

Table 3 (continued). A summary of all the oligomers utilised throughout the study, their sequence and a brief description

Name	Sequence (5' - 3')	Comments
RFS877	**GGCTACCGTCTCGCTTC**GGTGGAGGCGGTTCAGG	fwd, amplification of 3xFLAG to assemble pUC57C31:tcp830/*aacC4*:3xFLAG
RFS878	**GGCTACCGTCTCC**CTTGTTGCCCCTTCTCAGTCACTTGTC GTCATCGTCCT	rev, amplification of 3xFLAG to assemble pUC57C31:tcp830/*neo*:3xFLAG
RFS879	**GGCTACCGTCTCGCAAG**GTGACACAAGAATCTCT	fwd, amplification of *hph* gBlock to assemble pUC57C31:*hph*:3xFLAG
RFS880	**GGCTACCGTCTCC**CGCTCCGGGCGCTGTAT	rev, amplification of *hph* gBlock to assemble pUC57C31:*hph*:3xFLAG
RFS881	**GGCTACCGTCTCGAGCG**GGTGGAGGCGGTTCAGG	fwd, amplification of 3xFLAG to assemble pUC57C31:*hph*:3xFLAG
RFS882	**GGCTACCGTCTCC**ATGTTTTTTCCTCCATTTTTTTTATTTACT TGTCGTCATCGTCCT	rev, amplification of 3xFLAG to assemble pUC57C31:*hph*:3xFLAG
RFS883	**GGCTACCGTCTCGACAT**ATGCTGCGGCCCGTCGA	fwd, amplification of *gusA* gBlock to assemble pUC57C31:*gusA*:3xFLAG
RFS884	**GGCTACCGTCTCC**CTGCTTCCCGCCCTGCT	rev, amplification of gusA gBlock to assemble pUC57C31:*gusA*:3xFLAG
RFS885	**GGCTACCGTCTCGGCAG**GGTGGAGGCGGTTCAGG	fwd, amplification of 3xFLAG to assemble pUC57C31:*gusA*:3xFLAG
RFS886	**GGCTACCGTCTCCTGAC**TCACTTGTCGTCATCGT	rev, amplification of 3xFLAG to assemble pUC57C31:*gusA*:3xFLAG
RFS888	*CTTGTTGCCTCCTTAGCAGG*	fwd, amplification of pRFSdCas9 backbone for dCas13d plasmid assembly (Gibson overlap with RFS889)
RFS889	***CCTGCTAAGGAGGCAACAAG***ATGATCGAGAAGAAGAAG AG	fwd, amp of dCas13d gene for dCas13d plasmid assembly (Gibson overlap with RFS888)
RFS890	*TTAACTATTCCCGGAGACCT*	rev, amp of dCas13d gene for dCas13d plasmid assembly (Gibson overlap with RFS891)
RFS892	GGTGTATCCAACGGCGTCAG	rev, amp of tcp830p DR gblock
RFS893	***AACTGGTCGGGGTTTGAAAC***AGAGACGTTATATTCCCCA G	fwd, amp of *cat-ccdb* for dCas13d plasmid assembly (Gibson overlap with RFS896)
RFS896	*GTTTCAAACCCCGACCAGTT*	rev, amp of tcp830p DR for dCas13d plasmid assembly (Gibson overlap with RFS893)
RFS897	**GGCTACCGTCTCGCTTT**CCGGCCAGCCTCGCAGA	rev, amplification of tcp830/*neo* gBlock to assemble pUC57C31:tcp830/*neo*:3xFLAG
JEC62	atggGGAAGGAACCAAACAACAAC	Oligo Annealing: fwd, dCas9 targetless sgRNA

Table 3 (continued). A summary of all the oligomers utilised throughout the study, their sequence and a brief description

Name	Sequence (5' - 3')	Comments
JEC63	aaacGTTGTTGTTTGGTTCCTTCC	Oligo Annealing: fwd, dCas9 targetless sgRNA
JEC78	atggGAGAAGCTGACCGATGAGCT	Oligo Annealing: fwd, dCas9 *aacC4*-targeting sgRNA
JEC79	aaacAGCTCATCGGTCAGCTTCTC	Oligo Annealing: rev, dCas9 *aacC4*-targeting sgRNA
*Restriction sites are underlined, non-homologous sequences are in **bold**, lowercase text indicates an overhang for Golden Gate cloning and *italics* indicates Gibson overlaps		

Table 3 (continued). A summary of all the oligomers utilised throughout the study, their sequence and a brief description

2.1.4 Media and Buffers

All media were autoclaved at ~120 °C for 30-60 minutes prior to use.

Luria Broth Agar media

Per litre of deionised water; 10 g tryptone (Sigma Aldrich), 5 g yeast extract (Sigma Aldrich), 5 g NaCl (Sigma Aldrich), 5g agar (Oxoid)

SFM media

Per litre of deionised water; 20 g mannitol (Sigma Aldrich), 20 g Soya Flour (AA Baits), 20 g agar (Oxoid), 2g $MgCl_2$ (Sigma Aldrich), 1 g $CaCl_2$ (SLS)

PBS buffer

Per litre of deionised water; 8 g NaCl (Sigma Aldrich), 1.44 g Na_2HPO_4 (Sigma Aldrich), 0. 245 g KH_2PO_4 (AnalytiChem), 0.2 g KCl (ThermoFisher). Adjusted to pH 7.4 by titrating 0.1 M HCl.

Minimal Media Agar

Per litre of deionised water, 0.5 g L-asparagine (Sigma Aldrich), 0.5 g K_2HPO_4 (ThermoFisher), 0.2 g $MgSO_4 \cdot 7H_2O$ (Sigma Aldrich), 0.01 g $FeSO_4 \cdot 7H_2O$ (Fluorochem), 10 g agar (Oxoid), adjusted to pH 7 by titrating 0.1 M HCl. Before pouring, supplement with 10 g/L glucose (Sigma Aldrich).

ISP-2 liquid media

Per litre of deionised water; 4 g yeast extract (Sigma Aldrich), 4 g dextrose (Oxoid), 10 g malt extract (Oxoid)

Bovine serum albumin (BSA) standard solution

Per 100 µL of deionised water, 100 µg (1 µg/µL) bovine serum albumin (Sigma Aldrich)

Lysis buffer

Per litre of deionised water, 50 mM sodium phosphate buffer – 28.9 mM sodium phosphate dibasic heptahydrate (VWR) and 21.1 mM sodium phosphate monobasic monohydrate (Acros), 0.15 M NaCl (Honeywell Fluka). Take 50 mL of buffer and add 500 mg (10 mg/mL) lysozyme from chicken egg white (Sigma Aldrich) and one tablet of Pierce™ Protease Inhibitor Tablets, EDTA-free (ThermoFisher).

Running buffer (10X)

Per litre of deionised water, 0.25 M Tris base (ThermoFisher), 1.92 M glycine (Sigma Aldrich), 1% w/v SDS (10 g) (Severn Biotech). Dilute to 1X before use.

Transfer buffer (10X)

Per litre of deionised water, 0.25 M Tris base (ThermoFisher), 1.92 M glycine (Sigma Aldrich). Before use, dilute to 1X with 100 mL 10X transfer buffer, 200 mL methanol (20%)(Sigma Aldrich) and 700 µL dH_2O, store the at 4℃ and use within 1 week.

Tris buffered saline (TBS)(10X) / TBST (+ tween)

Per litre of deionised water, 0.2 M Tris base (ThermoFisher), 1.5 M NaCl (Honeywell Fluka), adjust to pH 7.6 by titrating with 0.1 M HCl. To make 1 L of 1X TBST use 100 mL of 10X TBS, 900 mL dH_2O and 1 mL of Tween-20 (National Diagnostics), use within 2 weeks

5% Blocking solution

Per 100 mL of 1X TBST, 5g of non-fat dairy milk (50 g/L), make fresh for each use

2.1.5 Antibiotics and Additional reagents

2.1.5.1 Antibiotics

For all antibiotics, after dissolving, stock solutions were filter sterilised using a 0.22 µm filter (Starlab) and stored at -20 °C. Table 4 shows the antibiotics used.

Antibiotics	Stock Concentration	Solvent	Working Concentration	Manufacturer
Apramycin	50 mg/mL	dH_2O	50 µg/mL	TOKU-E
Hygromycin B	50 mg/mL	dH_2O	50 µg/mL	Invitrogen
Carbenicillin	100 mg/mL	dH_2O	100 µg/mL	Formedium
Chloramphenicol	25 mg/mL	100% EtOH	25 µg/mL	Formedium
Kanamycin	50 mg/mL	dH_2O	40 µg/mL	Cayman Chemical Company
Nalidixic acid	25 mg/mL	0.3 M NaOH	25 µg/mL	Cayman Chemical Company

Table 4. Summary of Antibiotics, stock concentrations, solvents, working concentrations and manufacturer. All of the reagents were filter sterilised (excluding chloramphenicol) using a 0.22 µm filter (Starlab).

2.1.5.2 Additional Reagents

Reagent	Stock Concentration	Solvent	Storage	Working Concentration	Manufacturer
X-Gluc	250 mM	dH_2O	-20°C	125 µM	X-Gluc DIRECT
Anhydrotetracycline (aTc)	50 mg/mL	Ethanol	-20°C	50 µg/mL	ThermoFisher
Glucose	500 g/L	dH_2O	Room Temp	10 g/L	Sigma Aldrich

Table 5. Summary of reagents used, stock concentrations, solvents, working concentrations and manufacturer. All of the reagents were filter sterilised using a 0.22 µm filter (Starlab) before use.

2.2 Methods

All PCR reactions utilised primers, gBlocks and spacer oligonucleotides ordered from Integrated DNA technologies (IDT), and all enzymes, ligase buffers, polymerases, ligases and plasmid extraction kits were acquired from New England Biolabs (NEB), unless stated otherwise.

2.2.1 Construction of the pKanHygGus and pKan, pHyg and pGus Reporter Plasmids

The cloning strategy for generating the pKanHygGus and the pKan, pHyg and pGus reporter plasmids is diagrammatically explained in Figure 8. Starting with PCR amplification of the 3xFLAG tag and the three gBlocks containing each individual gene, the relevant products were isolated on a 2% agarose gel using the QIAquick Gel Extraction Kit (QIAGEN) as per manufacturer's instructions, terminal BsmBI restriction sites encoded on the primers were used to generate 4 nt overlaps for cloning. All of the Golden Gate cloning reactions were performed used a 1:1 insert:backbone ratio with 100 ng backbone (in this case, pUC57 C31) and the relevant volume of insert, as well as 2 µL T4 buffer, 1 µL BsmBI, 1 µL T4 ligase up to 20 µL total with dH_2O, the mixture was incubated for 10 cycles of 37°C for 10 minutes (for BsmBI digestion) and 16°C for 10 minutes (for ligation). The assembled plasmid was heat shocked into a 50 µL aliquot of calcium-competent NEB® 5-α cells using 5 µL of the Golden Gate mixture (or 10 ng of plasmid); where a 20 minute incubation of the cells and plasmid on ice was followed by a 45 second heat shock at 42 °C and subsequently an hour of rejuvenation in 300 µL LB shaking at 37 °C 200 rpm before plating onto LBA + selective marker – in this case carbenicillin. These placeholder plasmids (with the exception of the *neo* plasmid) are not the same as the single gene plasmids referenced later because they do not yet contain the *aacC4* promoter to express the gene. These plasmids were purified from 4 mL of a 10 mL overnight culture grown with carbenicillin using the Monarch® Plasmid Miniprep kits (NEB) as per manufacturer's instructions and the flagged gene sequences (including the *aacC4* promoter) were amplified by PCR. BsmBI cut sites on the primers generated the 4 nt overlaps that were used in Golden Gate cloning into the pUC57 C31 plasmid to generate the pKanHygGus plasmid. The pKanHygGus plasmid was confirmed by sequencing by SourceBioscience. Using the pKanHygGus plasmid as a template, keeping the RBS of each gene intact and ensuring the *aacC4* promoter remains on the backbone, primers (displayed in Figure 8) were used to amplify out the mono-cistronic pKan, pHyg and pGus reporter plasmids which were ligated and transformed into calcium-competent NEB® 5-α where, after plasmid extraction, they were confirmed through an NdeI restriction digest. All steps to the assembly are shown in Figure A4-9. Cultures containing all of the reporter plasmids were stored in 20% glycerol at -80°C for long term storage.

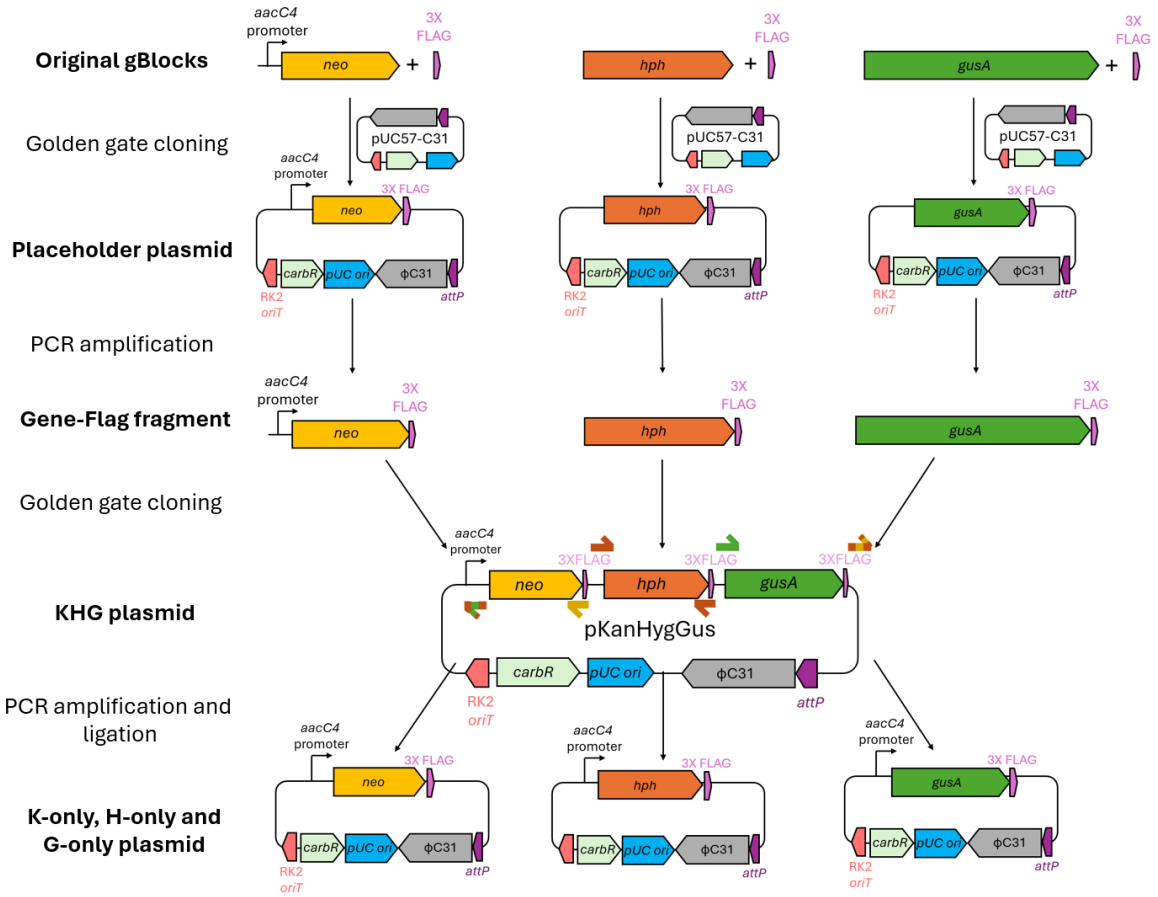


Figure 8. A simplified process of the construction of the pKanHygGus plasmid as well as the single-gene reporter plasmids. The primers used to amplify out the mono-cistronic reporter plasmids are colour coded with the rightmost primer on the diagram used for both the pKan and pHyg amplifications and the leftmost primer used for both pHyg and pGus amplifications.

