Towards a Cipher for Antibiotic Production in *Streptomyces*

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Intellectual Property

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.

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Abstract

Antimicrobial resistance (AMR) is an increasing threat that requires antibiotics of a new class to tackle the current resistance mechanisms. *Streptomyces* bacteria are prolific antibiotic producers, with approximately two thirds of all clinical antibiotics being derived from this genus (Barbuto Ferraiuolo *et al*, 2021). It has been estimated that *Streptomyces* species have the ability to produce 150,000 bioactive compounds beyond those currently known, and these are likely to include novel antibiotics, some of which may be useful in tackling AMR. These secondary metabolites are encoded by biosynthetic gene clusters (BGCs) that are silent (predicted natural products not expressed) or cryptic (unknown natural products). These BGCs are not expressed under standard laboratory conditions, complicating the study of these natural products with the aim of identifying new, clinically-useful antibiotics. One approach, known as OSMAC (One Strain, Many Compounds), to bypass this issue is by identifying growth conditions that may stimulate BGCs to produce secondary metabolites. This work implemented the OSMAC approach supported by efficient reporter platform GUS to interrogate activation of BGC expression in *Streptomyces albidoflavus (albus)* and *Streptomyces venezueale* under different cultivation conditions. This study found that approximately 62% of BGCs in these strains could be activated by the alteration of simple conditions such as the media of cultivation. Using the results of this study and a preceding study of this approach in *Streptomyces coelicolor*, a small number of conditions efficient in activating the bulk of tested BGCs can be identified. Establishing a platform of a few conditions to test under OSMAC could allow the use of this approach as an efficient platform for antibiotic discovery attempts.

Table of Abbreviations

Abbreviation	**Definition**
AMR	Antimicrobial Resistance
BGC	Biosynthetic Gene Cluster
DMSO	Dimethyl Sulfoxide
GUS	β-glucuronidase
HiTES	High-Throughput Elicitor Screens
OSMAC	One Strain, Many Compounds
PCR	Polymerase Chain Reaction
UTR	Untranslated Region
X-Gluc	5-Bromo-4-chloro-3-indolyl-β-D-Glucuronic Acid

Introduction

Antimicrobials are a crucial group of drugs, particularly so in the healthcare setting, used to treat a multitude of diseases caused by microbial infections. However, many antimicrobials are becoming increasingly less effective as resistance develops in target microorganisms. This issue is driven by the selection pressure applied when exposing the microorganisms to these drugs and exacerbated by the overuse of antimicrobials (Hughes, 2014). Resistance to antimicrobials can arise by various mechanisms, some of which confer multidrug resistance, making multiple different drugs obsolete against these microorganisms (Naghavi *et al*, 2024). Antimicrobial resistance (AMR) poses a considerable threat to human life and health as many infectious diseases could become untreatable. In 2019 there were approximately 4.95 million deaths associated with bacterial AMR worldwide (Murray *et al*, 2022) and the death toll per year will likely rise alongside the prevalence of resistance if no antimicrobials of new classes/mechanisms of action are introduced to tackle this issue. If the resistance problem continues on its current trajectory without intervention, it is predicted that globally in 2050 1.91 million deaths will be directly attributable to AMR and a further 8.22 million deaths associated with AMR (Naghavi *et al*, 2024).

The vast majority of clinically-deployed antibacterial drugs come from microorganisms (Barbuto Ferraiuolo *et al*, 2021). Despite this, few such antibiotics have been discovered in the recent decades as many efforts in this area simply lead to the re-discovery of known compounds (‘replication’) (Valiquette and Laupland, 2015). The timeline of antibiotic discovery and emergence of resistance is seen below in Figure 1. The synthesis of natural products (also known as secondary metabolites), such as antibiotics, is directed by clusters of genes (biosynthetic gene clusters [BGCs]) that encode the necessary components such as regulatory genes and core biosynthetic genes. However, many microorganisms’ antimicrobial compounds are yet to be elucidated due to many biosynthetic gene clusters (BGCs) appearing to be cryptic, encoding an unknown compound, or silent, wherein the expected encoded secondary metabolite is not produced (Yang *et al*, 2023). This has been recognised by analysing the genome of the microorganism and comparing the present BGCs to recovered secondary metabolites. Stimulating the expression of these silent or cryptic BGCs could result in the discovery and production of new antimicrobials that are not subject to existing resistance mechanism found in pathogenic bacteria, and which could therefore help to tackle the rising AMR issue (Yang *et al*, 2023).

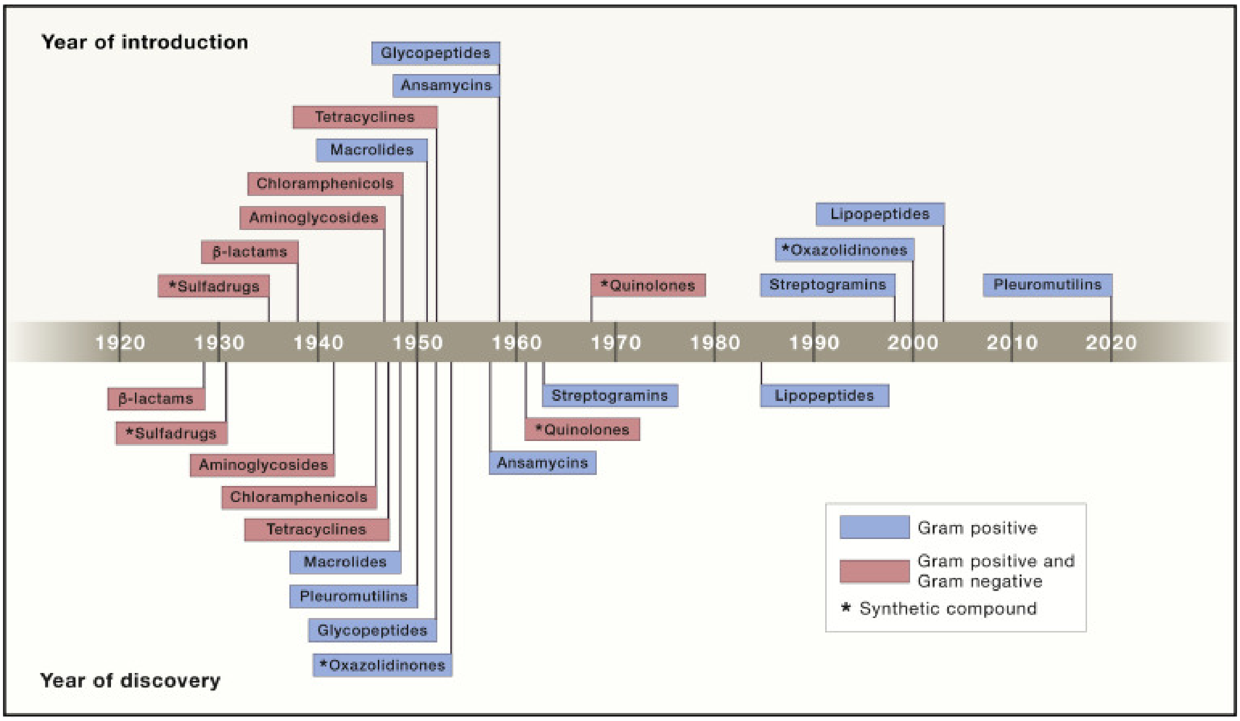


Figure 1. A timeline of antibiotic discovery (Lewis, 2020). The figure shows the year of discovery and clinical introduction for common antibiotics of different classes. Antibiotics with a narrow spectrum of activity (exclusively against Gram positive bacteria) are highlighted in blue, whilst broad spectrum agents are highlighted in pink. Drugs that are synthetic or semi-synthetic are indicated with an asterix.

Early antibiotics were frequently discovered using the Waksman platform, simply cultivating soil microorganisms and screening for antibiotic activity. The use of the Waksman platform eventually exhausted all antibiotics able to be discovered using this simple of a method. This lack of new discoveries led to the implementation of more complex approaches such as the production of synthetic or semi-synthetic antibiotics. However, these approaches did little to fulfil the urgent need for new antibiotics and failed to match the previous rate of introduction of clinical antibiotics (Muteeb *et al*, 2023).

The *Streptomyces* genus of bacteria are talented antibiotic producers, approximately two thirds of all clinically-deployed antibiotics are derived from this genus, including streptomycin, tetracyclines, and erythromycin (Barbuto Ferraiuolo *et al*, 2021). *Streptomyces* species have a complex lifecycle including the stages: germination, vegetative mycelia, aerial mycelia, and sporulation (Lo Grasso *et al*, 2016). This lifecycle is depicted in Figure 2 below. Each stage of the lifecycle has been seen to have a different profile of secondary metabolites as different pathways are activated to be advantageous in this stage of life (Manteca and Yagüe, 2018). It has been noted that more antibiotics are typically produced in the sporulation stage (Čihák *et al*, 2017). Most *Streptomyces* species are unable to progress past the vegetative mycelia phase in liquid culture (Manteca and Yagüe, 2018), affecting their secondary metabolite production.

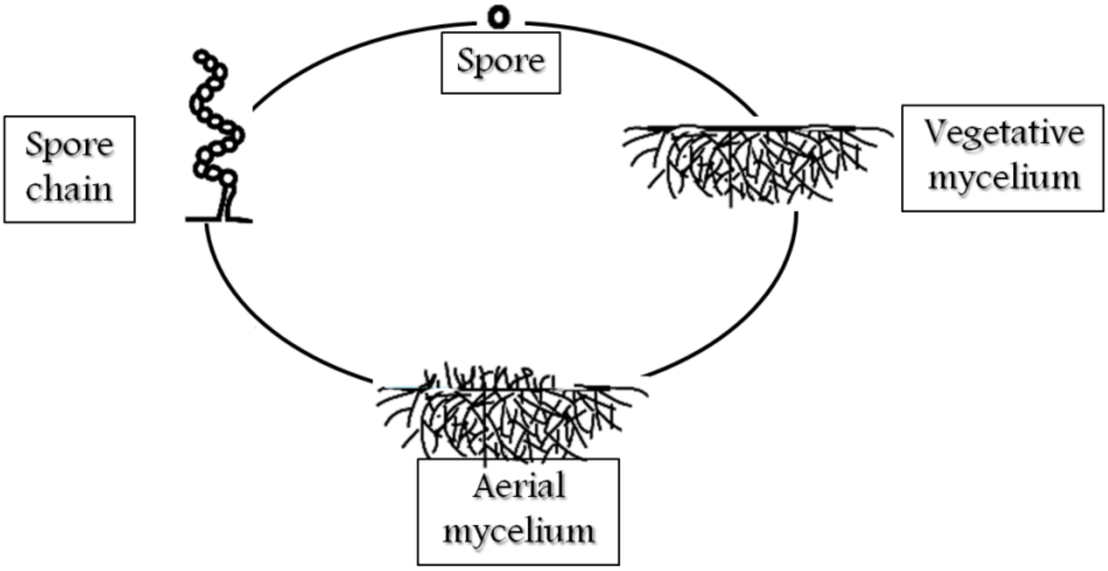


Figure 2. The lifecycle of *Streptomyces* (Lo Grasso *et al*, 2016). On solid media, spores germinate into vegetative mycelium, which grows into aerial mycelium. Next the *Streptomyces* may sporulate, producing spore chains from which individual spores may disperse to begin the lifecycle again (Lo Grasso *et al*, 2016).