2.2.2 Construction of the pRFSdCas13d Plasmid

pRFSdCas13d plasmid assembly is displayed in Figure 9. The pRFSdCas9 plasmid, previously constructed by the Seipke lab, contains a ΦBT1 integrase, RK2 *oriT* and *attP* integrase attachment site for *Streptomyces* spp. integration, these features were maintained in the pRFSdCas13d plasmid backbone. Amplification of the pRFSdCas9 backbone excluded the long stretch containing the dCas9 coding region, *cat-ccdB* cassette and sgRNA tail respectively so that it could be replaced with the pRFSdCas13d equivalents. A *Streptomyces* spp. codon optimised dCas13d gene, a fragment encoding both the inducible tcp830 promoter and dCas13d direct repeat, and a *cat-ccdB* cassette with BsmBI restriction sites were amplified using primers exhibiting 20 nt overlaps for Gibson assembly. 100 ng of dCas9 backbone and a 1:1 backbone:insert ratio of the three fragments were mixed alongside 10 µL of Gibson Assembly® Master Mix (NEB) up to 20 µL dH_2O and incubated at 50 °C for 1 hour before heat shock transformation into calcium-competent OneShot *ccdB* Survival 2T1^R for safe growth in the presence of the CcdB toxin and selected for with apramycin. Stocks were stored in 20% glycerol at -80°C. The validity of the plasmid was confirmed SourceBioscience and by restriction digest with KpnI. The gels used in the assembly are shown in Figure A1-2.

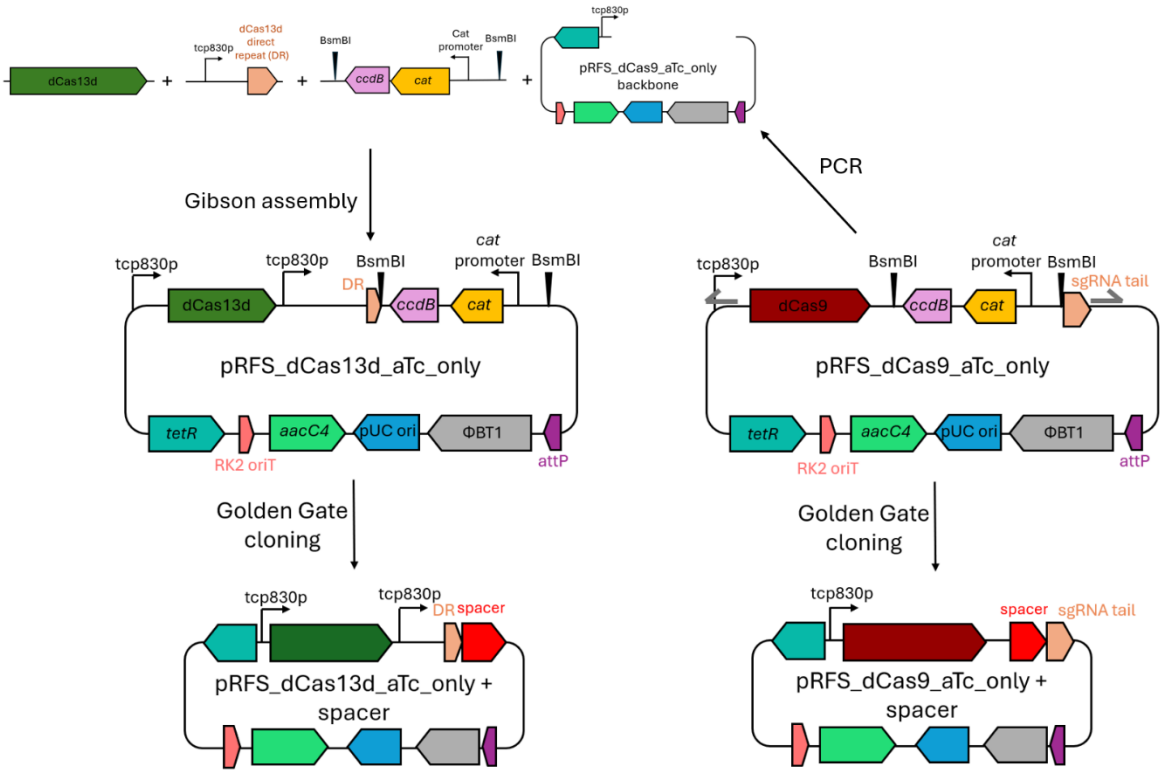


Figure 9. A simplified process of the pRFSdCas13d plasmid construction from the pRFSdCas9 backbone, a dCas13d gene fragment, a fragment containing the aTc-inducible *tcp830* promoter (tcp830p) and dCas13d DR and a fragment with the *cat-ccdb* cassette and BsmBI restriction sites using Gibson assembly. Also shown is the Golden Gate cloning of the sgRNA spacer which removes the *cat-ccdb* counterselection cassette , downstream of the DR and promoter using BsmBI restriction sites.

2.2.3 sgRNA Spacer Integration into the dCas9 and dCas13d Plasmids

The step to introduce an sgRNA spacer is simplified in Figure 9. The sgRNA spacer sequences were designed as a pair of single-stranded complementary oligonucleotides (20 nt long for pRFSdCas9 and 28 nt long for pRFSdCas13d) that contain an additional 4 nt overhang on each end. 5 µL of 20 µM forward oligo and 5 µL of 20 µM reverse oligo were mixed in 90 µL dH_2O and heated at 95 °C for 5 minutes before ramping down to 4 °C at 0.1 °C/sec in order to anneal the oligos. The hybridised oligonucleotides will showcase 4 nt 5' and 3' overlaps complementary to the overlaps created during BsmBI digestion of the pRFSdCas13d plasmid for Golden Gate assembly with the sgRNAs, where 0.5 µL of the annealed oligo mix was used in a typical reaction (detailed above). n on a 0.8% agarose gel and the plasmid size was compared against the unedited dCas plasmids (which is 1.4 kilobases larger)(Figure 10A). Stocks of the sgRNA-containing plasmids were made and stored in 20% glycerol at -80°C.

2.2.4 Mobilisation of the Reporter Plasmids and dCas Plasmids into *S. albidoflavus* J1074

With the vision of having *S. albidoflavus* J1074 strains containing the four reporter plasmids also containing the unique targeting dCas13d and dCas9 systems along with their relevant targetless controls, it made sense to first conjugate in the reporter plasmids and then the Cas plasmids to reduce the number of conjugations required.

The validated reporter plasmids were first transformed into 50 µL aliquots of calcium-competent ET12567/pUZ8002 cells by heat shock as previously described and plated onto LBA + kanamycin + chloramphenicol + carbenicillin. Overnight cultures were used to makes stocks of the strains and also 1 mL of overnight was used to inoculate a 30 mL flask of LB (with antibiotics) to begin the conjugation process. The cells were grown up to OD_{600} = 0.6 and subsequently harvested at 4000 x *g*, washed with 10 mL LB three times and resuspended in 1 mL LB where roughly ~10^8 spores or ~100 µL of *S. albidoflavus* J1074 stock were mixed in. The mixture was further resuspended in 300 µL LB to be plated onto SFM and incubated at 30 °C. Plates were incubated for 16-18 hours before flooding with 1 mL H_2O, 20 µL nalidixic acid and 50 µL of kanamycin, hygromycin or X-Gluc for the relevant reporter (carbenicillin cannot effectively kill *Streptomyces* spp.) and grown for a further 4 days at 30 °C. The nalidixic acid will selectively kill all of the ET12567/pUZ8002 cells on the plate, and the additional antibiotic will kill the J1074 cells that have not successfully integrated the reporter plasmid (or in the case of the pGus plasmid, X-Gluc was used to select for strains producing a blue pigment).

In order to isolate single colonies from the conjugation plate, cells were streaked out onto SFM + nalidixic acid + the relevant selective marker for 5 days thereupon the individual colonies could be easily picked and spread onto SFM + marker for 5 days to develop a full lawn of spores. Using a sterile cotton bud and 2 mL of 20% glycerol, the spores were harvested from the surface and collected into a cryo-vial using wide-bore pipette tips for long term storage at -80°C.

The reporter-containing *S. albidoflavus* J1074 strains were utilised in another round of conjugation (as described above) alongside ET12567/pUZ8002 cells containing the relevant targeting and control pRFSdCas9 or pRFSdCas13d plasmids where apramycin was used as a selective marker. So as to ascertain the concentration of the *S. albidoflavus* J1074 stocks hosting both a reporter sequence and the dCas system, the stocks were serially diluted in PBS and three sets of three 10 µL volumes were plated onto LBA plates at 10^{-6}, 10^{-7}, 10^{-8} dilutions and grown at 30 °C for 2 days. The colony forming units per mL (CFU/mL) was determined by counting and averaging the colonies on plates where the spots showed between 5-50 colonies and then multiplying this number by the dilution factor and volume plated (0.01 mL). From here on, when a number of spores is referred to, it is in reference to the colony forming units present.

2.2.5 Genomic DNA Extraction and sgRNA Confirmation

Growing *Streptomyces* spp. in 10 mL of ISP-2 media + apramycin shaking at 200 rpm for just 24 hours at 30 °C is sufficient to yield a pellet for genomic DNA (gDNA) extraction. The cells were pelleted at 4000 x *g* for 20 minutes before the pellet was resuspended in 600 µL buffer P1 and transferred to a 2 mL Eppendorf tube before the cells are lysed using 40 µL of 100 mg/mL Lysozyme from chicken egg white (Sigma Aldrich). For DNA purification, 500 µL 25:24:1 Phenol:Chloroform:Isoamyl Alcohol (ThermoFisher) was added to the lysed mixture which next was centrifuged at 16,000 x *g* for 10 minutes and the top layer was extracted into a new Eppendorf tube. After 3 rounds of this purification, 2.5x volume of 100% ethanol was added to the tube and the DNA was pelleted at 16,000 x *g* for 20 minutes. The pellet was washed with three rounds of 70% ethanol which was eventually removed using a pipette tip, the pellet was then left to fully air dry in the fume hood before finally being resuspended in 100 µL of sterile dH_2O and its concentration determined using Nanodrop2000 (ThermoFisher).

While the presence of the reporter genes could easily be determined phenotypically, the presence of the intact sgRNA region in the final strain was also confirmed by PCR (Figure A10-11)

2.2.6 Gene Silencing Plate Assay

The silencing assays were performed on minimal media agar supplemented with 10 g/L glucose which is not ideal for cell growth but will highlight even subtle phenotypic changes in terms of growth under an antibiotic or X-Gluc expression. Precise spore concentrations of were prepared in a 96-well plate, diluting into PBS, using the CFU/mL values established previously, and 10 µL spots containing 10^5, 10^4 and 10^3 spores were dropped onto the plate and left to dry. On each plate, the targeting dCas9 and dCas13d strains as well as their targetless controls were included. The plates were then incubated at 30 °C for 4 days before photos were taken. To begin with an apramycin minimum inhibitory concentration (MIC) assay was set up with 10 and 25 µg/mL of apramycin, and aTc values ranging from 5 ng/mL to 500 ng/mL to determine the ideal concentration of aTc for dCas13d activity. This aTc value was carried forward into plates containing kanamycin, hygromycin and X-Gluc to test the three-gene operon.

2.2.7 Western Blot Analysis

For liquid growth, approximately 10^8 spores were inoculated into 50 mL ISP-2 either uninduced or induced with 100 ng/mL aTc and cultures were incubated at 30 °C, 200 rpm for 18 h. Mycelia were harvested by centrifugation of all 50 mL of culture at 4000 x *g* for 20 mins and 100 mg of cells were resuspended in 200 µL lysis buffer (at a minimum of 400 µL buffer used per sample). Cell suspensions were lysed by vortex mixing in 100 mg of 0.1-mm-diamteter glass breads (PoweLyzer) for 30 min at 2000 rpm and 37 °C and subsequently centrifuged at 16, 000 x *g* for 20 minutes to obtain the supernatant. The protein concentration of the supernatant was determined in a Bradford assay and a normalised 63 µg was loaded on a 12.5% acrylamide SDS-PAGE and then transferred to a nitrocellulose membrane for Western blot analysis. The membrane was blocked with a 5% non-fat dairy milk (NFDM) solution rocking at 50 rpm at room temperature for an hour, probed with an HRP-conjugated DYKDDDDK (FLAG) tag monoclonal antibody (HRP-66008)(Proteintech) where it was diluted 1:10,000, in TBST + 5% NFDM; and finally the signals were detected with Pierce 1-Step Ultra TMB blotting solution (ThermoScientific).

3. Results

3.1 Construction of an Integrative, Customisable RNA-targeting dCas13d Plasmid

The design of a *Streptomyces* spp.-optimized dCas13d plasmid was guided by three fundamental principles: stable integration into the host genome, a modular and easily customisable sgRNA region, and tight inducible control over dCas13d and its associated sgRNA expression to minimise leaky knockdown. The backbone of the pRFSdCas9 plasmid was maintained as the backbone for the pRFSdCas13d plasmid meaning it has a high copy number pUC origin of replication permits large plasmid yields which is useful during cloning and the reliable apramycin resistance gene (*aacC4*) to provide a strong selectable marker. To facilitate integration into the host genome, an RK2 *oriT* allows the plasmid to be transferred from *E. coli* into the *Streptomyces* strain where a ΦBT1 integrase encoded on the plasmid will mediate site-specific recombination between the *attP* site on the plasmid and the *attB* site located on the *Streptomyces* spp. chromosome to stably and irreversibly integrate a single copy of the plasmid into the host genome. A *Streptomyces* spp. codon-optimised nuclease-deficient *Rfx*Cas13d gene was introduced under the control of the anhydrotetracycline(aTc)-inducible tcp830 promoter (tcp830p) to tightly control its expression. Also in the plasmid, a *cat-ccdB* counter-selection cassette is flanked by two BsmBI restriction sites that produce specific 4 nt overhangs for spacer cloning, as shown in Figure 10B, this simplified cloning strategy means that any plasmids that haven't taken up the sgRNA will die due to the toxin CcdB toxin. Upstream of where the spacer will be introduced is a dCas13d-specific direct repeat (DR) region which acts a scaffold for dCas13d binding, this sgRNA region is also held under the control of an aTc-inducible tcp830 promoter after a study investigating dCas13d activity in *E. coli* found expression of the sgRNA alone was sufficient to cause a degree of knockdown due to antisense binding to the target transcript (42). The sgRNAs for dCas13d contained an upstream DR and spacers 28 nt long, as per *Rfx*Cas13d specifications denoted in previous papers, that targeted the start codon and ribosome binding site (RBS) of a gene for the highest knockdown efficiency with as minimal off-target hits elsewhere in the genome as possible (42, 45). The dCas9 sgRNAs had a downstream sgRNA tail and spacers were 20 nt long and targeted within the first 100 nt of each operon on the template strand for optimal transcriptional knockdown (30, 53). Construction of the pRFSdCas13d plasmid was validated not only with a restriction digest (Figure 10A) but was confirmed through whole plasmid sequencing by SourceBioscience.