Despite their suspected potential, very few novel antibiotics with clinical potential have been discovered from *Streptomyces* since the 1960s. The first whole *Streptomyces* genome (that of a *Streptomyces coelicolor* strain) was released in 2002 (Bentley *et al*, 2002). The publication of the first genome sparked the acknowledgement of cryptic and silent BGCs by making apparent the gap between the size of the genome and its known metabolites (Ziemert *et al*, 2016). This was facilitated by the use of software such as antiSMASH, first released in 2010, to predict a microorganism's BGCs and identify potential silent clusters by comparing the genome to sequences of known BGCs (Blin *et al*, 2016).

These advancements revealed that *Streptomyces* harbours a very large number of silent or cryptic BGCs that are yet to be investigated. It has been estimated that *Streptomyces* has the ability to produce approximately 150,000 more bioactive compounds than all known secondary metabolites from this genus (Lacey and Rutledge, 2022). This indicates that the vast majority of BGCs that produce secondary metabolites remain inactive under standard laboratory conditions, potentially due to these not being the conditions in which the pathways for these secondary metabolites are activated in the host's natural environment. Due to this discrepancy, there may be a large reservoir of antibiotics yet to be discovered (Lacey and Rutledge, 2022).

This study investigated two species of *Streptomyces*: *Streptomyces albidoflavus* (*albus*) and *Streptomyces venezuelae*, both of which harbour well characterised and poorly characterised (including silent/cryptic) BGCs. They are known antibiotic producers, with *S. venezuelae* producing clinically-used antibiotic chloramphenicol (Ehrlich *et al*, 1948) and *S. albus* producing surugamide (Xu *et al*, 2017). These traits and their ease of cultivation in laboratory were the primary reasons that these species were chosen for this study. *S. venezuelae* is one of the few *Streptomyces* species able to sporulate in liquid media (Gomez-Escribano *et al*, 2021). *S. venezuelae* was originally isolated from soil in Venezuela in 1948 (Ehrlich *et al*, 1948). The original isolation of *S. albus* is less clear but is believed to be by Rossi-Doria in 1891 (Pridham and Lyons, 1961). antiSMASH 7.0 predicts *S. venezuelae* and *S. albus* to encode 32 and 23 BGCs, respectively, many of which are poorly characterised.

The expression of a BGC is often controlled by interlacing cascades of cluster-situated regulators and global regulators (van Wezel and McDowall, 2011). Over 12% of *Streptomyces* proteins are considered to have a regulatory function (van Wezel and McDowall, 2011). Understanding and targeting these regulatory factors could facilitate the activation of cryptic or silent BGCs and thus have important applications in the discovery of new antibiotics. A key factor in the expression of a BGC is the promoter region(s) that control the larger biosynthetic genes within its cluster, as the successful activation of the promoter(s) often results in BGC expression, and hence natural product production. Furthermore, the activation of the promoter can reflect the production of its secondary metabolite (van Wezel and McDowall, 2011), an idea utilised in this study to increase the throughput of researching multiple silent BGCs for antibiotic production.

There have been many methods used for investigating the secondary metabolites of silent BGCs of bacteria. Some approaches include heterologous expression, in which a heterologous host is used to express the chosen BGC. Studying a BGC in this manner is primarily an advantage when the original host is difficult to grow/study in laboratory conditions, although this is not an issue for the majority of *Streptomyces* species, as most can be cultivated in the laboratory (Shepherd *et al*, 2010). Heterologous approaches have been seen to have some success in activating silent BGCs. Li *et al* (2023) reported utilising heterologous expression to aid in the discovery of antimicrobial naphthocyclinones from *Streptomyces eurocidicus*. Although this approach is useful at activating secondary metabolite production from silent BGCs, it is not a viable approach for antibiotic discovery due to the amount of work required for the activation of a single BGC, where antibiotic discovery would require the testing of many BGCs to find a novel antibiotic (Kadjo and Eustáquio, 2023). This is especially the case as it is not always straightforward to predict the secondary metabolite encoded by a BGC, as it requires comparing to known databases, and a novel antibiotic may not be able to be predicted if it has little similarity to known clusters. Therefore, it is unlikely that a BGC of a novel antibiotic class would be identified. Consequently, it can be difficult to accurately target a worthwhile BGC, hence the need for the testing of multiple with minimal work (Lee et al, 2020).

Overall, heterologous approaches are largely unsuited to the vast regulatory network of *Streptomyces* species and require too much knowledge and work for a single BGC to be an efficient method of antibiotic discovery (Kadjo and Eustáquio, 2023). These issues can be avoided by mining natural products directly from their native producers, which can involve genetic approaches such as the insertion of a known constitutive or inducible promoter, which has seen success in activating silent BGCs in *Streptomyces* (Mao *et al*, 2019). However, this approach requires that the BGC of interest must be within a single operon (Mao *et al*, 2019). Another method of investigating BGCs within their host is by utilising induction-based approaches, which involve investigating the conditions under which the native host activates the BGC, such as the approach taken by Hemphill *et al* (2017) to discover new fusarielin metabolites from *Fusarium tricinctum*. One of the most simple induction-based approach of attempting to activate biosynthetic gene clusters in the native host is known as the OSMAC (One Strain, MAny Compounds) approach (Bode *et al*, 2002), which is based on the idea that a single strain has the ability to produce many secondary metabolites, some of which may not be expressed in standard cultivation conditions. OSMAC involves testing the microorganism of interest in various conditions in an attempt to trigger the production of different secondary metabolites. OSMAC is advantageous primarily for its simplicity, allowing many BGCs to be investigated with minimal work, which is ideal for antibiotic discovery. Furthermore, OSMAC does not require any information about the genome of the microorganism and can be used to test for activation of multiple BGCs simultaneously. Another advantage is that OSMAC is highly customisable and can incorporate various offshoots of approaches (such as including a reporter system) depending on the microorganism, target BGC(s), or desired reporter system. (Pan *et al*, 2019).

The OSMAC approach involves different strategies of stressing the microorganism, such as changing medium composition (including liquid vs solid media) or co-cultivation with other strain(s). Standard laboratory cultivation of a microorganism typically focuses on mimicking the microorganism’s natural environment and optimising growth, including by providing plenty of nutrients/precursors (Zhang *et al*, 2017). However, many BGCs are simply not expressed in order to prevent energy expenditure in environments where it is not advantageous to have the BGCs activated (Tomm *et al*, 2019). Therefore, putting the microorganism under stress such as nutrient deprivation can result in the activation of different metabolic pathways as a stress response, and potentially result in the production of secondary metabolites (Machushynets *et al*, 2019). Altering the pH and temperature of a culture can alter a microorganism's metabolic profile as it impacts enzyme activity, as enzymes are far less active and can even denature in conditions that are not the optimum temperature/pH. The cultivation pH also impacts the membrane permeability, hence affecting the uptake of substrates into the cell (Pan *et al*, 2019). Also, high temperatures can result in the upregulation of the transcription of stress response factors (Bursy *et al*, 2008). The physical conditions of the culture may influence the secondary metabolites that are produced. For example, agitation of a liquid culture may increase the availability of nutrients and oxygen throughout the culture, enhancing the growth of the microorganism, as well as subjecting the culture to mechanical stress (Ding *et al*, 2017). The OSMAC approach has already seen some success: in the seminal study outlining the OSMAC principle, Bode *et al* (2002) discovered 10 extra metabolites produced by *A. ochraceus* by following the OSMAC approach, altering salinity, temperature and vessel types (Romano *et al*, 2018).

OSMAC can also factor in additional approaches rather than the simpler culture conditions (temperature or media composition, for example) such as scaffolds, which have been successful in activating BGCs, likely by facilitating biofilm formation or disrupting the clumping of fungal cells (Tomm *et al*, 2019). Furthermore, co-culture as an extension of OSMAC can be an effective method for activating BGCs, as the interactions between microorganisms play a crucial role in their natural environment, which would most likely comprise a dynamic network of interactions. These interactions can influence the metabolic pathways involved in increasing the microorganism's fitness, which could include upregulating or entirely activating a BGC. Co-culture can trigger the activation of the BGC through causing nutrient changes, producing signalling molecules, antibiosis (such as by using cytotoxic molecules), or direct physical contact (Romano *et al*, 2018). This approach has already shown some success, such as the discovery of two novel butryolactones due to the co-cultivation of the fungal strain *A. terreus* with *B. cereus* and *B. subtilis* (Chen *et al*., 2015). Co-culture can allow many different interactions to be screened simultaneously, although this requires further investigation to determine the specific cause(s) of any BGC activation. In addition to this, the results of co-culture can be difficult to replicate due to varying levels of microbial growth in each screening (Covington *et al*, 2021). BGC activation may occur due to co-culture as an adaptation to increase competitiveness in the presence of compounds that indicate the presence of potential hosts to colonise or potential competitors for resources (Romano *et al*, 2018).