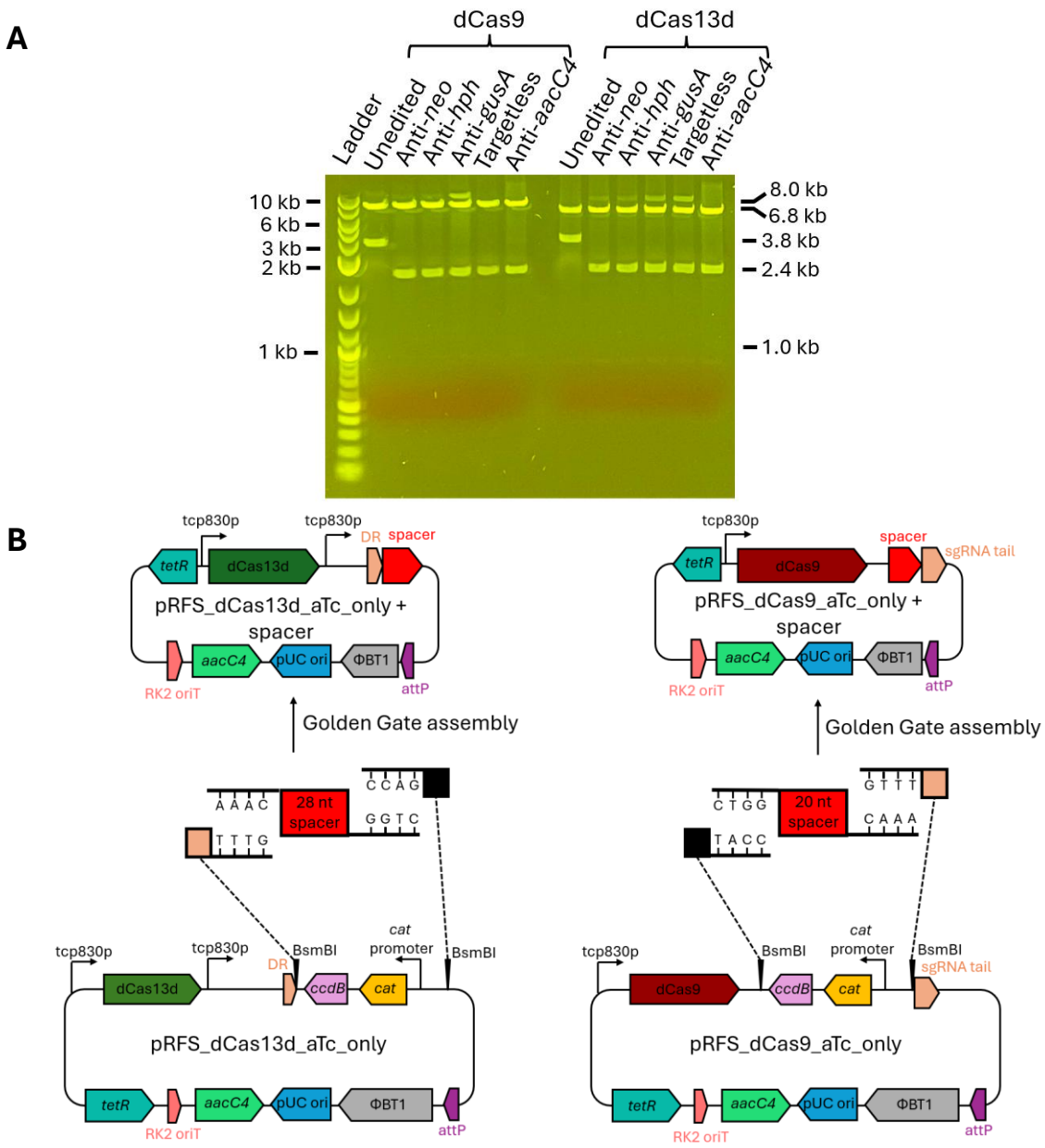


Figure 10. A graphical depiction of the sgRNA cloning process. **A**. Agarose gel showing a restriction digest of the various sgRNA-containing dCas9 and dCas13d plasmids. For the dCas9 plasmid, the unedited plasmid with the *cat-ccdB* cassette intact yields an 8021 and a 3778 bp band and after Golden Gate cloning it shows an 8021 and 2374 bp band. The unedited dCas13d plasmid yields a 6818 and 3795 bp band and the spacer-containing versions show a 6818 bp band and 2399 bp band. The gel used the NEB 1 kb plus ladder and a 0.8 % agarose gel. **B.** A cartoon representation of the Golden Gate reaction to clone an annealed spacer sequence with 4 nt overlaps into the complementary sites found in dCas9 and dCas13d plasmids after BsmBI digestion. The spacer will replace the *cat-ccdB* counter-selection cassette.

3.2 Initial dCas13d Testing Against an Artificial Single Gene

The functionality of dCas13d was initially assessed in *Streptomyces albidoflavus* J1074 by targeting the apramycin resistance cassette encoded on the same pRFSdCas9 or pRFSdCas13d plasmid in the presence and absence of apramycin. Glucose minimal medium agar was selected for this experiment as a harsh condition that would presumably more effectively reveal a phenotypic difference between silenced and unsilenced treatments. Spores for each strain were titred and serial dilution was used to spot 10 µL containing 10^5,10^4 and 10^3 spores onto agar plates. An apramycin concentration screen was performed to identify an appropriate concentration of apramycin for the experiment which identified apramycin 25 ug/mL as the most appropriate (Figure 11B). Next, the concentration of aTc, was optimised over a range of 0-500 ng/mL using plates containing 10 ug/mL apramycin, where the effects of aTc in particular would be more pronounced (Figure 11C). aTc appeared to have a deleterious effect on growth at >250 ng/mL when paired with 10 µg/mL apramycin so subsequently, up to 100 ng/mL of aTc was tested with 25 µg/mL apramycin (Figure 11D). Silencing of the *aacC4* gene using dCas9 and dCas13d (using spacers displayed in Figure 11A) should cause cell death in the presence of apramycin and aTc while otherwise no effect should be apparent, no effect should be seen for the strains harbouring a targetless sgRNA. At an optimum aTc concentration of 100 ng/mL, dCas13d silencing was clearly effective, showing a significant reduction in growth when compared to the targetless control.

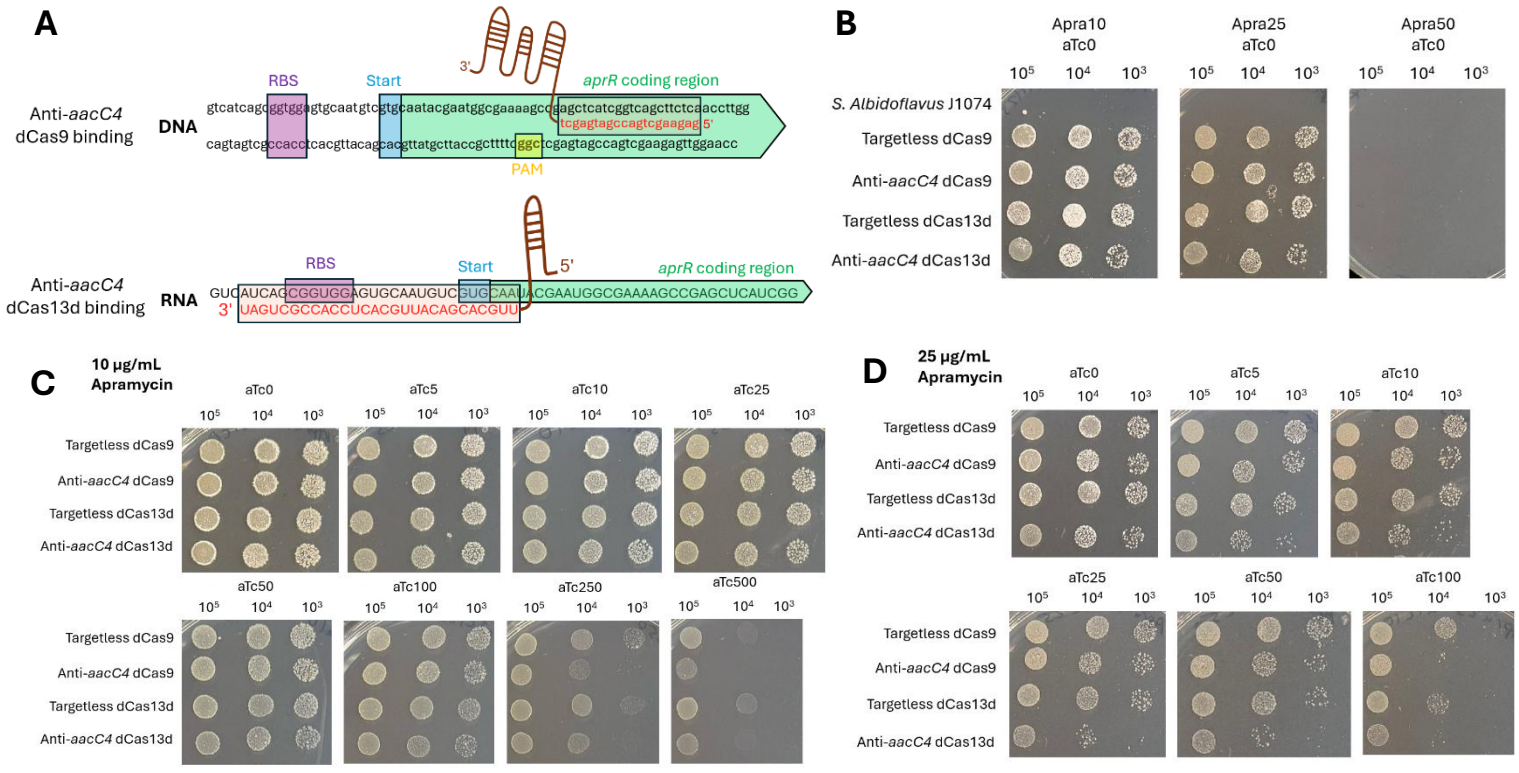


Figure 11. Silencing of the artificial *1* gene in *Streptomyces albidoflavus* J1074. **A.** A diagrammatic explanation of the region of DNA targeted by the anti-*aacC4* dCas9 sgRNA and RNA by the anti-*aacC4* dCas13d sgRNA respectively. **B.** Glucose minimal media agar containing up to 10, 25 and 50 µg/mL of apramycin alongside the four experimental strains and an *S. albidoflavus* J1074 control to determine the optimum apramycin concentration. **C.** *aacC4* silencing assay on glucose minimal media agar containing 10 µg/mL apramycin and up to 500 ng/mL aTc. **D.** *aacC4* silencing on glucose minimal media containing 25 µg/mL and up to 100 ng/mL aTc.

3.3 dCas13d Targeting an Artificial Multi-gene Operon

Previous work showed that dCas13d could silence a fluorescent reporter in *E. coli* with less impact on other operon genes compared to dCas9 (42). Motivated by this, a synthetic three-gene operon, under the *aacC4* promoter, where each gene had a unique ribosome binding site, was designed and assembled it into what is referred to as the integrative pKanHygGus plasmid (Figure 12A). The kanamycin resistance gene (*neo*), hygromycin resistance gene (*hph*), and the *gusA* gene encoding β-glucuronidase (which forms a blue precipitate in the presence of X-Gluc) were chosen for their easily selectable phenotypes, and each was tagged at the C-terminus with a 3xFLAG epitope. Additionally, this trio was favoured due to their distinct protein sizes that could easily be distinguished via Western Blot - with the tag included the protein sizesrange from 32.68 kDa for KanR-3xFLAG, 40.68 kDa for HygR-3xFLAG, up to 72 kDa for GusA-3xFLAG. The plasmid backbone contains the *carbR* gene for selection as well as an RK2 *oriT,* ΦC31 integrase and *attP* attachment site to facilitate efficient conjugation into *Streptomyces* spp. The pKanHygGus plasmid was verified by whole plasmid sequencing by SourceBioscience before it was used as a template to construct the monocistronic reporter plasmids: pKan, pHyg and pGus (Figure 12B). With the future goal of applying dCas13d to investigate large multi-gene operons across the genomes of *Streptomyces* spp., the single-gene variants would provide a point of comparison for knockdown efficiency for mono and tri-gene conditions. All of the conjugated *Streptomyces albidoflavus* strains were confirmed phenotypically (Figure A13).

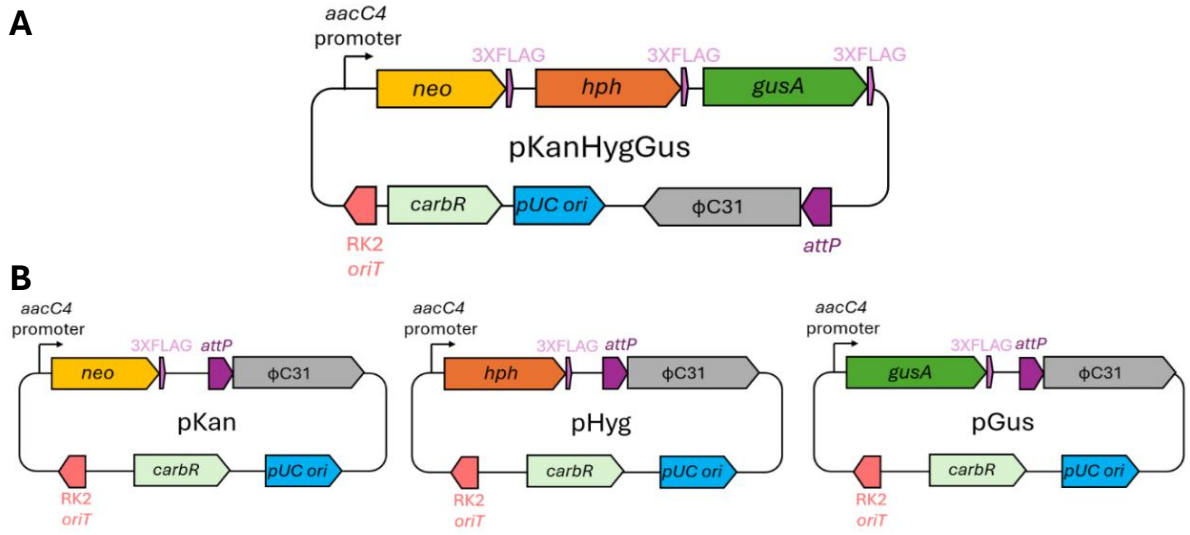


Figure 12. A diagrammatic representation of the tricistronic and monocistronic reporter plasmids. All plasmids contain the same backbone with the *carbR* gene for selection along with the pUC *ori*, RK2 *oriT*, ΦC31 integrase and the *attP* attachment site for integration into *Streptomyces* spp. **A.** The pKanHygGus plasmid contains all three of the *neo*, *hph* and *gusA* genes with a unique RBS, under expression of the same *aacC4* promoter. **B.** The single-gene variants have just the one gene under expression of the *aacC4* promoter with their RBS from the pKanHygGus preserved. **C (overleaf).** Glucose minimal media control assays to determine the ideal working concentration of kanamycin, hygromycin and X-Gluc for future induced assays. Native *S. albidoflavus* J1074 without the reporter or a dCas plasmid was included as a negative control.

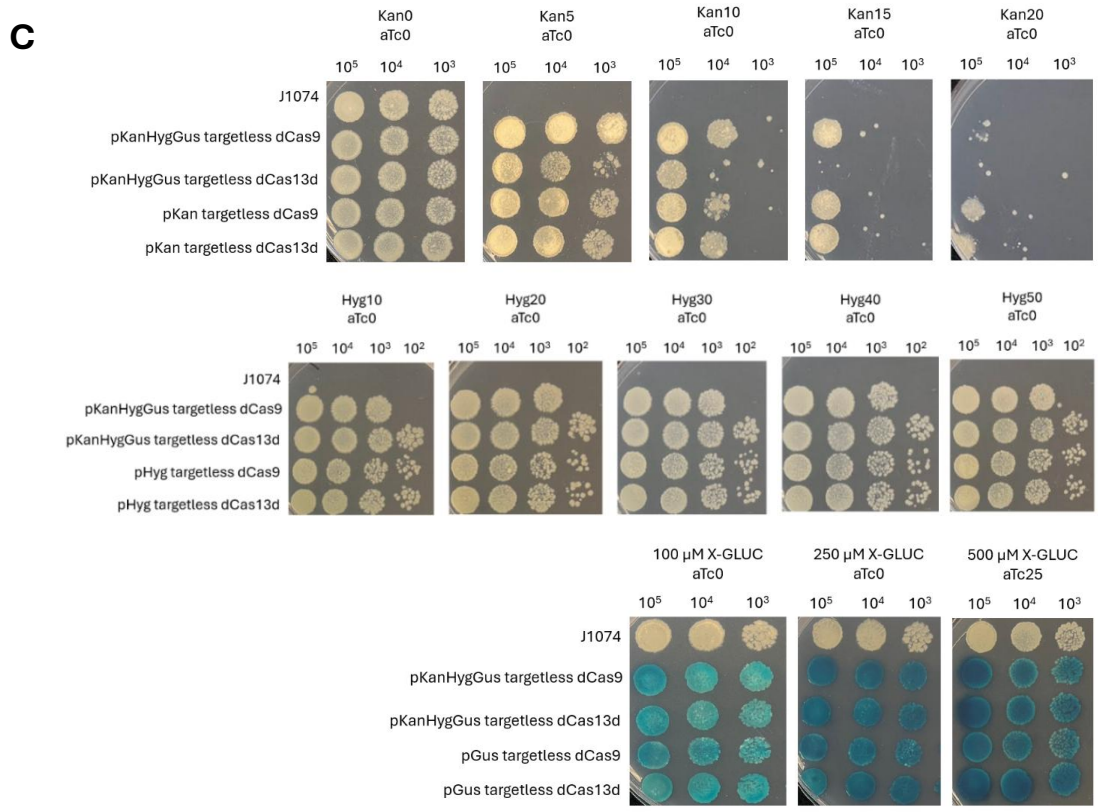


Figure 12 (continued). A diagrammatic representation of the tricistronic and monocistronic reporter plasmids. C. Glucose minimal media control assays to determine the ideal working concentration of kanamycin, hygromycin and X-Gluc for future induced assays. Native *S. albidoflavus* J1074 without the reporter or a dCas plasmid was included as a negative control.