Much of co-culture's success may be due to the diffusible molecules produced by the other present microorganisms. Another method of BGC activation focuses solely on the molecules that may activate the production of a secondary metabolite. Elicitors are molecules that affect the microorganism's metabolic processes, such as antibiotics or enzyme inhibitors. Elicitors can include diverse range of signalling molecules that can act in response to many regulators that indirectly control natural product production (Xu *et al*, 2017). As there are a vast number of elicitors that could activate a BGC, utilising this approach for BGC activation can require up to thousands of chemicals for screening, which may be greatly laborious or even unfeasible. To streamline this approach, High-Throughput Elicitor Screens (HiTESs) was created. This is an approach using elicitors to activate silent biosynthetic pathways that typically uses a reporter gene. HiTES involves subjecting small amounts of the culture to a diverse library of elicitors, and then analysing the results by mass spectrometry, or different assays depending on the reporter used (Xu *et al*, 2017). The use of elicitors and other key conditions that can be changed with the goal of activating silent/cryptic BGCs can be seen in Figure 3 below.

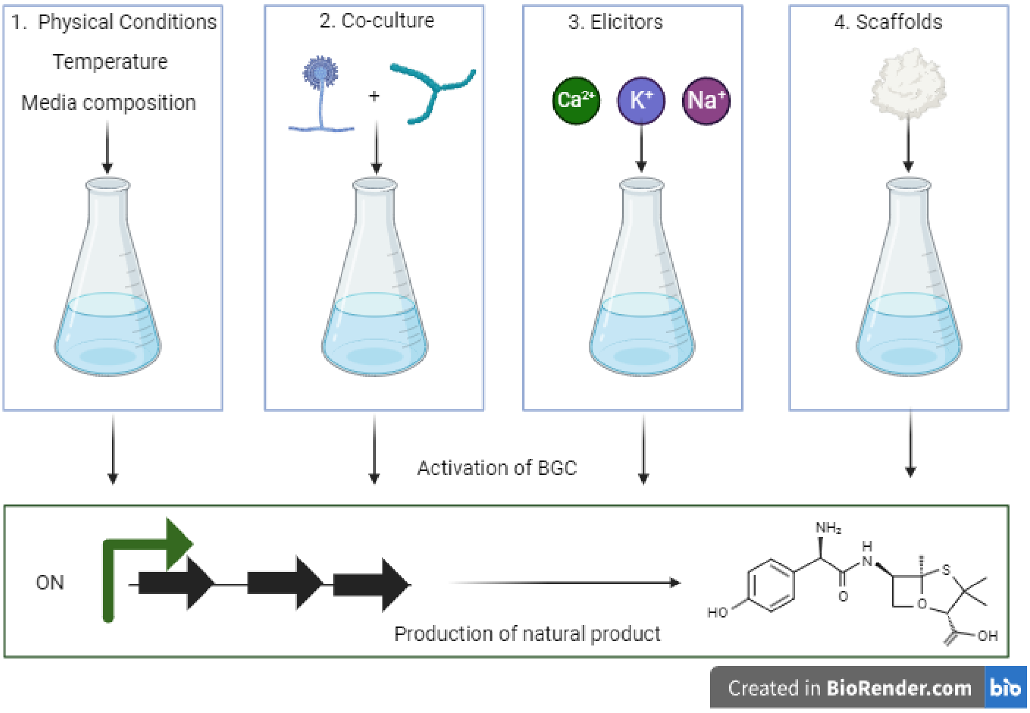


Figure 3. Conditions that may be altered within the OSMAC approach. Four types of conditions that can be altered in an attempt to trigger BGC activation and the corresponding secondary metabolite production. These conditions are simple cultivation conditions (such as temperature and media composition), co-culture with other microorganisms, the addition of elicitors (such as antibiotics), and the introduction of scaffolds. Created in BioRender.

The major limitation of the OSMAC approach is its empirical nature of randomly altering cultivation conditions, of which there are an unlimited number of changes and combinations that can be made (Pan *et al*, 2019). This is the major reason that this approach has not been viable or efficient for antibiotic discovery. Elucidating the conditions with the highest success rate would make the OSMAC method much more useful. Furthermore, traditional OSMAC lacks a link between the expression of a BGC to the secondary metabolite produced, leaving the BGC itself poorly characterised and providing little information about the class of BGC activated by certain conditions, and so being unable to inform future approaches (Pan *et al*, 2019). To link antibiotic production to its gene cluster and identify BGC activation, the GUS reporter system can be implemented. The GUS approach involves fusing the promoter of a BGC of interest to a gene (*gusA*) encoding the enzyme β-glucuronidase (GUS), which produces a blue pigment (if cultivated in/on media containing a suitable substrate for this enzyme) when activated, allowing easy and simple recognition of the activation of the BGC promoter (Myronovskyi *et al*, 2011). The active GUS enzyme does not require any cofactors for activity and tolerates a large variety of culture conditions, making it especially suitable for OSMAC. Furthermore, it can be used for different screening formats by using different substrates, such as 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) for spectrophotometric screening, and 4-methylumbelliferyl-β-d-glucuronide (MUG) for fluorometric screening, which allows the gene expression to be quantified. In addition to this, a positive GUS signal is visible to the naked eye and so does not require any special equipment to identify activation. This reporter system is especially suited to *Streptomyces* as most species of the genus do not naturally encode this enzyme (Myronovskyi *et al*, 2011).

In a recent study undertaken by PhD student Diaaeldin Elimam *et al* within the same laboratory as this study, the GUS system was utilised in the OSMAC testing of *Streptomyces coelicolor*, where it was found to be efficient and reliable. However, its use in this species identified a flaw of the GUS reporter system, as wildtype *S. coelicolor* produces the blue pigment actinorhodin, making it unsuitable for GUS assays as GUS activation would not be as easily identified. Due to this, a mutant strain without blue pigment had to be utilised but showed a slightly different metabolic repertoire to the host, indicating that using mutant strains to tackle any blue pigment production may produce results that are not entirely reflective of the wildtype species. This is a downfall of this reporter system, as many bacterial species may encode a blue pigment, meaning a deletion strain, which may differ in GUS activation to the wildtype, or the additional step of neutralising the natural pigment would be required for GUS testing. The workflow of utilising this reporter system to inform BGC activation in the OSMAC approach can be seen below in Figure 4.

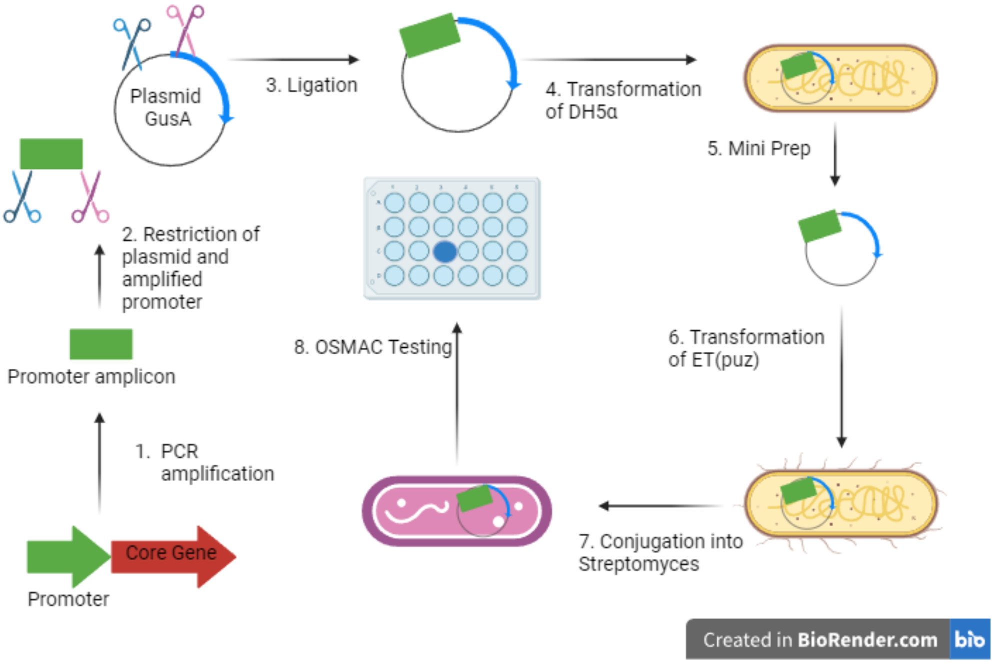


Figure 4. Workflow of the OSMAC approach in *Streptomyces* utilising the GUS reporter system. The full workflow of introducing a promoter-GUS fusion to the original *Streptomyces* strain to create a reporter strain for the OSMAC approach. The promoter of interest is chosen by using antiSMASH 7.0 software to select a BGC predicted to potentially produce an antibiotic and then selecting a promoter utilising BPROM (Solovyev and Salamov, 2011), antiSMASH, and any existing studies that have previously identified a promoter for this gene cluster. The chosen promoter is amplified by PCR. This amplicon and the vector plasmid are restricted with the same enzymes and ligated together, before being introduced into DH5α. The plasmid is then extracted from the DH5α cells to produce a high yield of stable promoter-GUS fusion vector. This is then used to transform ET12567 (pUZ8002), a conjugation donor which introduces the integrative plasmid into *Streptomyces* to create a reporter strain for the promoter of interest. Created in BioRender.

Despite its success, this specific approach of utilising the OSMAC method with a reporter could be considered to have the limitation of only allowing one BGC to be investigated per strain but allows the BGC to immediately be linked to the natural product upon signal, and once engineered, the multiple strains could easily undergo the condition testing simultaneously. The use of the reporter system also prevents the expenditure of additional time and resources on secondary metabolite extraction when the BGC remains inactive, as the lack of activation can be readily identified. Despite the fact that some genetic manipulation is required, the manipulation involved is relatively simple and well defined, and much easier and more effective than many other genetic methods of investigating BGCs and linking them to their natural product (Myronovskyi *et al*, 2011). Furthermore, this method does not require a great deal of annotation of the genome. A sequence of the microorganism allows the use of prediction software such as antiSMASH, NCBI BLAST, and BPROM, which can identify likely potential antimicrobial-producing BGCs and the promoter(s) of these biosynthetic genes, meaning that lesser characterised species could also be investigated with this approach.

The framework of utilising the GUS reporter system alongside OSMAC in investigating *Streptomyces* BGCs has been extensively researched in *S. coelicolor* by Elimam *et al* (personal communication). The utilisation of growth under two different temperatures: 28°C (normal growth temperature for *Streptomyces*) and 37°C combined with growth on 11 different types of agars was found to be successful in the activation of 80% of BGCs, indicating that it may be a simple, accessible, and reliable framework for elucidating the natural products encoded by cryptic BGCs (Elimam, personal communication).