To first find the ideal concentration of kanamycin, hygromycin and X-Gluc for the assay, using the targetless sgRNA strains as a control varying concentrations were tested in the glucose minimal media agar setting (seen in Figure 12C). The results showed that kanamycin had a much stronger effect on the cells than anticipated and as such for *neo* silencing assays, 10 µg/mL using spore spots of 10^5 10^4 10^3 were settled on, hygromycin on the other hand displayed a low bactericidal effect so after further testing, 100 µg/mL and lower spore densities of 10^4 10^3 10^2 were chosen for *hph* knockdown, finally 125 µM X-Gluc and 10^5 10^4 10^3 spore concentrations would be used for *gusA* testing. For each of the assays a wildtype *S. albidoflavus* J1074 stock was used as a negative control and confirmed the efficiency of kanamycin/hygromycin killing and X-Gluc expression. In order to confirm that the dCas9 and dCas13d systems could successfully knock down the three flagged genes, a glucose minimal media assay was performed in the presence of kanamycin, hygromycin or X-Gluc to the previously stated concentrations. In the case of *neo* and *hph* knockdown, the plates should show reduced growth when the systems are induced in the presence of kanamycin and hygromycin respectively while for *gusA*, should manifest as a reduction in blue colouration. As seen in Figure 13, 100 ng/mL aTc was supplemented into the glucose minimal media as well as kanamycin, hygromycin and X-Gluc however the silencing was not very pronounced. An argument could be made for the anti-*neo* dCas13d and anti-*gusA* dCas9 strains showing a visible change, but the majority showed no significant changes when comparing the uninduced plates to the induced. Optimisations with different concentrations were attempted however no significant silencing results were produced in regard to the tri-cistronic operon plate assay (Figure A15-17).

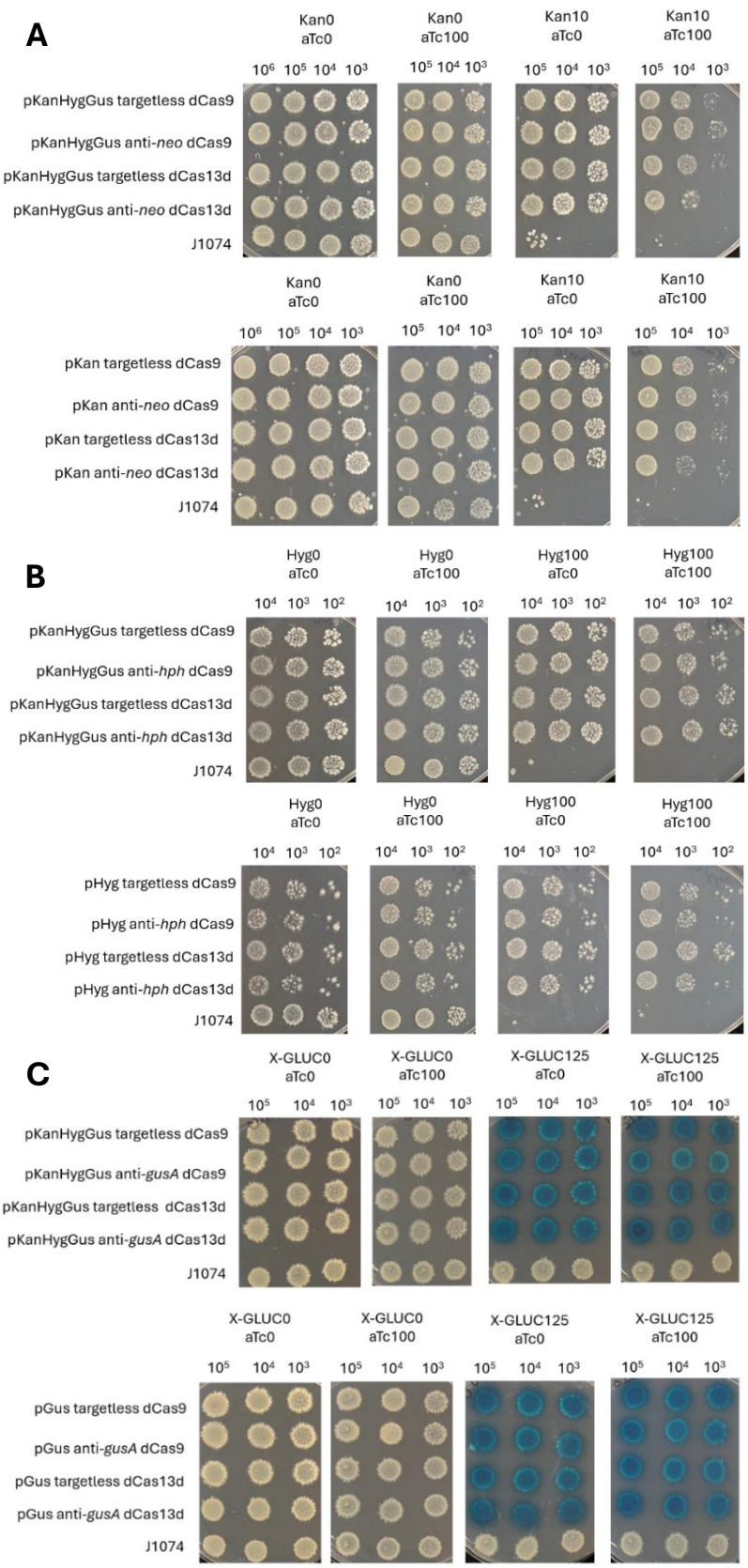


Figure 13. Attempted silencing of the *neo*, *hph* and *gusA* genes in both the tricistronic and monocistronic operons hosted in *Streptomyces albidoflavus* J1074 on glucose minimal media agar. 100 nM aTc was used for the induced plates. Native J1074 is present as a negative control across all plates **A.** Attempted silencing of *neo* gene in the pKanHygGus and pKan strains in the presence of 10 µg/mL kanamycin **B.** Attempting silencing of the *hph* gene in the pKanHygGus and pHyg strains in the presence of 100 µg/mL hygromycin. **C.** Attempted silencing of the *gusA* gene in the pKanHygGus and pGus strains in the presence of 125 µM X-Gluc.

Western Blot of the *S. albidoflavus* J1074 strains harbouring the pKanHygGus plasmid and gene-targeting sgRNAs was performed. Cultures were grown in 50 mL of nutrient-rich ISP-2 media for 18 hours in the presence or absence of 100 ng/mL aTc. This growth period was selected based on prior observations of the lab group that cells in these conditions typically reached the end of the exponential growth phase after ~24 hours and overgrowth may impact the inducers effect. 63 µg of each protein extract per sample was first run on an SDS-PAGE (Figure 14A) and then transferred to a nitrocellulose membrane.

Looking at the Western Blot seen in Figure 14B, the only protein visible on the membrane was the GusA-3xFLAG protein that is 72 kDa in size, the 40.68 kDa HygR-3xFLAG and 32.68 kDa KanR-3xFLAG were not present in the locations shown in the "Expected bands" lane. Focussing solely on the GusA protein bands, they are much less abundant for the induced anti-*neo* and anti-*hph* strains which could suggest a downstream silencing effect and are barely present at all for the anti-*gusA* lanes, but as seen in the gel, the overall levels of protein does not appear to be reliable and even the uninduced anti-*gusA* cultures have little to no protein present on the membrane. This method offers provide a relatively straightforward, easily interpretable technique to directly monitor the level of protein expression and silencing as opposed to using an indirect assay (e.g. fluorescent proteins) however the results do not reflect this.

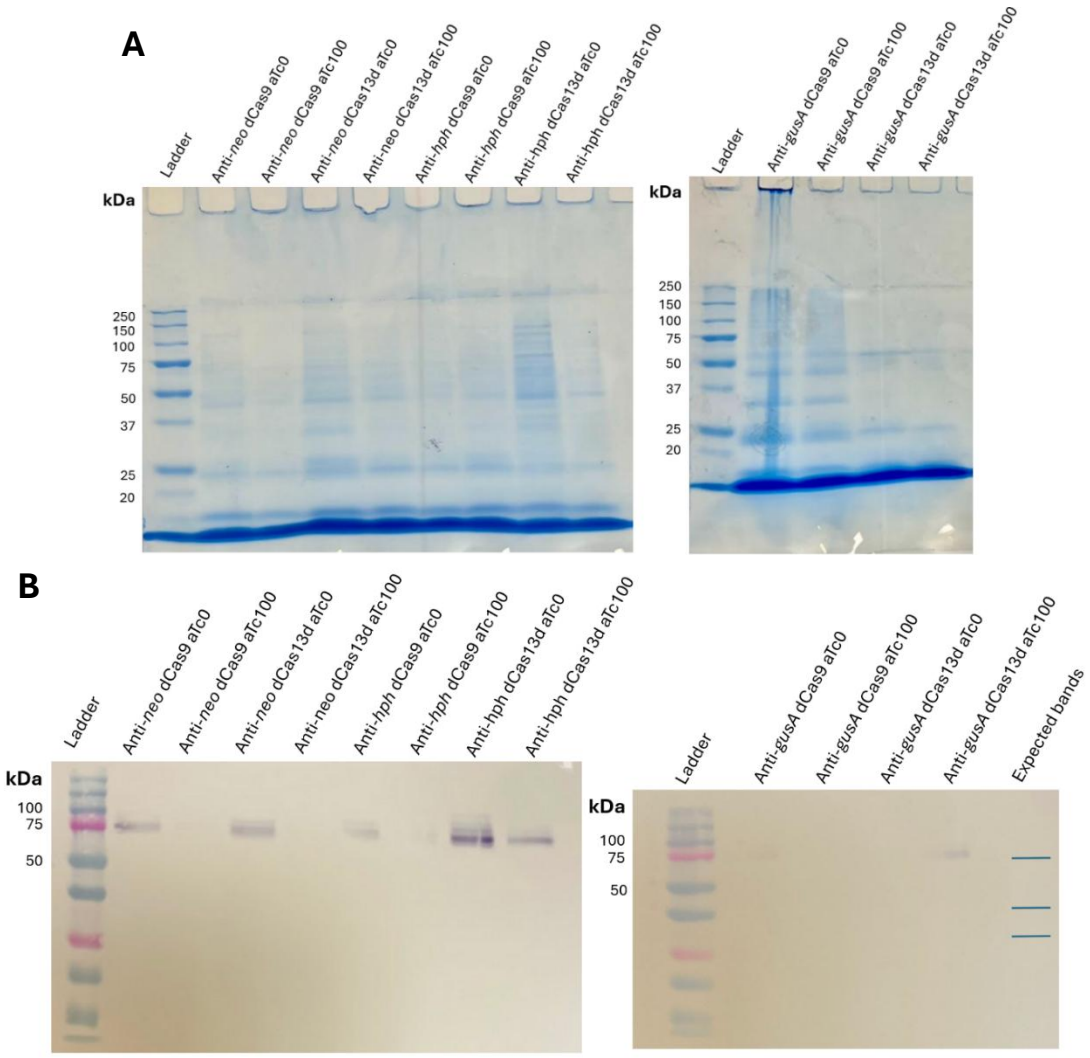


Figure 14. SDS-PAGE and Western Blot to track protein expression of the KanR, HygR and GusA proteins across uninduced and induced cultures containing anti-*neo*, anti-*hph* and anti-gusA dCas9 and dCas13d strains grown in ISP-2 for 18 hours. **A.** 12.5% acrylamide SDS-PAGE containing 63 µg of protein per lane (calculated in a Bradford assay) with Precision Plus Protein Dual Color Standard (BioRad) as the ladder. **B.** A nitrocellulose membrane 63 µg of protein per lane using Precision Plus Protein Dual Color Standard (BioRad) ladder.

Overall, this study took the first steps to establishing dCas13d as a potential component in the *Streptomyces* spp. functional genomics toolbox, constructing the first dCas13d plasmid optimised for integration and activity in *Streptomyces* spp. and proving that dCas13d can silence a gene to impact fitness in *S. albidoflavus* J1074, however the more ambitious, artificial three-gene operon studies were not as conclusive.

4. Discussion

As antimicrobial resistance poses an escalating global health crisis and closes in on the limited arsenal of antibiotics, so too rises the demand for novel antimicrobial agents with unique mechanisms of action. Recent advances in genome sequencing has provided us with the opportunity to revisit the microbial taxa that once served as prolific sources of antimicrobials with a new foundation of understanding. In order to fully unlock this potential, the development of robust functional genomics tools to first strengthen our understanding of these organisms is a necessary step to streamline the discovery of new bioactive compounds and in turn accelerate the pipeline for next-generation antibiotics.

4.1 Evaluation of Experimental Procedure

The exciting potential dCas13d combined with the reputation for secondary metabolite production of the *Streptomyces* genus is what inspired us to undertake this project. Existing high-throughput bacterial functional genomics tools such as CRISPRi-seq show limitations in its ability to study gene function as it is limited to operon level knockdown and Tn-seq lacks the targetability, titratability and reliability to be a standalone tool, this project takes the first steps in building up dCas13d for application in *Streptomyces* spp. to fill in these gaps.

This study confidently achieved the simplest and yet most important goal of the project, which was to show that a codon-optimised dCas13d can silence a gene at the translational level in *Streptomyces albidoflavus* J1074. The first *Streptomyces* spp.-compatible dCas13d plasmid was constructed and showed its ability to cause lethal silencing by knocking down the apramycin resistance gene in the presence of apramycin. The next objective was to characterise the most compelling draw of dCas13d; can it knockdown individual genes with a reduced impact on the downstream genes present within an operon? For this, an artificial three-gene operon in the form of the pKanHygGus plasmid was constructed with tagged genes to permit direct quantitative study of silencing on a Western Blot. The pKanHygGus plasmid alongside three single-gene variants (pKan, pHyg, pGus) were integrated into *S. albidoflavus* J1074 alongside dCas9 or dCas13d. However, the study failed to achieve confident silencing of the tricistronic operon genes with both dCas9 and dCas13d in both plate assays and a quantitative Western Blot which halted any further study.

To substantiate plasmid design, sgRNA engineering and the factors utilised in the assays - many factors were considered.

It stands to reason that dCas13d and dCas9 without the sgRNA present should not impact the host at all (such was shown for dCas13d by (42)) so why hold expression under control of an inducible promoter? The Cas protein is the strongest factor involved in knocking down a gene, so controlling the level of Cas protein would allow us to control the level of silencing, which is an important factor for future applications to study essential genes where total knockdown would be lethal. For dCas9, the DNA-targeting sgRNA poses no risk however for dCas13d, the complementary ssRNA spacer of the sgRNA can act as a form of antisense RNA knockdown and

has been shown to impact gene expression, for this reason, in the pRFSdCas13d plasmid, the sgRNA is also under control of an inducible promoter to prevent leaky knockdown (42). An anhydrotetracycline-inducible system involving a *tetR* resistance gene and tcp830 promoter was chosen as it is the standard for dCas9 and dCas13d experiments, offering tight control and low leakiness, it was also readily available to us from previous work by the Seipke lab with CRISPRi-seq (26).

The sgRNAs were designed based on the optimum variables validated in previous studies (Figure A14). For dCas9 sgRNAs, 20 nt spacers were engineered to target the template within the first 100 nt of the start codon where the orientation and PAM presence (a "CGG" PAM was used for every sgRNA as it was the most abundant in the high G/C genome) was confirmed by CRISPy-web (30, 54). For dCas13d, a 28 nt spacers targeting the ribosome binding site and start codon were designed (42). One sgRNA was used per gene simply to streamline the experiments and in the design it was ensured that no major off-targets were present by running each sgRNA through the NCBI Nucleotide BLAST software against the *S. albidoflavus* J1074 genome (55). Experimentally, the integration of each spacer was confirmed via restriction digest (seen in Figure 10A) and later on the presence of the Cas plasmids in *S. albidoflavus* J1074 was not only verified phenotypically through apramycin resistance but also by PCR of genomic DNA (Figure A10 and Figure A11).

The pKanHygGus plasmid was confirmed not only by a restriction digest (Figure A7) but sequenced by SourceBioscience, the monocistronic plasmids (pKan, pHyg, pGus) used this validated pKanHygGus plasmid as a template during construction and were confirmed by a restriction digest (Figure A9). Each gene was proven to be active during phenotypic selection (Figure A13). Despite this, the powerful growth inhibition under kanamycin and substandard effects of hygromycin made perfecting each assay rather difficult so perhaps utilising a redesigned three-gene operon with different genes, maintaining the different protein sizes for Western Blot study, may yield more satisfactory results in future experiments.