If this framework is seen to be successful across the genus, it could substantially facilitate the search for new antibiotics and the elucidation of cryptic BGCs. To assess the success of the framework as an efficient approach to antibiotic discovery in the *Streptomyces* genus at large, the OSMAC framework was used in *Streptomyces albidoflavus* (*albus*) and *Streptomyces venezuelae*, species picked due to being phylogenetically distant from *S. coelicolor* and each other, hence allowing the study to best represent the extent of the genus. These are species with a number of both well characterised and poorly characterised (including silent/cryptic) BGCs. They are known antibiotic producers, with *S. venezuelae* producing clinically-used antibiotic chloramphenicol (Ehrlich *et al*, 1948) and *S. albus* producing surugamide (Xu *et al*, 2017). The *Streptomyces* species in this study do not naturally produce a blue pigment, and so no additional work is needed to utilise the GUS reporter system. Many of their silent BGCs are predicted by antiSMASH to have the potential to produce antibiotics of multiple different classes and so they have great potential to encode novel natural products. The aim of this study was thus to test the success of OSMAC method in activating silent BGCs in *S. albus* and *S. venezuelae*, and analysing if this activation profile matches the results seen in *S. coelicolor*. Combined, these results could hopefully reveal the minimum number of conditions that can activate the bulk of BGCs, creating a framework of conditions to inform future studies and making antibiotic discovery efforts as efficient as possible.

Materials and Methods

Bacterial Strains and Plasmids

Strains used for plasmid propagation, conjugation, and assembly of GUS reporter platform, and the vectors employed in this study are listed in Table 1 and 2. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth or on Luria-Bertani agar (LBA), with liquid cultures subject to vigorous aeration at 180 rpm in an Eppendorf New Brunswick Innova 43 Incubator Shaker. *Streptomyces* strains were grown at 28°C on Soya Flour Mannitol (SFM) agar, also known as Mannitol Soya (MS) agar, unless stated otherwise.

Table 1: *E. coli* and *Streptomyces* strains used in this study.

Strain	**Description/Application**	**Source**
***Escherichia coli* strains:**		
DH5α (Jung *et al*, 2010)	*fhuA2 lac(del)U169 phoA glnV44 Φ80, lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17.* Used for plasmids propagation prior to transformation into conjugal strains. Chemically competent.	Life Technologies
ET12567 (pUZ8002) (Larcombe *et al*, 2024)	*Methylation-deficient strain (dam-dcm-hsdM-),* carrying the non-transmissible RK2-derivative (pUZ8002). Used for conjugation. Chemically competent and resistant to kanamycin and chloramphenicol.	Life Science Market
BW25113 (Grenier *et al*, 2014)	Wildtype E. coli strain, used to test for production of chloramphenicol due to its antibiotic sensitivity.	Keio collection
Streptomyces strains:		
Streptomyces albidoflavus (albus) J1074 (Parent strain) (Chater and Wilde, 1980)	A mutant of the *S. albus* G strain. Lacks an active SalI restriction modification system. Used for generation of GUS reporter strains specific to chosen BGCs	Seipke Laboratory
S. venezuelae ATCC 10712 (Parent strain) (Ehrlich *et al*, 1948)	Descendent of the Caracas/Yale/Burkholder strain. Carries a 158-kb linear plasmid. Used for generation of GUS reporter strains specific to chosen BGCs	Seipke Laboratory

Table 2: Vectors and plasmids used in this study.

Plasmid	Description/Application	Source
pGUSHL4aadA (Myronovskyi *et al*, 2011)	pTESa marker-free integrative vector containing promoter-less *gusA*, helical linker HL4, poly-linker for cloning genes of interest, and spectinomycin resistance cassette *aadA*. This plasmid was used to express *gusA* from the promoter region of interest, and to integrate this construct into the host chromosome. This plasmid uses the ΦC31 integrase system to integrate into the *attB* site of the *Streptomyces* chromosome.	O'Neill Laboratory
pSETGUSTTG (Myronovskyi *et al*, 2011)	Derivative of pSET152 containing 2.0-kb BamHI fragment of *gusA* cloned into BamHI site, with TTG start codon instead of ATG and under control of *lac* promoter. This plasmid was integrated into each original host species at the chromosome's *attB* site and used as positive controls for GUS expression as *gusA* is constitutively expressed due to this plasmid.	O'Neill Laboratory
pGUSHL4aadA:SurE promoter	Restricted (by XbaI and EcorV) pGUSHL4aadA with the promoter for SurE (Xu *et al*, 2017) from *S. albus* ligated upstream of *gusA* to create a transcriptional/translational fusion to be conjugated into *S. albus* for OSMAC testing.	This work
pGUSHL4aadA:Can promoter	Restricted (by XbaI and EcorV) pGUSHL4aadA with the promoter for Can (Olano *et al*, 2014 () from *S. albus* ligated upstream of *gusA* to create a transcriptional/translational fusion to be conjugated into *S. albus* for OSMAC testing.	This work
pGUSHL4aadA:R19 promoter	Restricted (by XbaI and EcorV) pGUSHL4aadA with the promoter for R19 from *S. albus* ligated upstream of *gusA* to create a transcriptional/translational fusion to be conjugated into *S. albus* for OSMAC testing.	This work
pGUSHL4aadA:7R promoter	Restricted (by XbaI and EcorV) pGUSHL4aadA with the promoter 7R for chloramphenicol (Fernández-Martínez *et al*, 2014) from *S. venezuelae* ligated upstream of *gusA* to create a transcriptional/translational fusion to be conjugated into *S. venezuelae* for OSMAC testing.	This work
pGUSHL4aadA:R6 promoter	Restricted (by XbaI and EcorV) pGUSHL4aadA with the promoter for R6 from *S. venezuelae* ligated upstream of *gusA* to create a transcriptional/translational fusion to be conjugated into *S. venezuelae* for OSMAC testing.	This work
pGUSHL4aadA:R7 promoter	Restricted (by XbaI and EcorV) pGUSHL4aadA with the promoter R7 for chloramphenicol from *S. venezuelae* ligated upstream of	This work

	gusA to create a transcriptional/translational fusion to be conjugated into *S. venezuelae* for OSMAC testing.	
pGUSHL4aadA:R11 promoter	Restricted (by XbaI and EcorV) pGUSHL4aadA with the promoter for R11 from *S. venezuelae* ligated upstream of *gusA* to create a transcriptional/translational fusion to be conjugated into *S. venezuelae* for OSMAC testing.	This work
pGUSHL4aadA:R20 promoter	Restricted (by XbaI and EcorV) pGUSHL4aadA with the promoter for R20 from *S. venezuelae* ligated upstream of *gusA* to create a transcriptional/translational fusion to be conjugated into *S. venezuelae* for OSMAC testing.	This work
pGUSHL4aadA:R32 promoter	Restricted (by XbaI and EcorV) pGUSHL4aadA with the promoter for R32 from *S. venezuelae* ligated upstream of *gusA* to create a transcriptional/translational fusion to be conjugated into *S. venezuelae* for OSMAC testing.	This work

Promoter selection for generation of fusion constructs

The web version of microbial genome-mining tool (antiSMASH 7.0) that utilises known BGCs to predict biosynthetic pathways involved in secondary metabolites production (Blin *et al*, 2023), was used to identify BGCs. All antiSMASH-predicted BGCs were predicted in the detection strictness ‘relaxed’ mode. Twelve BGCs were selected, seven from *S. venezuelae* and five from *S. albus*, but difficulties in the workflow resulted in two of these from *S. albus* and one from *S. venezuelae* being abandoned, to leave eight in total in the investigation. Some of these BGCs were selected due to previous research stating their activation with the aim of replicating this activation as a control for the framework and further investigating the conditions of their activation. Other BGCs chosen were silent but predicted to produce antibiotics or cryptic. These BGCs were selected to test the framework’s success in activating silent BGCs, as this could be utilised for antibiotic discovery.

The promoter regions chosen for some BGCs were based on published data for known promoters as was the case for 7R for chloramphenicol in *S. venezuelae* (Fernández-Martínez *et al*, 2014), and the promoter for surugamide (SurE) in *S. albus* (Xu *et al*, 2017). Other BGCs with no such publications had promoters designed at a potential regulatory gene upstream of the biosynthetic gene, as predicted using a combination of antiSMASH and BPROM software. The promoter fragments were designed to include the first few codons of the target gene to ensure that a GUS signal could be produced when the gene is activated. Another promoter for chloramphenicol, the SV R7 reporter construct was designed this way. This construct includes a *gusA* fusion with a different predicted promoter region (the antiSMASH-labelled transcription binding site before the core biosynthetic genes) to the Fernández-Martínez *et al* (2014) promoter.

Molecular biology

Genomic DNA extraction was carried out using the Monarch® Genomic DNA Purification Kit according to the manufacturer’s protocol (New England Biolabs, 2025). PCR amplification (primers can be found in Table 3 below) was completed using GoTaq® Green Master Mix according to the manufacturer’s instructions (Promega, 2024). PCR DNA fragments were purified using the Monarch® DNA Gel Extraction Kit following the manufacturer's instructions (New England Biolabs, 2024). Double restriction was completed using NEB XbaI and EcoRV restriction enzymes and their combined protocols ((New England Biolabs, 2025). DNA fragments were purified using the Monarch® PCR & DNA Cleanup Kit according to the manufacturer’s instructions (New England Biolabs, 2024). Ligation was completed using NEB Quick Ligase and its protocol (New England Biolabs, 2025). Plasmids were isolated using the QIAprep Spin Miniprep Kit following the manufacturer’s protocol (QIAGEN, 2025).

Table 3: Oligonucleotide primers used in this study. All primers provided by IDT. Primers highlighted in red did not produce successful reporter plasmids and so these regions did not undergo GUS testing. The restriction sites are in lowercase.