Experiments by Adler et al, 2025 demonstrate that flexibility in the concentration of aTc is needed for each inhibition study, balancing background toxicity of the inducer while ensuring a strong level of target inhibition, during their phage inhibition experiments they ranged from 10 ng/mL aTc to 100 ng/mL of anhydrotetracycline (56). Meanwhile Cardiff et al, 2024 found that dCas13d activity improved up to 200 ng/mL aTc in *E. coli* when supressing a fluorescent reporter (42). With that said, the results in Figure 11C or Figure 12A show that there is clearly a deleterious effect of aTc on the targetless-sgRNA strains at concentrations above 100 ng/mL aTc; for future functional genomics applications, using a toxic concentration of aTc would be detrimental to the point of the experiments which is to investigate gene fitness so the experiments didn't cross this threshold. However, it is still worth taking into consideration that, while dCas9 only has to modulate the single integrated operon in the host genome at one stable site, dCas13d has to target all of the mRNA transcripts produced by said operon, so the level of induction may have to be tailored to the level of gene expression as not to over silence or under silence. After many repeats attempting to optimise the concentrations (Figure A15-17), it stands to reason that the aTc concentration was not at fault.

On the whole, with every step of the process validated along the way, one explanation is that the sgRNAs were ineffective. Perhaps the dCas13d sgRNAs needed to be planted more centrally on the RBS to act with higher efficiency, however due to the tight packing of the genes within the artificial operon, the sgRNAs were designed as such to reduce the risk of occluding the upstream coding region and stop codon. Regardless, it would be interesting to see whether a different set of sgRNAs would have a more pronounced silencing effect. Alternatively, suboptimal knockdown

by the Cas proteins could result in partial expression of the genes where even a low level of protein is sufficient to provide resistance or blue colouration, this may have an especially strong impact depending on the half-life of each protein.

Quantitative Westen Blotting would offer a method to quantitatively monitor protein expression based on the band intensity that would give a direct reading as to the effects of silencing and allow a direct comparison of the protein expression of induced and uninduced samples on the same membrane. Due to time constraints however there was not much time to optimise this protocol. A controlled 10^8 spores were inoculated in nutrient-rich ISP-2 for 18 hours induced with 100 ng/mL of aTc, which was proven to be an effective induction concentration while not drastically impacting growth. This growth time was chosen as previous work by the Seipke lab group found that, with these settings, cell growth peaked at around 24 hours, and overgrowing the cells may diminish the impact of aTc. In hindsight, the cells should have been incubated for longer as the protein yields were not significant enough to run 100 µg of protein on each lane and the level of protein between each sample fluctuated greatly (Figure 14A). It could also be said that in a more nutrient-rich growth medium, a higher concentration of aTc could possibly be utilised which may permit a wider distinction between induced and uninduced samples.

4.2 The Future of dCas13d in *Streptomyces* spp.

We proved that dCas13d could silence a gene and influence fitness with efficiency comparable to the well-established dCas9 system in *S. albidoflavus* J1074, a host chosen for a few key reasons. Namely, the fact that it is a phylogenetically more ancient strain relative to its genus, which makes it an ideal host to extrapolate dCas13d functionality to other *Streptomyces* species (57). Also, among the *Streptomyces* genus, it is relatively fast growing and has a more minimised genome with less secondary metabolite clusters, it also does not have any products that would intrude on using *gusA* as a reporter (whereas the common pigmented products of other strains would make interpreting the GusA colouration assay more difficult). With that said, a smaller future goal will be to confirm dCas13d functionality in some other the other most common model strains such as *S. coelicolor*.

The ability to target RNA and silence translation is what makes dCas13d such a compelling tool to add to the functional genomics toolkit, avoiding complex regulatory pathways and offering the potential to knock down genes with reduced polar effects - wherein silencing one individual gene will impact the other genes in the operon. Initial experiments using fluorescent reporters in *E. coli* showed that dCas13d could silence genes with reduced polar effects compared to dCas9 but still encounters some polar effects particularly on downstream genes due to translational coupling (42). Translational coupling is yet to be fully understood but it is predicted to arise due to a combination of different mechanisms, perhaps the idea that ribosomes reaching the stop codon of an upstream gene will remain loosely bound and re-initiate at the nearby downstream RBS or if not, they may disassemble locally to be in a high concentration to quickly reassemble and bind the downstream RBS (58). Additionally, it has been suggested that translation of an upstream gene can unfold inhibitory secondary structures in the downstream transcript region that may block initiation (58). The distance between the stop codon of the upstream gene and start codon of the downstream gene is proportional to the level of coupling, with the most efficient coupling occurring with -4 to +3 nt spacing and then as spacing is increased to ~+40 nt the coupling is reduced by up to 10-fold (58). Cardiff et al, 2024 found that inhibiting translation of an upstream gene using dCas13d had a significant impact on the expression of the downstream gene on the same transcript while the inverse showed a much lower impact on upstream genes when knocking down a downstream gene (42). In *Streptomyces* spp., a genus

synonymous for having dense gene clusters and for having a high G/C genome capable of forming stable RNA secondary structures, the presence of translational coupling may pose a constraint to dCas13d application. Therefore, future experiments should centre around dissecting the efficacy of dCas13d knockdown in the face of translational coupling, this could be by testing activity against more complex operons such as the artificial three-gene operon pitched in this study or instead focusing on a smaller gene clusters found natively in *Streptomyces* spp.

Bacterial RNA knockdown can also be performed with antisense RNA (asRNA), short sequences complementary to a target region that will form an RNA double helix with the transcript to disrupt the translation machinery. However, asRNA suffers from challenges including degradation by host nucleases, low binding stability, inconsistent inhibition and the fact that more complex enhanced asRNAs need to be synthesised extracellularly and delivered to the host, which can be particularly difficult depending on the host (59). dCas13d offers a targetable, titratable and efficient method of RNA knockdown that is easy to set up by simply swapping a new spacer into a plasmid, in fact, it has already been applied to high-throughput techniques, as seen in the application of phage-wide CRISPRi-art in 2025 (56). How well dCas13d will translate into bacteria and specifically, *Streptomyces* spp., is a question yet to be entirely answered but even if not as a stand-alone tool, it has already shown potential to pair with other functional genomics tools. An example of this is the pairing of dCas13d translational knockdown with CRISPR activation (CRISPRa) - wherein a nuclease-deficient Cas9 protein fused to a transcriptional activator will increase the downstream expression of an operon and dCas13d will knockdown an individual gene within the operon (42). The heightened expression of the operon means that when dCas13d knocks down its target gene, it will have a proportionally lower impact on the other genes within the operon while still effectively silencing the target gene (42). As a relative new tool discovered less than a decade ago, dCas13d must be characterised in greater detail before its capabilities are realised and it earns its place in the functional genomics toolkit.

5. Conclusion

In this thesis, dCas13d was proven capable of translational silencing in the complex, multicellular bacteria *Streptomyces albidoflavus* J1074, part of a genus renowned for being prolific antibiotic producers. However, future work needs to be done to better investigate the application of dCas13d against multi-gene operons, where it has the potential to silence individual genes with lower polar effects than the existing CRISPRi method but barriers such as translational coupling stand in its way. Taken together, dCas13d may have a strong role to play in the *Streptomyces* spp. functional genomics toolkit but we need to advance our understanding first.

5.1. References

1. Komaki H. Recent Progress of Reclassification of the Genus *Streptomyces*. Microorganisms. 2023;11(4).
2. Yague P, Lopez-Garcia MT, Rioseras B, Sanchez J, Manteca A. New insights on the development of *Streptomyces* and their relationships with secondary metabolite production. Curr Trends Microbiol. 2012;8:65-73.
3. Becher PG, Verschut V, Bibb MJ, Bush MJ, Molnar BP, Barane E, et al. Developmentally regulated volatiles geosmin and 2-*methylisoborneol* attract a soil arthropod to *Streptomyces* bacteria promoting spore dispersal. Nat Microbiol. 2020;5(6):821-9.
4. Shepherdson EM, Baglio CR, Elliot MA. *Streptomyces* behavior and competition in the natural environment. Curr Opin Microbiol. 2023;71:102257.
5. McCormick JR, Flardh K. Signals and regulators that govern *Streptomyces* development. FEMS Microbiol Rev. 2012;36(1):206-31.
6. Urem M, van Rossum T, Bucca G, Moolenaar GF, Laing E, Swiatek-Polatynska MA, et al. OsdR of *Streptomyces coelicolor* and the Dormancy Regulator DevR of Mycobacterium tuberculosis Control Overlapping Regulons. mSystems. 2016;1(3).
7. Alam K, Mazumder A, Sikdar S, Zhao YM, Hao J, Song C, et al. *Streptomyces*: The biofactory of secondary metabolites. Front Microbiol. 2022;13:968053.
8. Bentley SD, Chater KF, Cerdeno-Tarraga AM, Challis GL, Thomson NR, James KD, et al. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature. 2002;417(6885):141-7.
9. Liu Z, Zhao Y, Huang C, Luo Y. Recent Advances in Silent Gene Cluster Activation in *Streptomyces*. Front Bioeng Biotechnol. 2021;9:632230.
10. Quinn GA, Banat AM, Abdelhameed AM, Banat IM. *Streptomyces* from traditional medicine: sources of new innovations in antibiotic discovery. J Med Microbiol. 2020;69(8):1040-8.
11. Hutchings MI, Truman AW, Wilkinson B. Antibiotics: past, present and future. Curr Opin Microbiol. 2019;51:72-80.
12. Lee N, Hwang S, Kim J, Cho S, Palsson B, Cho BK. Mini review: Genome mining approaches for the identification of secondary metabolite biosynthetic gene clusters in *Streptomyces*. Comput Struct Biotechnol J. 2020;18:1548-56.
13. Collaborators GBDAR. Global burden of bacterial antimicrobial resistance 1990-2021: a systematic analysis with forecasts to 2050. Lancet. 2024;404(10459):1199-226.
14. Reygaert WC. An overview of the antimicrobial resistance mechanisms of bacteria. AIMS Microbiol. 2018;4(3):482-501.
15. Davies J. Where have All the Antibiotics Gone? Can J Infect Dis Med Microbiol. 2006;17(5):287-90.
16. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P T. 2015;40(4):277-83.
17. Poudel AN, Zhu S, Cooper N, Little P, Tarrant C, Hickman M, Yao G. The economic burden of antibiotic resistance: A systematic review and meta-analysis. PLoS One. 2023;18(5):e0285170.
18. Donald L, Pipite, A., Subramani, R., Owen, J., Keyzers, R.A. and Taufa, T. *Streptomyces*: Still the Biggest Producer of New Natural Secondary Metabolites, a Current Perspective. Microbiol Res. 2022;13, 418-465.
19. Blin K, Shaw S, Vader L, Szenei J, Reitz ZL, Augustijn HE, et al. antiSMASH 8.0: extended gene cluster detection capabilities and analyses of chemistry, enzymology, and regulation. Nucleic Acids Res. 2025;53(W1):W32-W8.
20. Nikolaidis M, Hesketh A, Frangou N, Mossialos D, Van de Peer Y, Oliver SG, Amoutzias GD. A panoramic view of the genomic landscape of the genus *Streptomyces*. Microb Genom. 2023;9(6).

21. Gust B, Challis GL, Fowler K, Kieser T, Chater KF. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci U S A. 2003;100(4):1541-6.
22. Cobb RE, Wang Y, Zhao H. High-efficiency multiplex genome editing of *Streptomyces* species using an engineered CRISPR/Cas system. ACS Synth Biol. 2015;4(6):723-8.
23. Gust B, Chandra G, Jakimowicz D, Yuqing T, Bruton CJ, Chater KF. Lambda red-mediated genetic manipulation of antibiotic-producing *Streptomyces*. Adv Appl Microbiol. 2004;54:107-28.
24. Zaburannyi N, Rabyk M, Ostash B, Fedorenko V, Luzhetskyy A. Insights into naturally minimised *Streptomyces albus* J1074 genome. BMC Genomics. 2014;15:97.
25. Xu Z, Wang Y, Chater KF, Ou HY, Xu HH, Deng Z, Tao M. Large-Scale Transposition Mutagenesis of *Streptomyces coelicolor* Identifies Hundreds of Genes Influencing Antibiotic Biosynthesis. Appl Environ Microbiol. 2017;83(6).
26. Clarke JE, Faulkner TR, Seipke RF. A platform for CRISPRi-seq in *Streptomyces albidoflavus*. mBio. 2026:e0306525.
27. Fernandez-Garcia G, Valdes-Chiara P, Villazan-Gamonal P, Alonso-Fernandez S, Manteca A. Essential Genes Discovery in Microorganisms by Transposon-Directed Sequencing (Tn-Seq): Experimental Approaches, Major Goals, and Future Perspectives. Int J Mol Sci. 2024;25(20).
28. van Opijnen T, Bodi KL, Camilli A. Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. Nat Methods. 2009;6(10):767-72.
29. Fernandez-Martinez LT, Del Sol R, Evans MC, Fielding S, Herron PR, Chandra G, Dyson PJ. A transposon insertion single-gene knockout library and new ordered cosmid library for the model organism *Streptomyces coelicolor* A3(2). Antonie Van Leeuwenhoek. 2011;99(3):515-22.
30. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell. 2013;152(5):1173-83.
31. Ameruoso A, Villegas Kcam MC, Cohen KP, Chappell J. Activating natural product synthesis using CRISPR interference and activation systems in *Streptomyces*. Nucleic Acids Res. 2022;50(13):7751-60.
32. Marraffini LA, Sontheimer EJ. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. Nat Rev Genet. 2010;11(3):181-90.
33. Makarova KS, Wolf YI, Iranzo J, Shmakov SA, Alkhnbashi OS, Brouns SJJ, et al. Evolutionary classification of CRISPR-Cas systems: a burst of class 2 and derived variants. Nat Rev Microbiol. 2020;18(2):67-83.
34. Shmakov S, Abudayyeh OO, Makarova KS, Wolf YI, Gootenberg JS, Semenova E, et al. Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems. Mol Cell. 2015;60(3):385-97.
35. O'Connell MR. Molecular Mechanisms of RNA Targeting by Cas13-containing Type VI CRISPR-Cas Systems. J Mol Biol. 2019;431(1):66-87.
36. Huynh N, Depner N, Larson R, King-Jones K. A versatile toolkit for CRISPR-Cas13-based RNA manipulation in Drosophila. Genome Biol. 2020;21(1):279.
37. Nunez JK, Lee AS, Engelman A, Doudna JA. Integrase-mediated spacer acquisition during CRISPR-Cas adaptive immunity. Nature. 2015;519(7542):193-8.
38. Perculija V, Lin J, Zhang B, Ouyang S. Functional Features and Current Applications of the RNA-Targeting Type VI CRISPR-Cas Systems. Adv Sci (Weinh). 2021;8(13):2004685.
39. Zhang B, Ye Y, Ye W, Perculija V, Jiang H, Chen Y, et al. Two HEPN domains dictate CRISPR RNA maturation and target cleavage in Cas13d. Nat Commun. 2019;10(1):2544.
40. Gleditzsch D, Pausch P, Muller-Esparza H, Ozcan A, Guo X, Bange G, Randau L. PAM identification by CRISPR-Cas effector complexes: diversified mechanisms and structures. RNA Biol. 2019;16(4):504-17.