Primer	**Sequence (5' to 3')**	**Source**
SA SurE FD	caacttctagaGGGCGCGCTGCTCAACGTGA	**Xu *et al*, 2017**
SA SurE RV	aaaagatatcTGCGTCCCCTGCGCCCAC	**Xu *et al*, 2017**
SA Can FD	caacttctagaAGGGGCTACCACAGTATTGGC	**Olano *et al*, 2014**
SA Can RV	aaaagatatcCGGTTGGGCACCTTGAGCTTC	**Olano *et al*, 2014**
SA R19 FD	caacttctagaGTTGCTGTACCCCGGAGTC	**This work**
SA R19 RV	aaaagatatcGGAGTGGAGTTCCGCGAA	**This work**
SV 7R FD	caacttctagaCCTGATCATGCGCTGGCTTAG	**Fernández-Martínez *et al*, 2014**
SV 7R RV	aaaagatatcAACTCGGTCTCGCTCTCCG	**Fernández-Martínez *et al*, 2014**
SV R6 FD	caacttctagaCCATCCCGCGCATCTGGTA	**This work**
SV R6 RV	aaaagatatcGGCCTTGTCGACGATCCG	**This work**
SV R7 FD	caacttctagaCCAGGAAATGCTCCGCGA	**This work**
SV R7 RV	aaaagatatcGTCGTCCGGGTCGAATGC	**This work**
SV R11 FD	caacttctagaCACCTCGACGAGAAGGTCG	**This work**
SV R11 RV	aaaagatatcGAAGGTGAGGGTCTCGTGCC	**This work**
SV R20 FD	caacttctagaGCTGCTTGCGAAGCTGATC	**This work**
SV R20 RV	aaaagatatcGTCGAATTCGGCGAACAGC	**This work**
SV R32 FD	caacttctagaGAAAAGGCTCCTCGGTACGGA	**This work**
SV R32 RV	aaaagatatcTCGTTCCGGTGACGCCT	**This work**
SA R5 FD	caacttctagaCTCGAAGGTGAGCAACTCCG	**This work**
SA R5 RV	aaaagatatcATCGTGCCCGTAGTGCAC	**This work**
SA R6 FD	caacttctagaGACAGTCCCTTCCGTGACGA	**This work**
SA R6 RV	aaaagatatcGAGGTGACCGGGACAGACG	**This work**
SV R5 FD	caacttctagaAAGGAGATGGTCGAGGACCTC	**This work**
SV R5 RV	aaaagatatcCCACATCTCGTCCGCTTCG	**This work**
SV R8 FD	caacttctagaTGCTCGCCTTCACCAACCC	**This work**
SV R8 RV	aaaagatatcATAACTGTTTCCCGGGGAGAC	**This work**
Plasmid FD	GCGCGAGCAGGGGAATTGATC	**Myronovskyi *et al*, 2011**

Transformation of a plasmid into *E. coli* DH5α was completed using chemically competent cells (provided by Elimam from the O'Neill lab) and the standard protocol. The ligated plasmid was added to *E. coli* DH5α cells at a 1:10 ratio and left to incubate on ice for 30 minutes, before this mixture was put under heat shock for 45 seconds at 42°C. This was followed by incubation on ice for two minutes, and then the addition of 950 µl SOC recovery media (composition in the Appendix) and incubation at 37°C shaking for 1 hour and 30 minutes. The mixture was centrifuged at approximately 1800 x *g* for 10 minutes, 600 µl of the supernatant was discarded and the pellet was suspended in the remaining supernatant. Up to 300 µl of this mixture was spread onto LB agar and incubated overnight at 37°C.

Conjugation into *Streptomyces* was performed following the ActinoBase protocol: “Conjugation using ET12567/pUZ8002” (ActinoBase, 2020).

Integration of GUS Reporters into the Genomes of Streptomyces

For each *gusA*-promoter fusion, an amplicon corresponding to the promoter of the BGC of interest was generated by PCR (oligonucleotide primers included in Table 3 above). The promoter fragments were digested with XbaI and EcoRV and ligated into similarly-digested plasmid pGUSHL4aadA to produce an in-frame transcriptional/translational fusion with a promoter-less *gusA.* These constructs were established in *E. coli* DH5α and their DNA sequence verified by sequencing the plasmid (Azenta Life Sciences, Essex) and aligning it to the desired sequence using Benchling (Benchling, 2025). Following conjugation using *E. coli* donor strain ET12567/pUZ8002 (Gust *et al*, 2003), the reporter constructs integrated into the *Streptomyces* chromosome. To confirm the integration of the plasmid construct into the chromosome, genomic DNA of each *gusA* strain was extracted and used for diagnostic PCR with oligonucleotide primers targeting the plasmid sequences with each promoter (these oligonucleotide primers are included in Table 3). For further 3 above) verification, these PCR products were extracted, sequenced (Azenta Life Sciences, Essex) and aligned with the desired sequences (Benchling, 2025).

Positive control strains for GUS signal (*S. albus* J1074 ΦC31 *attB*::pSETGUSTTG and *S. venezueale* ATCC 10712 ΦC31 *attB*::pSETGUSTTG), were generated by integrating pSET152-based plasmid containing *gusA* gene under control of the *lac* promoter, resulting in a constitutively expressed *gusA* gene. Parent strains without any modification were used as negative controls. They contained no empty vector.

Spore Collection

Spores were collected by allowing the *Streptomyces* strain to grow to sporulation (approximately five days). Spores were displaced using a cotton bud and suspended in sterile water. This was filtered through a cotton stuffed syringe and the spores pelleted by centrifuging at 4000x *g* for 10 minutes. The spores were resuspended in 40% glycerol to achieve a total spore count of between 5 × 10^5 and 1 × 10^6.

β-Glucuronidase (GUS) Reporter Assay

To test the effect of media composition (exact composition in the Appendix) on different BGC activation, 11 solid agar: three minimal media (NL5, PDA, and ISP4) and eight complex media (SDA, MHA-II, TSA, LBA, ISP1, ISP2, ISP3, GYM, and SFM) were used. The media was made to a final concentration of substrate X-Gluc of 200 µM dissolved in dimethyl sulfoxide (DMSO). In 24 well plates, 2 ml of medium per well was dispensed. Four types of liquid media (2X YT, LB, MH-II and ISP2) were also investigated, in both shaking and static conditions (shaking was only tested at 37°C due to the lack of a plate shaker for use at 28°C. Reporter strains spores were dispensed onto agar at approximately 2.5 × 10^7 spores/spot. Plates were incubated at either 28°C or 37°C for five days. The presence of blue (on colourless media) or green colour (on brown/ yellowish media) was interpreted as a GUS signal, and hence the expression of the promoter of interest.

Ethanol shock at concentrations of 2, 4, and 6% (Sekurova *et al*, 2016), osmotic stress utilising 2.5% and 5% NaCl media (Sun *et al*, 2017), and DMSO effect at concentrations of 1, 2, and 3% (Chen *et al*, 2000), were also assessed. However, all of these conditions were tested in NL5 media, which contains a small amount of NaCl, so the baseline for osmotic stress testing was above zero. In addition to this, the X-Gluc was dissolved in DMSO, and this solution was added to all media tested, so the baseline for DMSO testing was above zero. Antibiotics (rifampicin, triclosan, tetracycline, erythromycin, bacitracin, lincomycin, amphotericin, sulfadiazine, fluconazole, and cefradroxil) were examined as elicitors. These antibiotics were chosen in an attempt to cover antibiotics with a range of mechanisms of action, as different mechanisms are likely to trigger different metabolic pathways, hence having different potentials to activate natural products (Xu *et al*, 2017). Antibiotics were tested at final concentrations of 10 µM and 100 µM. All additional factors were tested on NL5 media, the media seen to have the most activation and clearest reporter signals (most visible blue colour) at 28°C.

pH values of 3.5 to 8.5 were also tested for their ability to activate secondary metabolite production. Plain NL5 has a pH of 7.5. The other pH values were obtained by adjusting the pH of the media using HCl or NaOH.

Evidence of Chloramphenicol Production

The reporter strain “7R” for chloramphenicol was dotted on two separate plates of NL5 agar (agar on which the reporter strain gave a GUS signal) and incubated at 28°C for three to five days until sporulation. An overlay of *E. coli* BW25113 (susceptible to chloramphenicol) or *E. coli* ET12567 (resistant to chloramphenicol) was made by cultivating overnight in LB broth at 37°C. This was made to a concentration of 10^8 cells/ml by achieving an optical density of 0.08-0.12 at 625nm. The cell mixture was added to cooled 0.5% soft agar (exact composition noted in the Appendix) at a ratio of 1:10. 7 ml of the cell-containing agar was overlayed on the reporter strain plates, which were then incubated at 37°C. The appearance or lack of a zone of inhibition in each *E. coli* strain could support the presence of chloramphenicol.

Results

Selection of BGCs and Promoters

BGCs that are predicted to produce antibiotics by antiSMASH analysis (by comparing the BGC sequences to known antibiotic clusters, as shown in Figure 4) or BGCs that were entirely cryptic were focused on. Some BGCs, such as chloramphenicol, had known promoters (Fernández-Martínez *et al*, 2014) that were used as positive controls. Most BGCs were selected due to their silent and under-characterised nature, and most chosen had a low similarity to their predicted most similar natural product, hence being more cryptic, and potentially encoding a novel secondary metabolite. These were chosen as they would be useful in testing the success of OSMAC in activating poorly characterised BGCs, and they also have the highest potential to lead to antibiotic discovery due to their distance from known compounds. Promoters for these were designed in the UTR before the core biosynthetic gene, or the predicted transcription binding site following predictions from antiSMASH and BPROM. The location of the promoter regions for which primers were designed can be seen below in Figures 5 and 6.

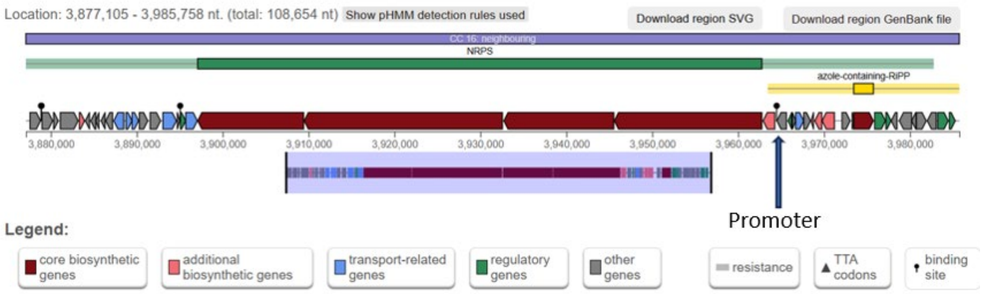
The *S. albus* genome is approximately 6.8 million base pairs, encoding 23 biosynthetic regions as predicted by antiSMASH 7.0. The *S. venezuelae* genome is approximately 8.2 million base pairs, encoding 32 biosynthetic regions. Twelve BGCs across both species were selected to form reporter constructs for GUS testing. These BGCs are listed in Table 4 below.

Table 4. The BGCs that were selected for reporter constructs. The species of origin and region number of the BGC is given, as is the most similar natural product to this cluster and the cluster's similarity to this product, and the class of the BGC. Regions with no similar compound given by antiSMASH are labelled 'cryptic'. The regions highlighted in blue are characterised and known to produce these specific secondary metabolites. For the BGCs highlighted in grey, the construction of a reporter plasmid was unsuccessful, as detailed in the paragraph below.

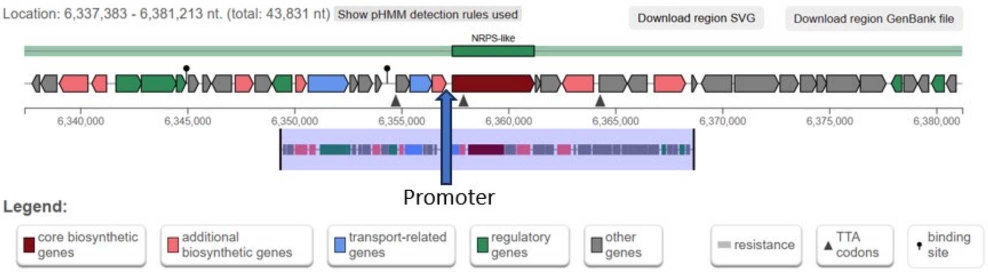
***Streptomyces* Species**	**BGC Number and Most Similar Predicted Product**	**Type of BGC**	**Similarity to Most Similar Predicted Product**
***S. albus* J1074**	**Region 5: Paulomycin**	**PKS-like**	**66%**
	Region 6: Cryptic	**RiPP-like**	**N/A**
	Region 15: Surugamide A/D	**NRPS**	**100%**
	Region 19: Minimycin	**NRPS-like**	**80%**
	Region 23: Candicidin	**PKS**	**100%**
***S. venezuelae* ATCC 10712**	**Region 5: Venezuelin**	**Lanthipeptide**	**100%**
	Region 6: Rebeccamycin	**Indole**	**25%**
	Region 7: Chloramphenicol	**NRP**	**100%**
	Region 8: Malacidin	**Lipopeptide**	**5%**
	Region 11: Lactonamycin	**Polyketide**	**10%**
	Region 20: Auricin	**PKS**	**55%**
	Region 32: Cryptic	**NRPS**	**N/A**

Some of the chosen BGCs were ultimately not included in GUS activity screening as they were unable to produce reporter strains. *S. albus* region 5, *S. albus* region 6, and *S. venezuelae* region 5 failed to produce a PCR amplicon to be ligated into the reporter plasmid. *S. venezuelae* region 8 produced a PCR amplicon that was able to be ligated into the plasmid, but the sequencing of the plasmid did not show the desired sequence.

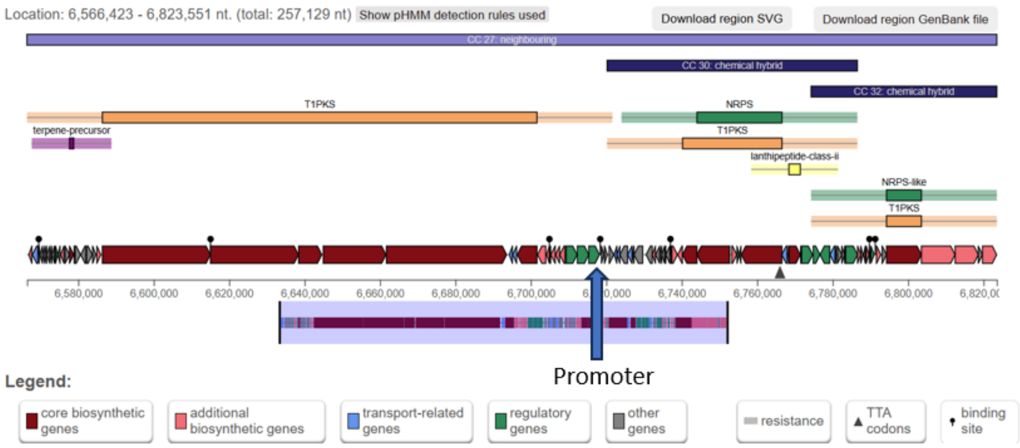
For each chosen BGC, it was engineered for the *gusA* gene to be fused with the gene likely to be the core biosynthetic gene for the natural product of interest, as predicted by antiSMASH. antiSMASH predictions for the BGCs of the successful reporter strains can be seen below in Figures 5 and 6.



1. **SurE: Region 15, known to produce surugamide**

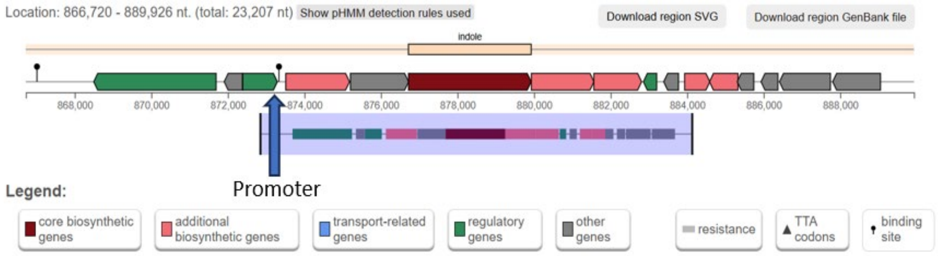


2. **R19: Region 19, known to produce minimycin**

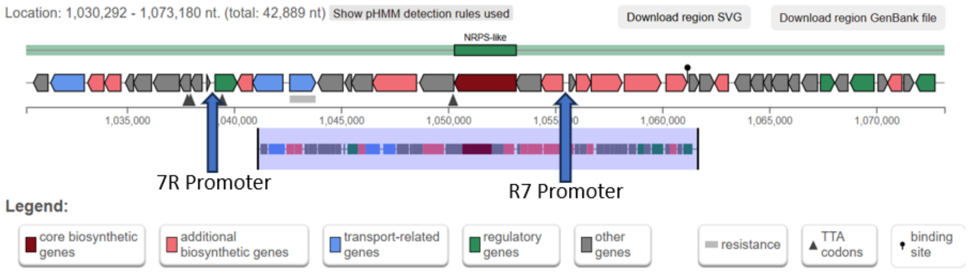


3. **Can: Region 23, known to produce candicidin BGC**

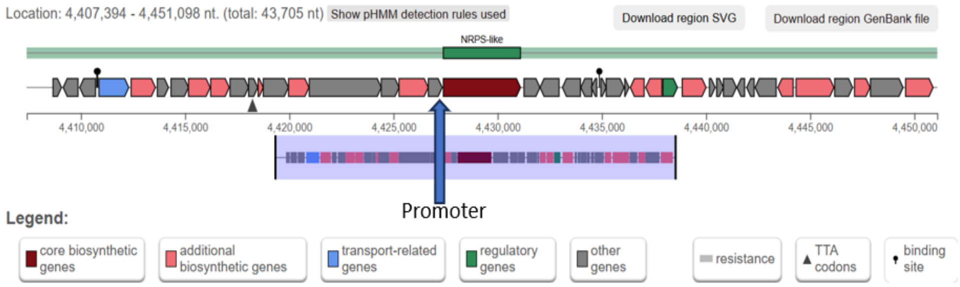
Figure 5. The antSMASH analysis for *S. albus* J1074 BGCs for which reporter strains were made, with the location of the promoters indicated with a blue arrow. Beneath each image is the title of the region it represents. The key for the types of genes can be found at the bottom of each image. The blue arrows indicate the position of the approximately 600bp promoter that was amplified and adjoined to *gusA* to create the reporter strain for the BGC.


Location: 866,720 - 889,926 nt. (total: 23,207 nt)

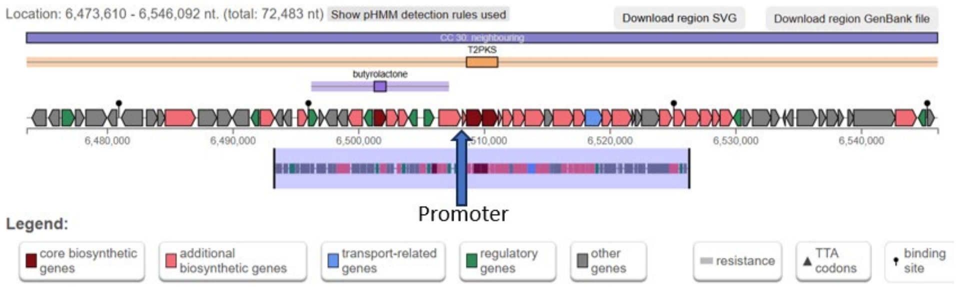
1. **R6: Region 6, predicted to produce a product similar to rebeccamycin**


Location: 1,030,292 - 1,073,180 nt. (total: 42,889 nt)

2. **7R and R7: Region 7, known to produce chloramphenicol**

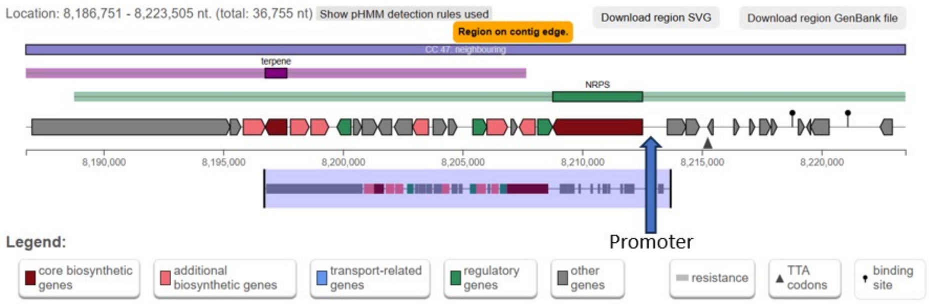

Location: 4,407,394 - 4,451,098 nt. (total: 43,705 nt)

3. **R11: Region 11, predicted to produce a product similar to lactonamycin**


Location: 6,473,610 - 6,546,092 nt. (total: 72,483 nt)

4. **R20: Region 20, predicted to produce auricin**

Figure 6. The antSMASH analysis for *S. venezuelae* ATCC 10712 BGCs for which reporter strains were made, with the location of the promoters indicated with a blue arrow. Beneath each image is the title of the region it represents.