41. Kuo HC, Prupes J, Chou CW, Finkelstein IJ. Massively parallel profiling of RNA-targeting CRISPR-Cas13d. Nat Commun. 2024;15(1):498.
42. Cardiff RAL, Faulkner ID, Beall JG, Carothers JM, Zalatan JG. CRISPR-Cas tools for simultaneous transcription & translation control in bacteria. Nucleic Acids Res. 2024;52(9):5406-19.
43. Yan WX, Chong S, Zhang H, Makarova KS, Koonin EV, Cheng DR, Scott DA. Cas13d Is a Compact RNA-Targeting Type VI CRISPR Effector Positively Modulated by a WYL-Domain-Containing Accessory Protein. Mol Cell. 2018;70(2):327-39 e5.
44. Zhang C, Konermann S, Brideau NJ, Lotfy P, Wu X, Novick SJ, et al. Structural Basis for the RNA-Guided Ribonuclease Activity of CRISPR-Cas13d. Cell. 2018;175(1):212-23 e17.
45. Zhao F, Zhang T, Sun X, Zhang X, Chen L, Wang H, et al. A strategy for Cas13 miniaturization based on the structure and AlphaFold. Nat Commun. 2023;14(1):5545.
46. Abramson J, Adler J, Dunger J, Evans R, Green T, Pritzel A, et al. Accurate structure prediction of biomolecular interactions with AlphaFold 3. Nature. 2024;630(8016):493-500.
47. Nam J, Jeong, Y.Y. & Seo, P.J. RfxCas13d Exhibits Collateral RNA Degradation Activity in Plant Systems. J Plant Biol. 2025;68, 291–299.
48. Shi P, Wu X. Programmable RNA targeting with CRISPR-Cas13. RNA Biol. 2024;21(1):1-9.
49. Yu L, Zou J, Hussain A, Jia R, Fan Y, Liu J, et al. Systemic evaluation of various CRISPR/Cas13 orthologs for knockdown of targeted transcripts in plants. Genome Biol. 2024;25(1):307.
50. Konermann S, Lotfy P, Brideau NJ, Oki J, Shokhirev MN, Hsu PD. Transcriptome Engineering with RNA-Targeting Type VI-D CRISPR Effectors. Cell. 2018;173(3):665-76 e14.
51. MacNeil DJ, Gewain KM, Ruby CL, Dezeny G, Gibbons PH, MacNeil T. Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. Gene. 1992;111(1):61-8.
52. Chater KF, Wilde LC. Restriction of a bacteriophage of *Streptomyces albus* G involving endonuclease SalI. J Bacteriol. 1976;128(2):644-50.
53. Radzisheuskaya A, Shlyueva D, Muller I, Helin K. Optimizing sgRNA position markedly improves the efficiency of CRISPR/dCas9-mediated transcriptional repression. Nucleic Acids Res. 2016;44(18):e141.
54. Blin K, Shaw S, Tong Y, Weber T. Designing sgRNAs for CRISPR-BEST base editing applications with CRISPy-web 2.0. Synth Syst Biotechnol. 2020;5(2):99-102.
55. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL. BLAST+: architecture and applications. BMC Bioinformatics. 2009;10:421.
56. Adler BA, Al-Shimary MJ, Patel JR, Armbruster EG, Colognori D, Charles EJ, et al. CRISPRi-ART enables functional genomics of diverse bacteriophages using RNA-binding dCas13d. Nat Microbiol. 2025;10(3):694-709.
57. Seipke RF. Strain-level diversity of secondary metabolism in *Streptomyces albus*. PLoS One. 2015;10(1):e0116457.
58. Brown KM, Wade JT. Translational coupling of neighboring genes in prokaryotes. J Bacteriol. 2025;207(10):e0025525.
59. Rasmussen LC, Sperling-Petersen HU, Mortensen KK. Hitting bacteria at the heart of the central dogma: sequence-specific inhibition. Microb Cell Fact. 2007;6:24.

6. Appendix

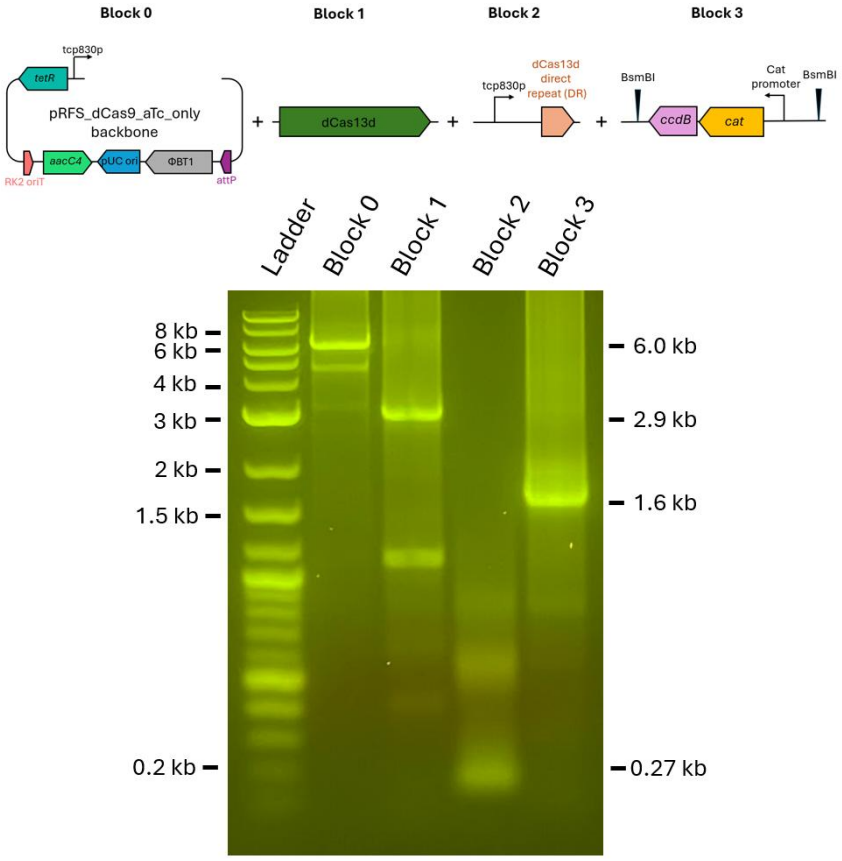


Figure A1. Agarose gel showing amplification of the components used to assemble the pRFSdCas13d plasmid in a Golden Gate reaction, including the pRFSdCas9 backbone (6014 bp), the dCas13d coding gene (2904 bp), the dCas13d direct repeat sequence (265 bp) and the sequence containing the *cat-ccdB* cassette (1598 bp). The gel used the NEB 1 kb plus ladder and a 1% agarose gel.

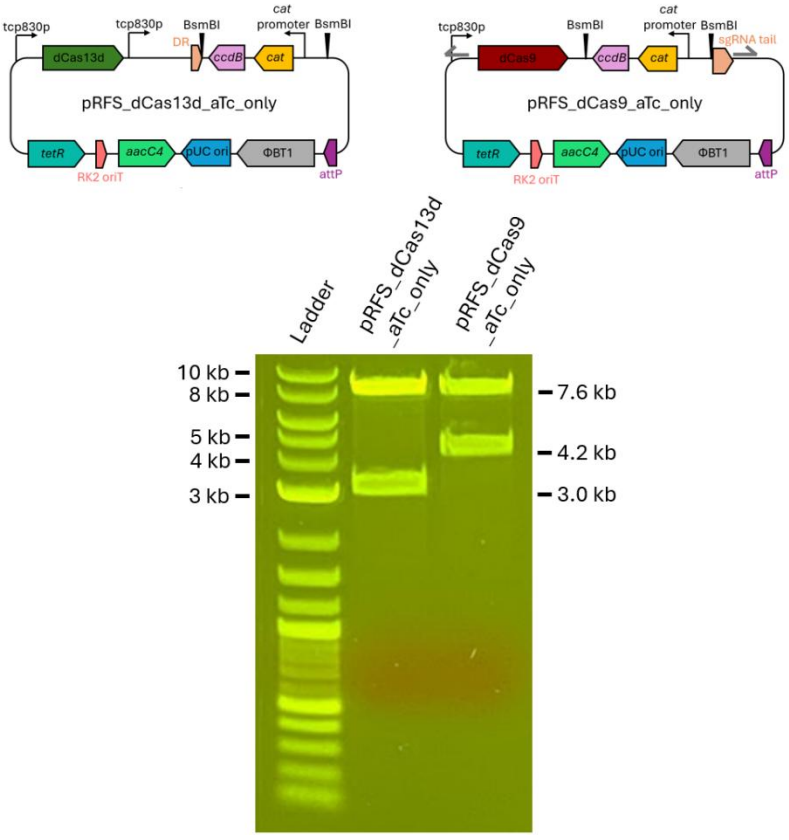


Figure A2. Agarose gel showing the restriction digestion of the pRFSdCas13d (yielding a 7642 bp and 2971 bp band) and pRFSdCas9 (yielding a 7625 and a 4174 bp band) plasmids using KpnI. The gel used the NEB 1 kb plus ladder and a 0.8 % agarose gel.

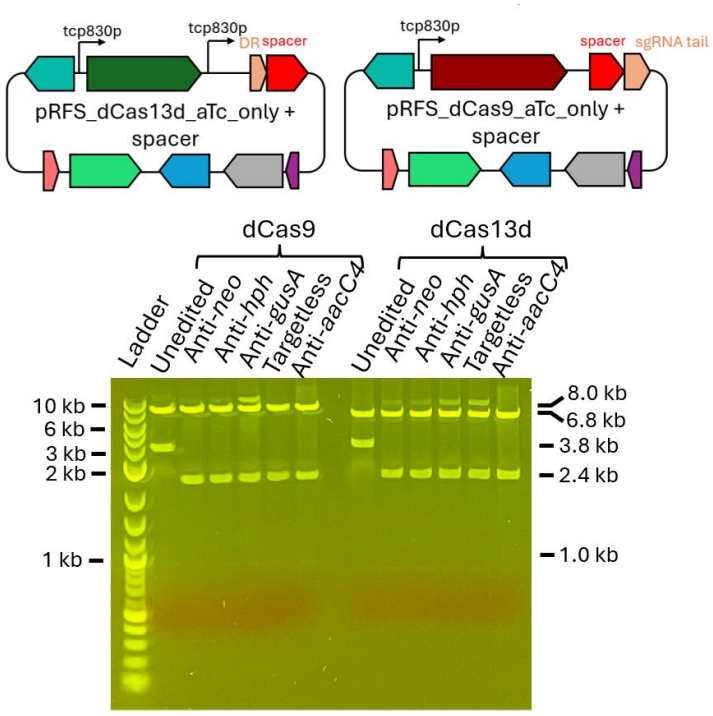


Figure A3. Agarose gel showing amplifications of the sgRNA-containing plasmids using the specific spacer as a primer binding site to confirm the correct spacer is present for each strain. dCas9 strains show a band of 4729 bp and dCas13d strains show a band of 4333 bp. The gel used the NEB 1 kb plus ladder and a 0.8 % agarose gel.

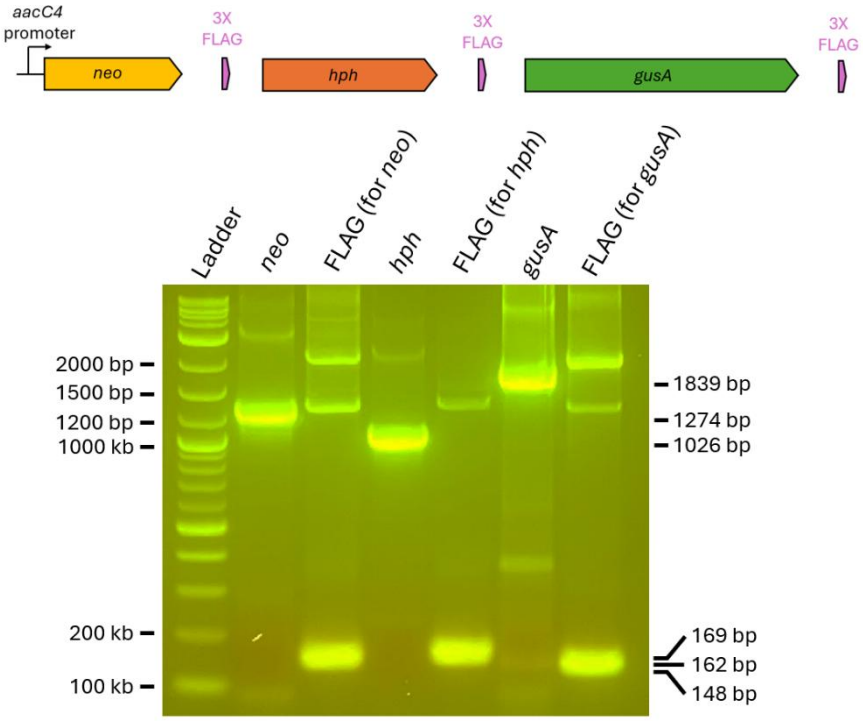


Figure A4. Agarose gel showing the amplification of *neo* (1274 bp), *hph* (1026 bp) and *gusA* (1839 bp) gblocks and the specific 3xFLAG sequences that will be ligated to the *neo* (162 bp), *hph*(169 bp) and *gusA* gene (148 bp). The gel used the NEB 1 kb plus ladder and a 2% agarose gel.

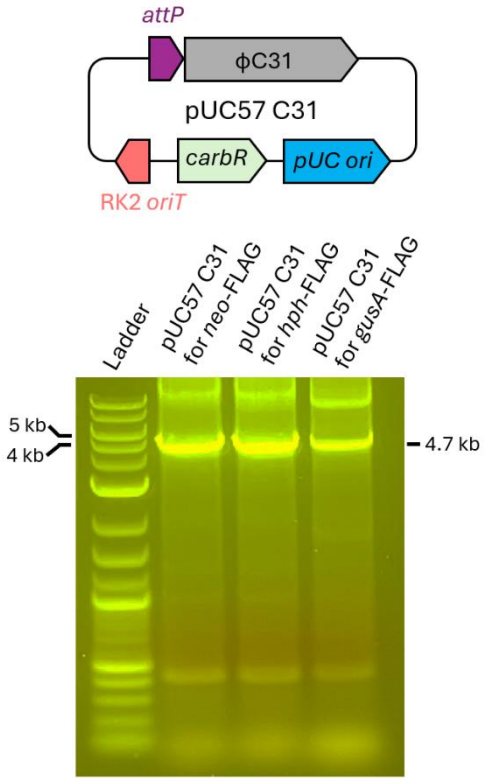


Figure A6. Agarose gel showing the amplification of the pUC57-C31 backbone. All of which are 4.7 kb long and the only difference is a 4 nt overhang encoded on each primer for Golden Gate assembly with the *neo* + FLAG, *hph* + FLAG or *gusA* + FLAG amplicons. The gel used the NEB 1 kb plus ladder and a 0.8% agarose gel.

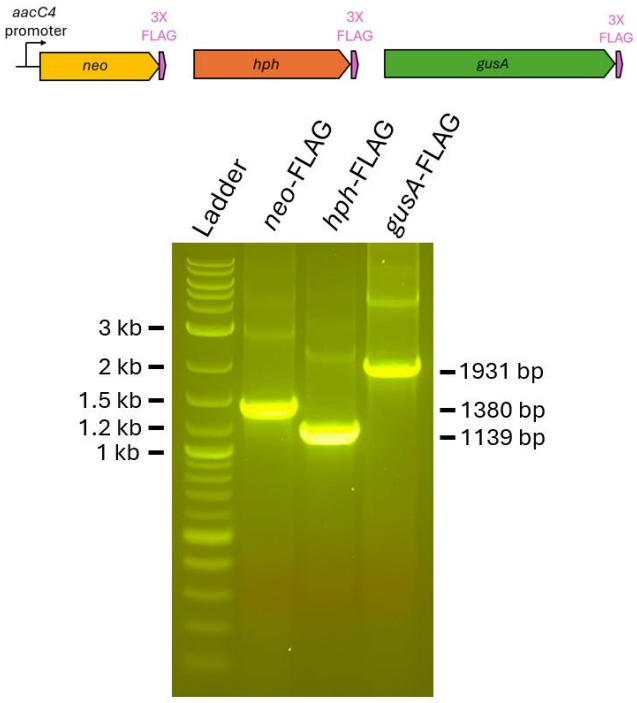


Figure A5. Agarose gel showing the amplification of *neo*-3xFLAG (1380 bp), *hph*-3xFLAG (1139 bp) and *gusA*-3xFLAG (1931 bp) from their respective placeholder pUC57-C31 plasmid. The gel used the NEB 1 kb plus ladder and a 1.5% agarose gel.

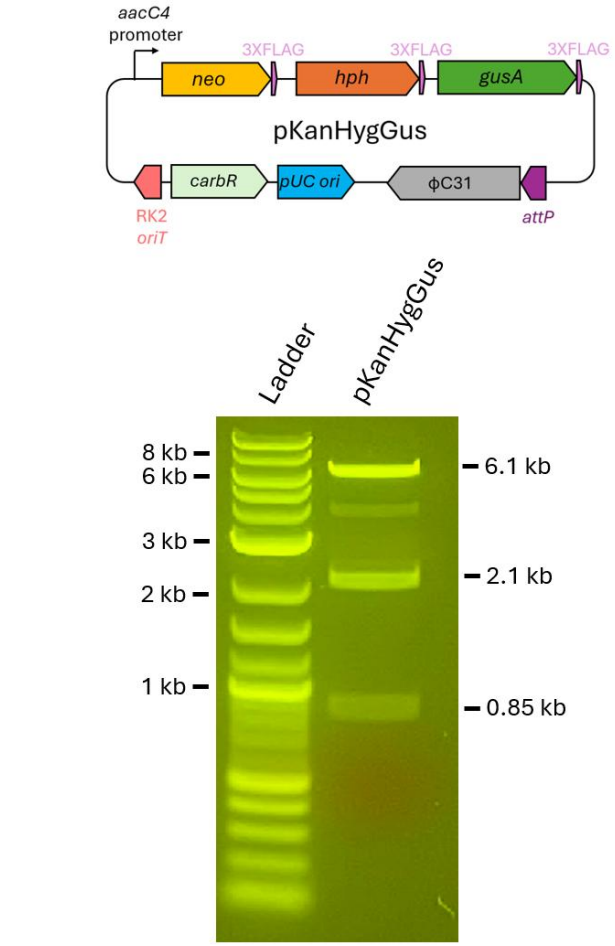


Figure A7. Agarose gel showing a restriction digest of the Golden Gate assembled pKanHygGus plasmid with the three reporter genes all tagged with a 3xFLAG and under expression of the *aacC4* promoter. The digest with NdeI enzyme yielded a 6130 bp, a 2052 bp and an 879 bp band. The gel used the NEB 1 kb plus ladder and a 1% agarose gel.

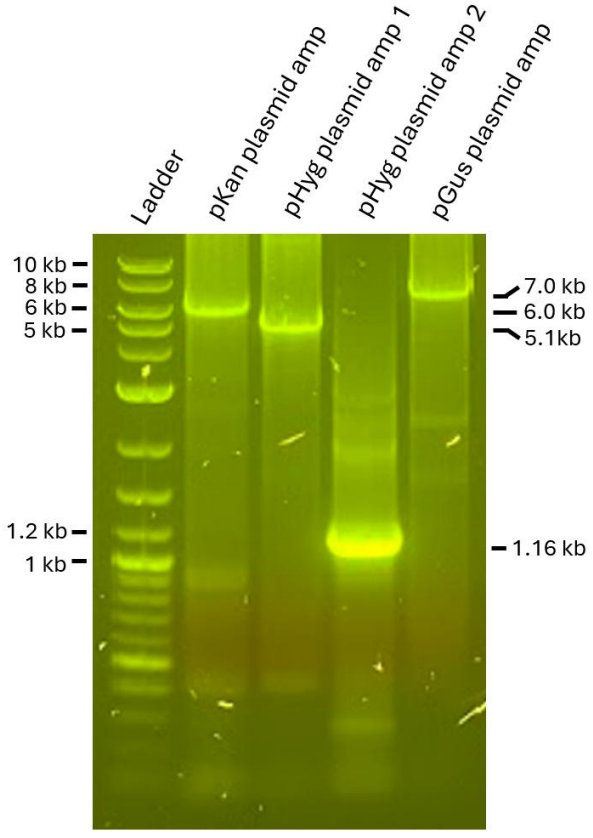


Figure A8. Agarose gel showing amplifications of the KHG plasmid to produce the monocistronic reporter plasmids. The pKan plasmid could be amplified using only one set of primers (6013 bp), as could the pGus plasmid (7035 bp) but for the pHyg plasmid, amp 1 contains the backbone (5087 bp) and amp 2 contains the *hph-3xFLAG* region (1157 bp) which were ligated together. The gel used the NEB 1 kb plus ladder and a 0.8% agarose gel.

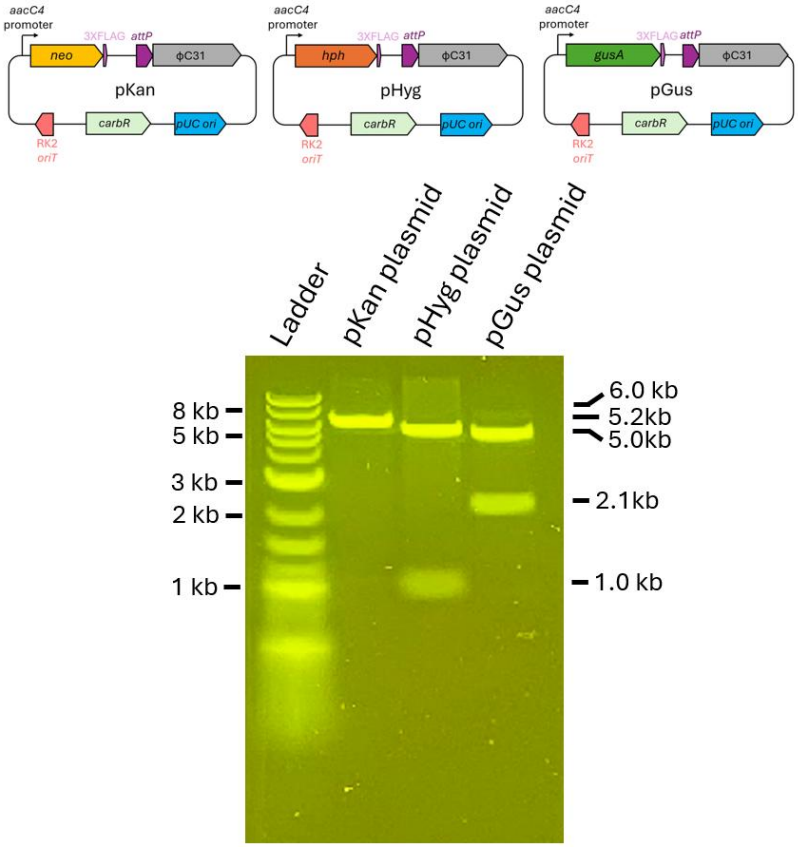


Figure A9. Agarose gel showing an NdeI restriction digest of the pKan, pHyg and pGus monocistronic reporter plasmids. The digest yielded a 5985 bp band for pKan, a 5207 and 981 bp band for pHyg, and a 4955 bp and 2052 bp band for pGus. The gel used the NEB 1 kb plus ladder and a 1 % agarose gel.

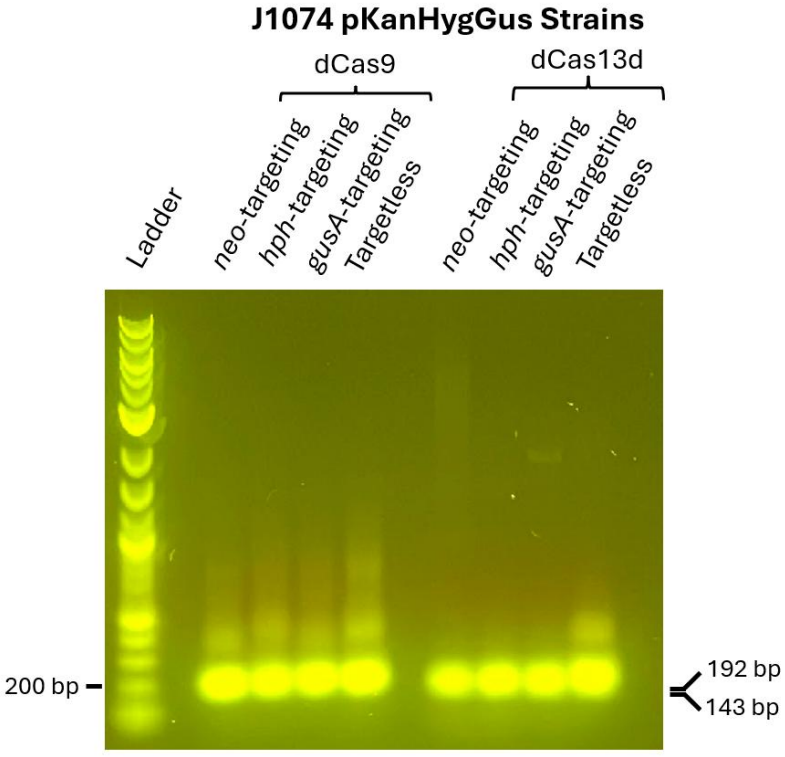


Figure A10. Agarose gel showing an amplification of J1074 pKanHygGus strains each containing a unique sgRNA. The dCas9 amplifications yield a 192 bp band and the dCas13d amplifications yield a 143 bp band. The gel used the NEB 1 kb plus ladder and a 1.5 % agarose gel.

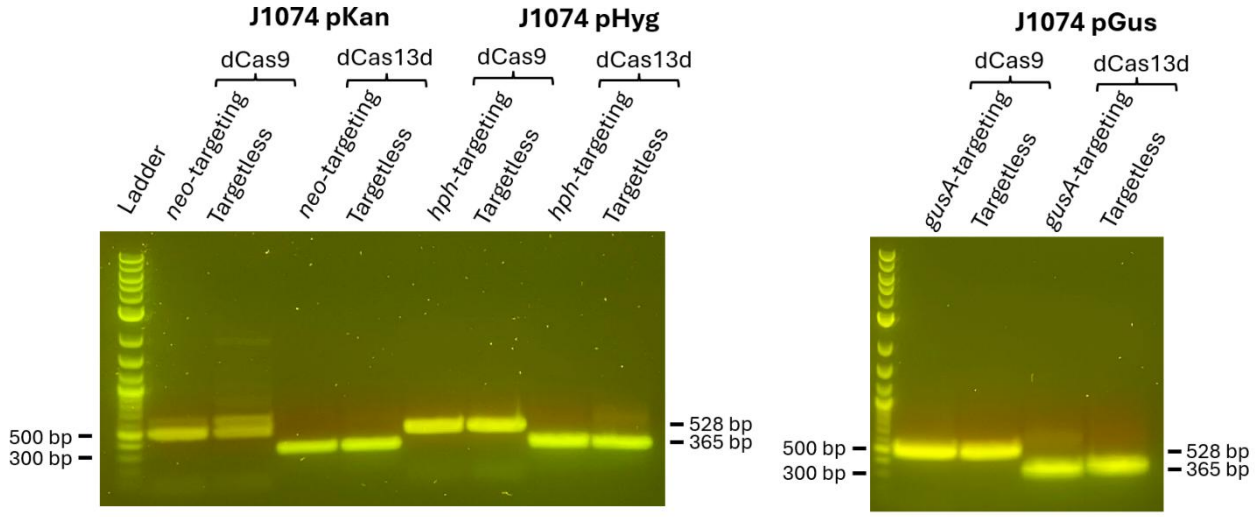


Figure A11. Agarose gels showing an amplification of J1074 pKan, pHyg and pGus strains each containing a unique sgRNA. The dCas9 amplifications yield a 528 bp band and the dCas13d amplifications yield a 365 bp band. Each gel used the NEB 1 kb plus ladder and a 1.5 % agarose gel.

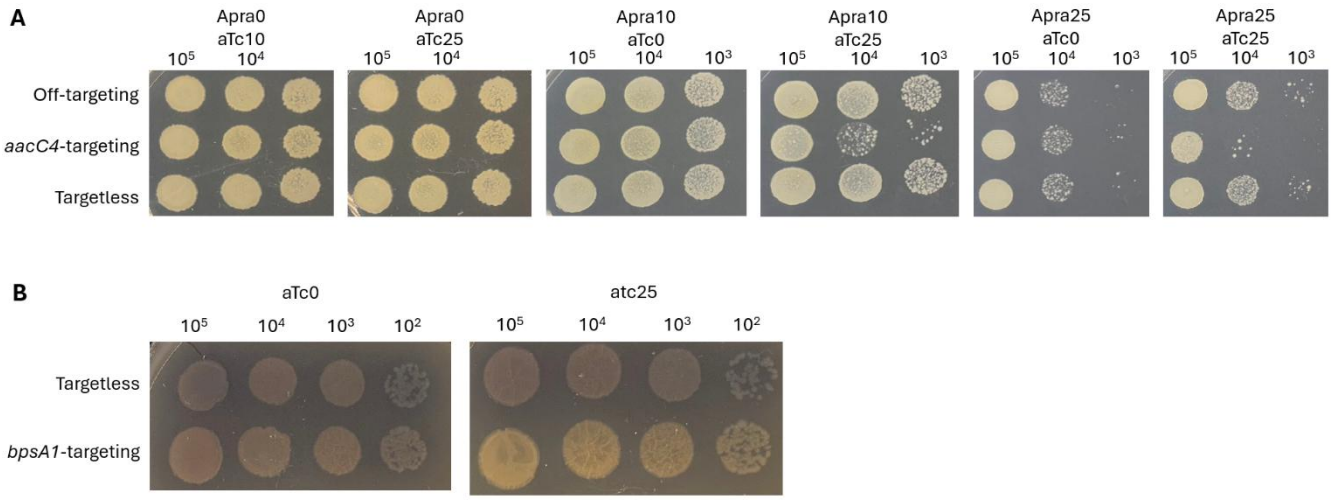


Figure A12. Using strains from (32). Confirmation of CRISPRi silencing in *S. albidoflavus* J1074. **A.** dCas9 silencing of the apramycin resistance gene (*aacC4*) to investigate the ability of dCas9 to affect fitness. The off-target sgRNA targets the ΦBT1 integrase and the targetless sgRNA doesn't hit anywhere in the genome. Performed on glucose minimal media agar. **B.** CRISPRi silencing of the indigoidine synthetase gene (*bpsA1*) on ISP-2 media.

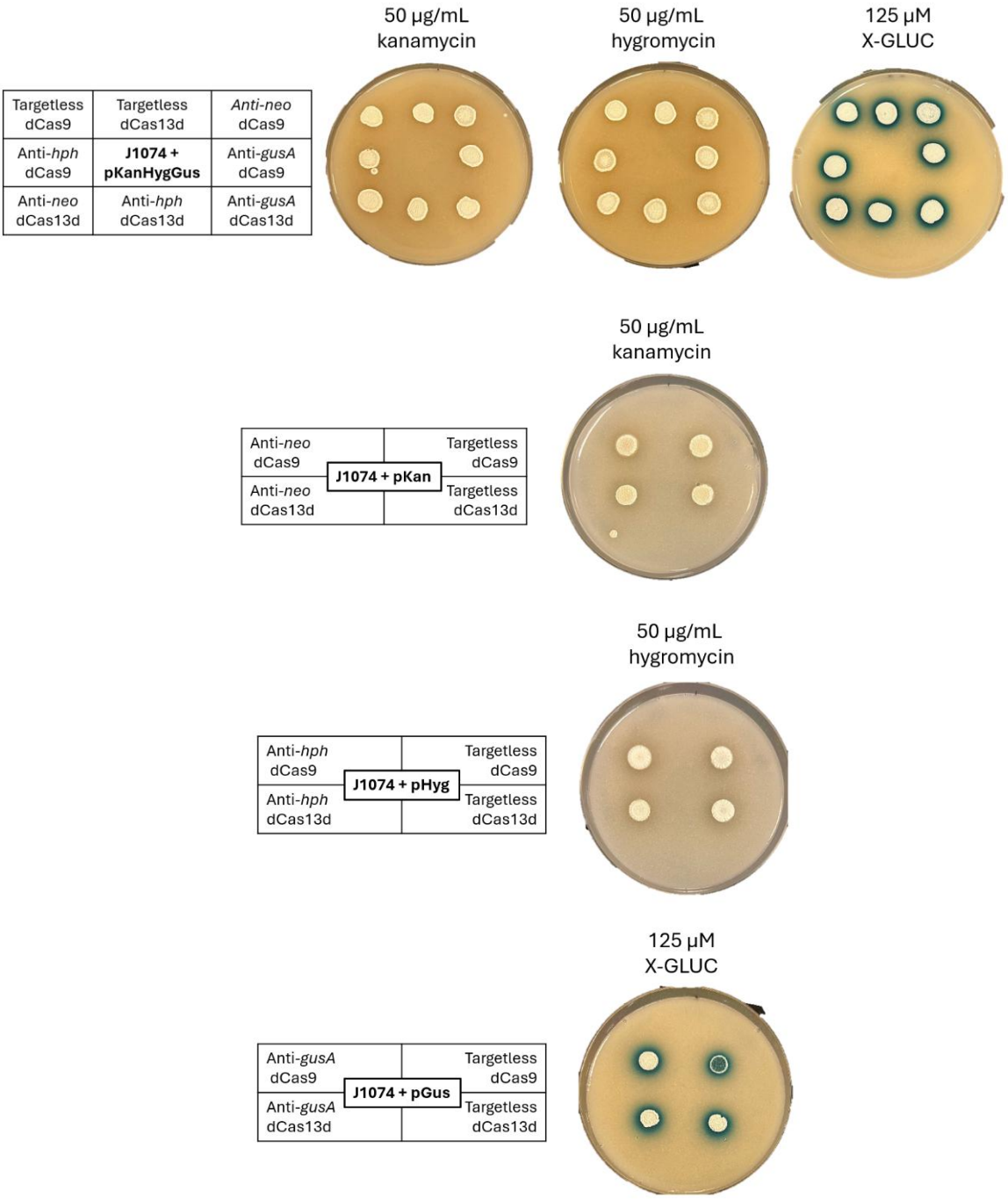


Figure A13. Phenotypic confirmation of every *S. albidoflavus* J1074 strain containing pKanHygGus, pKan, pHyg and pGus and dCas9 or dCas13d plasmid using the relevant selective marker.