5. **R32: Region 32, cryptic (no predicted product)**

Figure 6 *(continued).* The antSMASH analysis for *S. venezuelae* ATCC 10712 BGCs for which reporter strains were made, with the location of the promoters indicated with a blue arrow. Beneath each image is the title of the region it represents. The key for the types of genes can be found at the bottom of each image. The blue arrows indicate the position of the approximately 600bp promoter that was amplified and adjoined to *gusA* to create the reporter strain for the BGC.

Production of Reporter Strains

As shown above in Table 4, reporter strains for eight BGCs (three for *S. albus* and five for *S. venezuelae*), of which the chloramaphenicol region in *S. venezuelae* had two constructs generated using different potential promoters, were successfully generated following the protocols included in the Methods section. These constructs were verified by PCR and sequencing at both the miniprep stage and in the transconjugant (Azenta Life Sciences, Essex).

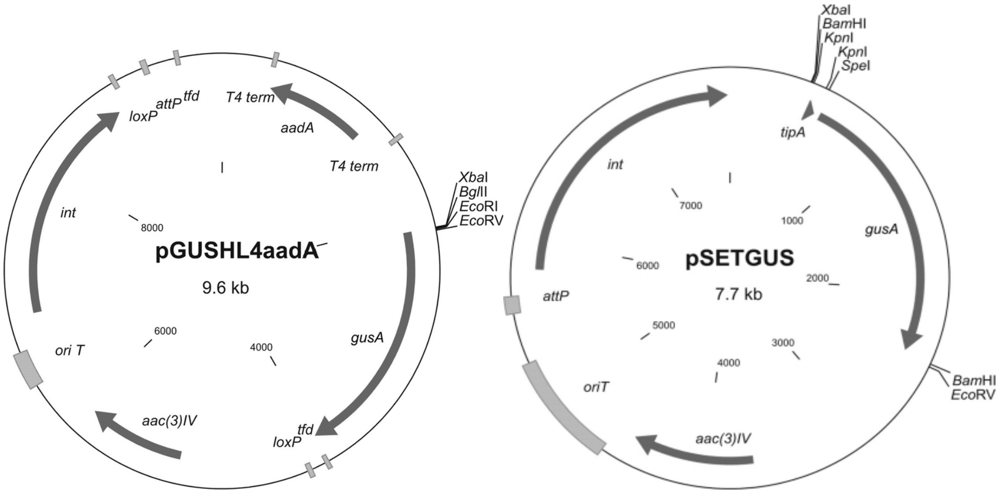


Figure 7. Plasmid vectors used in this study (Myronovskyi *et al*, 2011). Left is pGUSHL4aadA, an integrative vector containing promoter-less *gusA* and the spectinomycin resistance cassette aadA. This plasmid was used to fuse *gusA* to the promoter region of interest, and to integrate this construct into the host chromosome to test for BGC activation using this reporter system. Right is pSETGUS plasmid with a TTG start codon, as this is favoured in these species of *Streptomyces*. This plasmid was integrated into each original host species to generate positive controls for GUS expression as *gusA* is constitutively expressed from this plasmid.

Below demonstrates an example of the pGUSHL4aadA-promoter fusion found in the reporter strains for *Streptomyces* in this study.

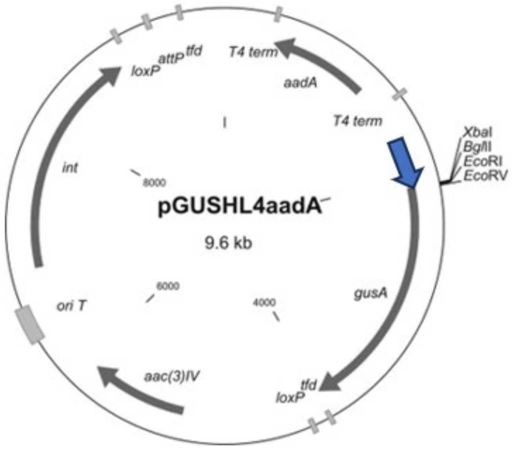


Figure 8. pGUSHL4aadA plasmid with the selected promoter (blue arrow) from the strain of interest adjoined to *gusA*. Restriction enzymes XbaI and EcorV cleave out the base pairs between these recognition sites, ahead of *gusA* and the promoter of interest (approximately 600bp in length) is ligated in at this point. This is the case for every reporter strain generated in this study.

Conditions Tested and Activation Profile

A large number of methods have been seen to activate secondary metabolism (Pan *et al*, 2019). However, this research intentionally did not investigate some avenues that have seen success due to their complexity and limited scalability potential. An example of a condition not investigated is co-culturing the microorganism of interest with other species, as this comes with a great deal of complexity, such as a lack of reproducibility due to the level of bacterial growth of each species differing between cultures (Covington et al, 2021). The approaches used in this study are those that are simpler and more accessible, as these are most feasible for the establishment of an antibiotic discovery framework. These are summarised below in Table 5.

Table 5. The cultivation conditions altered as part of the OSMAC approach. More information about the specific conditions tested under each section (solid media, liquid media, DMSO effect, ethanol shock, osmotic stress, antibiotics, pH, and temperature) is given in below.

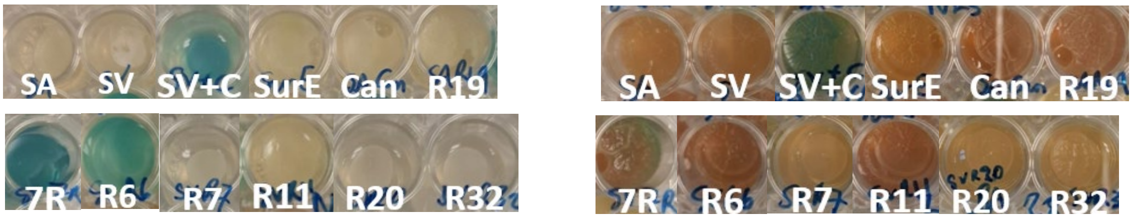
Condition	**Detail**
Solid Media	Eleven different types of solid media (all compositions in the Appendix): NL5, LBA, ISP1, ISP2, ISP3, ISP4, MHA-II, GYM, SDA, PDA and SFM.
Liquid Media	Four different types of liquid media. (all compositions in the Appendix): LB, 2X YT, MH-II and ISP1. These were tested in both static and shaking conditions.
DMSO Effect	NL5 agar was made with three different concentrations of DMSO: 1, 2 and 3%
Ethanol Shock	NL5 agar was made with three different concentrations of ethanol: 2, 4 and 6%.
Osmotic Stress	NL5 agar was made with different two concentrations of NaCl: 2.5% and 5%.
Antibiotics	Ten different antibiotics were added to NL5: rifampicin, triclosan, tetracycline, erythromycin, bacitracin, lincomycin, amphotericin, sulfadiazine, fluconazole, and cefradroxil. These were tested at two different concentrations (10 and 100 µM) for each antibiotic.
pH	NL5 agar was made to six different pH values: 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5.
Temperature	Each type of liquid and solid media was tested under two cultivation temperatures: 28°C and 37°C.

Solid Media

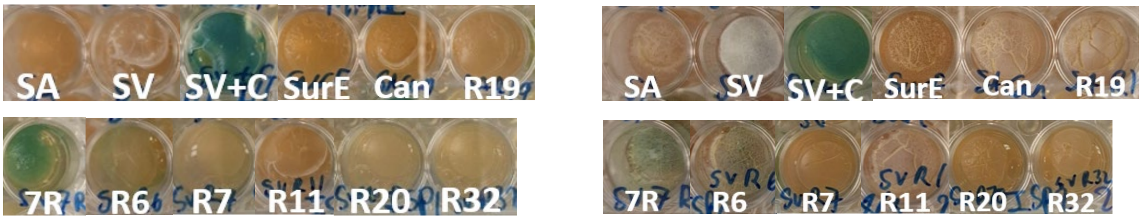
Different compositions of solid media were used as they would have different nutrient profiles and hence either provide the necessary nutrients for certain pathways or trigger a stress response that may activate BGCs due to nutrient deprivation (Hoskisson and Fernández-Martínez, 2018). Three reporter constructs out of the eight tested were activated by changing only the media composition. All media compositions were tested at both 28°C and 37°C, as the former is *Streptomyces*' ideal growth temperature (Berendsen *et al*, 2013), and the additional heat of 37°C could help to upregulate certain pathways (Thakur *et al*, 2009).

A GUS signal was observed in nearly all types of agar media tested for the *S. venezuelae* chloramphenicol reporter 7R, indicating that this antibiotic is likely being produced (Figure 6). This accords with previous studies, including the one that the promoter used was taken from: Fernández-Martínez *et al*, 2014. A different promoter for chloramphenicol (the one designed in this study) was also tested but did not show a signal under any conditions, suggesting that this designed construct did not adequately capture a promoter for this region. This absence of GUS activation also indicates that the lack of signal given by a reporter strain may not be reflective of the production of a secondary metabolite, as it may still be produced without showing a signal if *gusA* has not been adjoined to an accurate promoter.

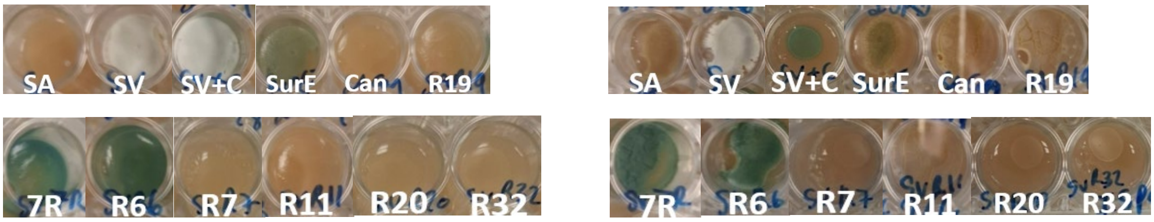
Cryptic region 6 in *S. venezuelae*, most similar compound being reported by antiSMASH 7.0 as Rebeccamycin, also showed a signal on all media except LBA. Surugamide in *S. albus* also activated on some agar (ISP3, ISP4, GYM, and SFM), as previously seen in the study the promoter region was taken from: Xu *et al* (2017). The signal for this region was seen a day or two after those in *S. venezuelae*, likely because *S. albus* takes longer to sporulate, and sporulation is often seen to be linked to an increased production of secondary metabolites, some of which are only produced in this stage (Čihák *et al*, 2017). This activation profile can be seen in Figure 9, Figure 10 and Table 6.