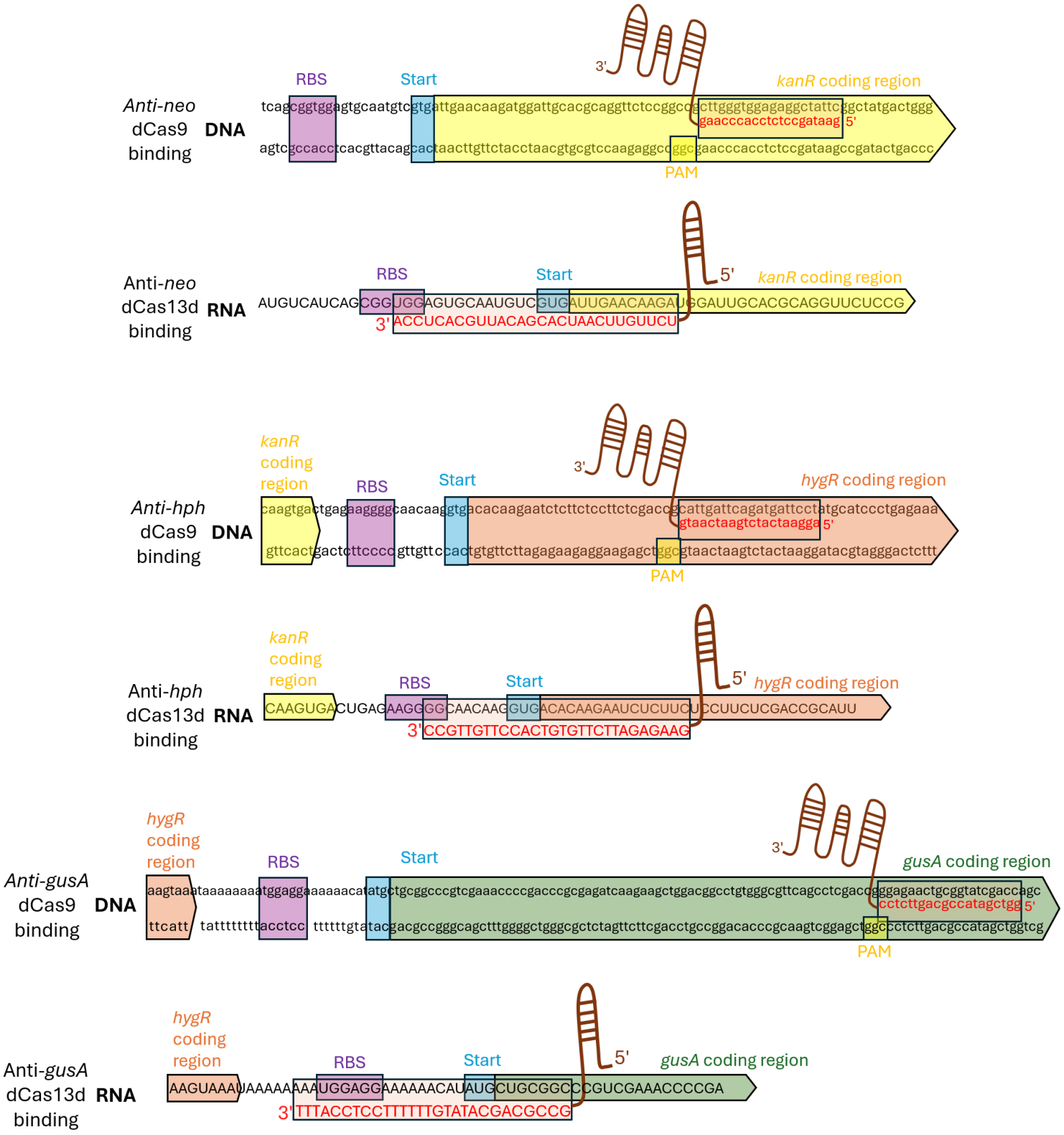


Figure A14. A diagrammatic representation of where the anti-*neo*, anti-*hph* and anti-*gusA* sgRNAs for dCas9 and dCas13d bound on the target gene.

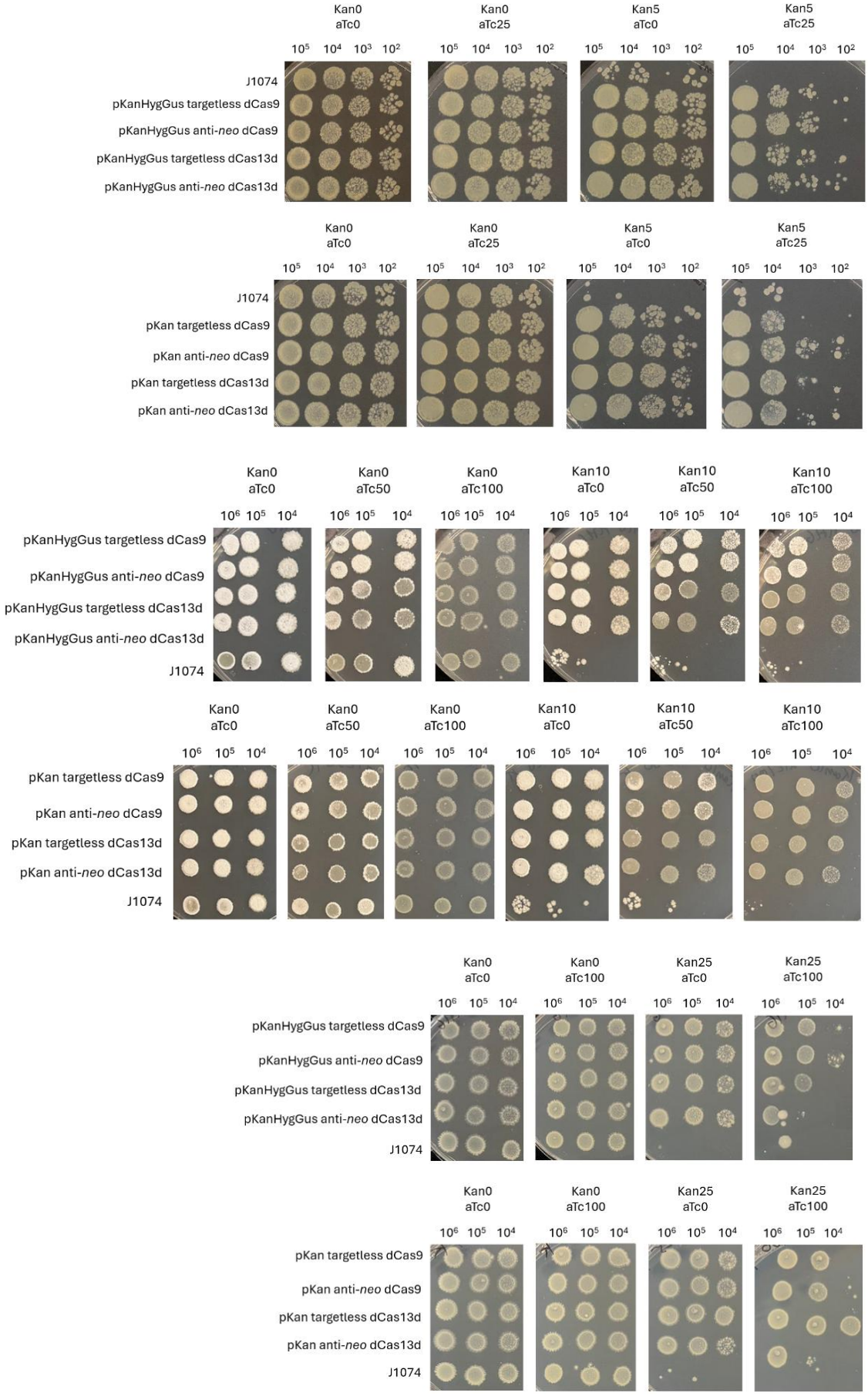


Figure A15. Glucose minimal media assays investigating knockdown of the *neo* gene. Plates vary between hosting 10^6-10^2 spores per dot. *S. albidoflavus* J1074 serves as a negative control.

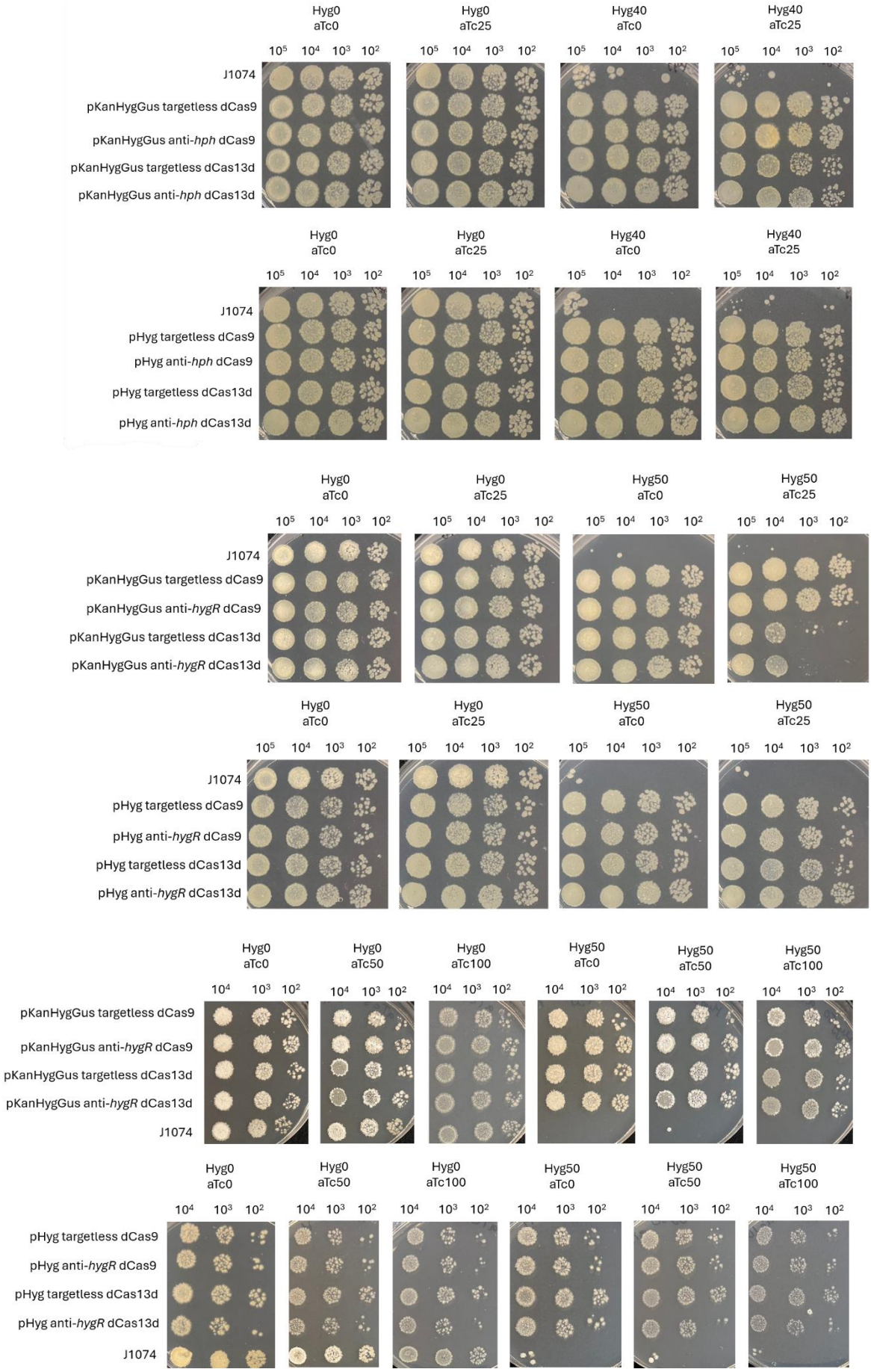


Figure A16. Glucose minimal media assays investigating knockdown of the *hph* gene. Plates vary between hosting 10^5-10^2 spores per dot. *S. albidoflavus* J1074 serves as a negative control.

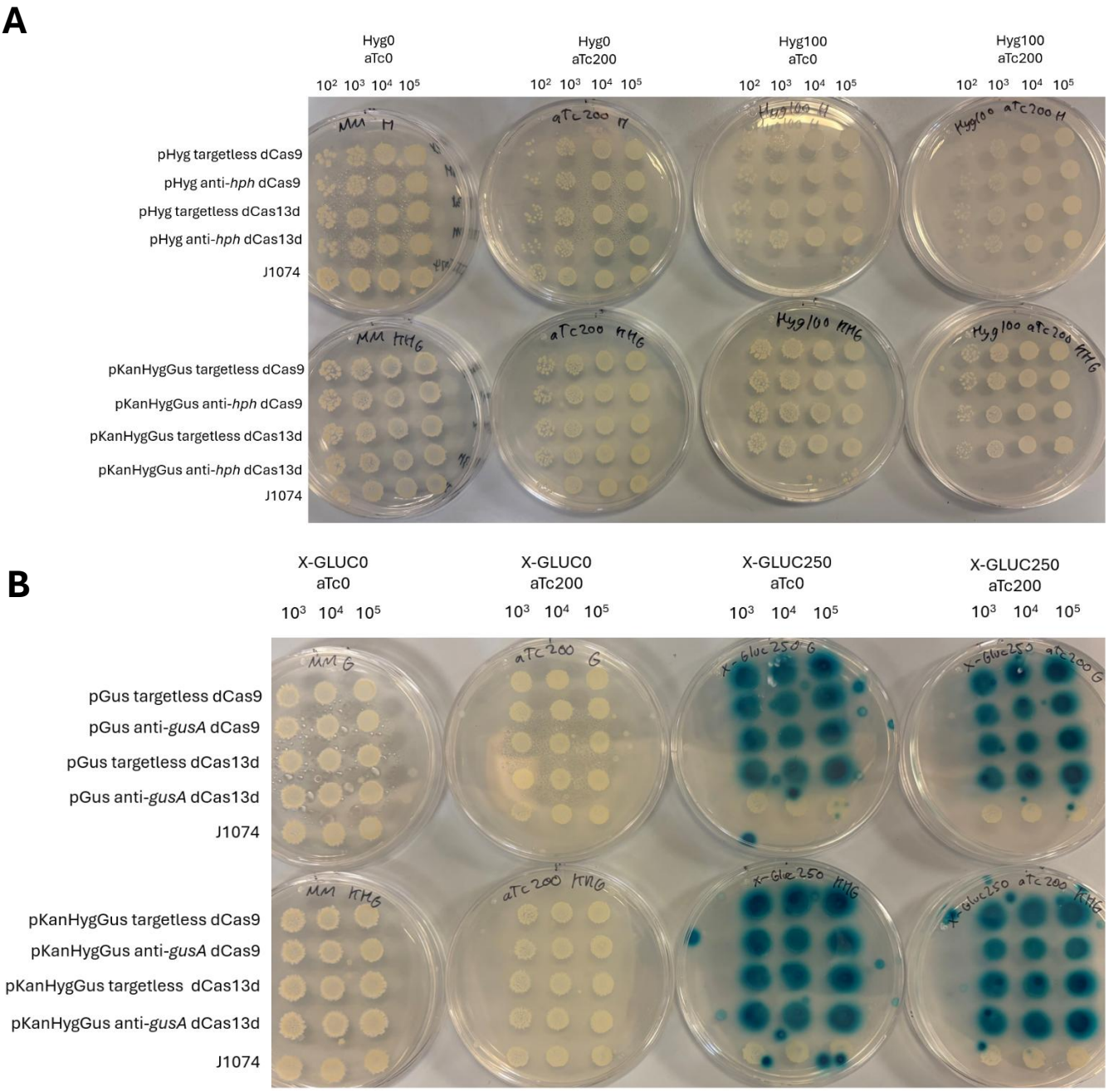


Figure A17. Glucose minimal media assay using 200 ng/mL of aTc inducer.
A. Knockdown of *hph* **B.** Knockdown of *gusA*