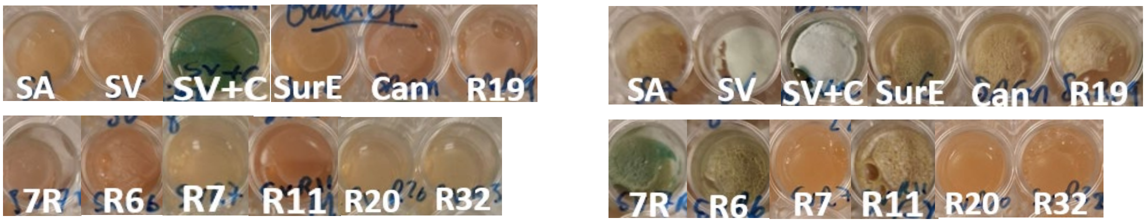
a. **NL5 (left) and LBA (right)**



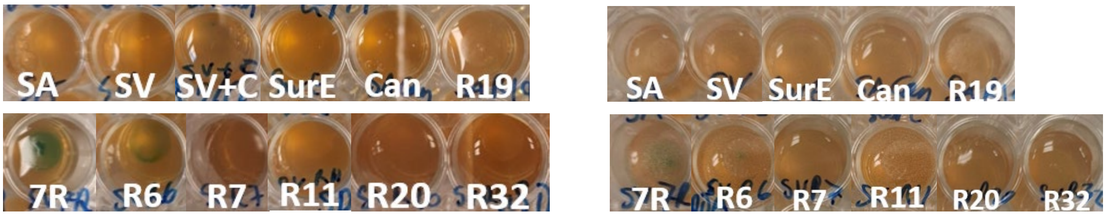
b. **ISP1 (left) and ISP2 (right)**



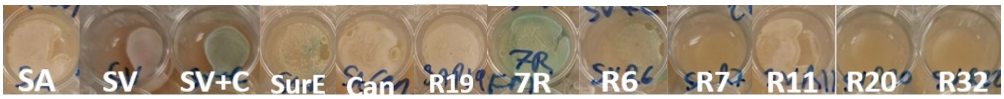
c. **ISP3 (left) and ISP4 (right)**



d. **MHAII (left) and GYM (right)**



e. **SDA (left) and PDA (right, no image of PDA positive control available)**

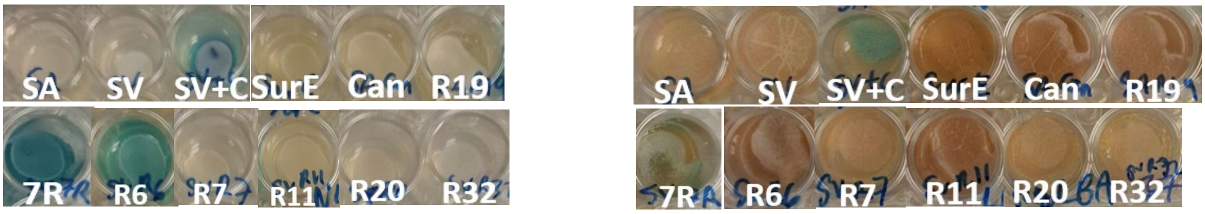


f. **SFM**

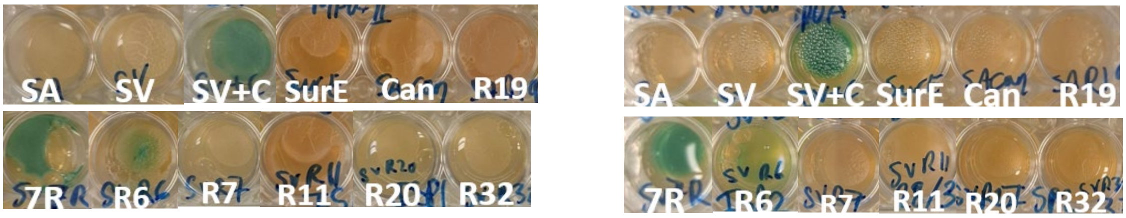
Figure 9. The reporter strains on different types of media at incubation temperature 28°C. Media type is specified in the captions beneath the sets of images (a to f). For this figure and all those following, “SA” is an abbreviation for the

Figure 9 ***(continued)*****.** *S. albus* parent strain and “SV” is an abbreviation for the *S. venezuelae*, both of which were used as negative controls. “SV+C” is the positive control strain of *S. venezuelae* (constitutive GUS activation). The positive control in *S. albus* was tested on plates that were not photographed, and so they could not be included in this study’s figures. Some SV positive controls may appear white due to the aerial mycelia, they showed a GUS reporter signal underneath the aerial mycelia which was more visible earlier in the incubation. All images are after at least three days of incubation, but exact incubation times may vary as the images with the clearest activation and visibility were used.

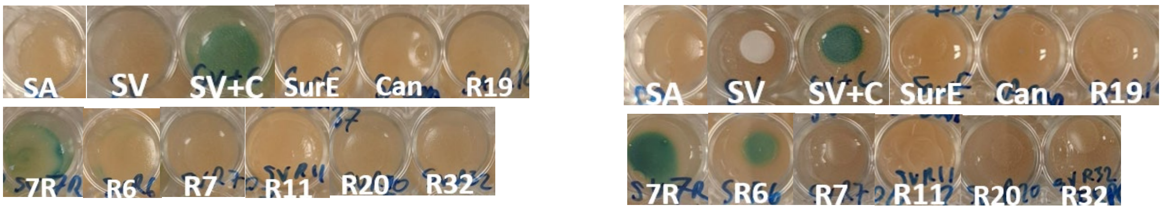
Reporter strains grown on the same selection of agar at incubation temperature 37°C can be seen in Figure 10 below.



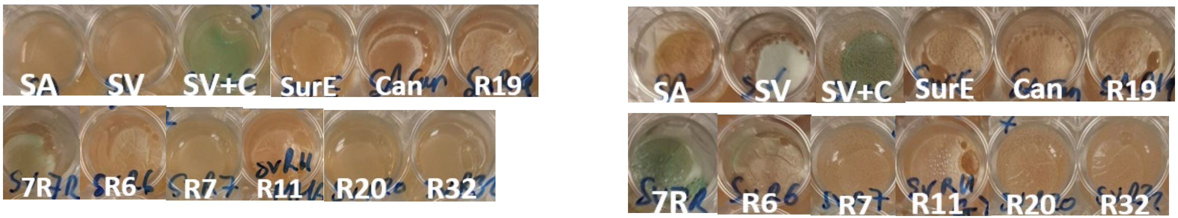
a. **NL5 (left) and LBA (right)**



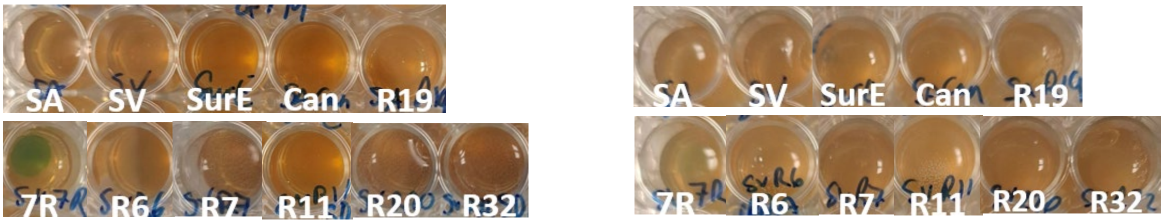
b. **ISP1 (left) and ISP2 (right)**



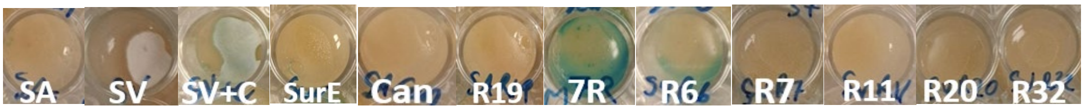
c. **ISP3 (left) and ISP4 (right)**



d. **MHAII (left) and GYM (right)**



e. **SDA (left) and PDA (left). No images of positive controls available.**



f. **SFM**

Figure 10. The reporter strains on different types of media at incubation temperature 37°C. Images are labelled a to k with the media type.

The activation of all reporter strains on different types of agar can be seen below in Table 6.

Table 6. GUS activation of the reporter strains on different types of solid media. Dark blue indicates a strong GUS signal and light blue indicates a weaker GUS signal. These signal colours are in comparison to the positive control and determined by eye and this is the case for all reporter signal readings in this study. Names of *S. albus* BGCs are highlighted green, *S. venezuelae* BGCs are highlighted in red.

	NL5		LBA		ISP1		ISP2		ISP3		ISP4		MHA-II		GYM		SDA		PDA		SFM	
	28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37
SurE									■	▨	▨	▨			▨						▨	
Can																						
R19																						
7R	■	■	▨	▨	■	■	■	■	■	■	■	■		▨	■	■	■	■	▨	■	■	■
R7																						
R6	■	■			▨	▨	■	■	■	■	■	■		▨	▨	▨	▨		▨		▨	▨
R11																						
R20																						
R32																						

Evidence of Chloramphenicol Production

The chloramphenicol reporter strain that produced a GUS signal on NL5 also produced a zone of inhibition in wildtype BW25113 *E. coli* overlay, as seen in Figure 11. This strain of *E. coli* is susceptible to most antibiotics, including chloramphenicol (Granier et al, 2014), and so this indicates that the *Streptomyces* reporter strain is producing an antibiotic to create this zone of inhibition. As this BW25113 *E. coli* is susceptible to many different antibiotics, this zone of inhibition simply supports the presence of an antibiotic and not specifically chloramphenicol. To supplement this, the *S. venezuelae* strain was tested against an overlay of ET12567 (pUZ8002) *E.*

coli, which is resistant to chloramphenicol. The *S. venezuelae* showed no zone of inhibition in this resistant *E. coli.* Thus, the presence of a zone of inhibition in chloramphenicol susceptible bacteria and the absence of this in chloramphenicol resistant bacteria together support the idea that this strain of *Streptomyces* is producing chloramphenicol. These results support the concept that GUS reporter activation in this strain is reflective of the activation of the targeted BGC and potentially indicative of the production of a secondary metabolite. This supports the validity of the GUS reporter system and it being representative of the expression of the intended BGC.

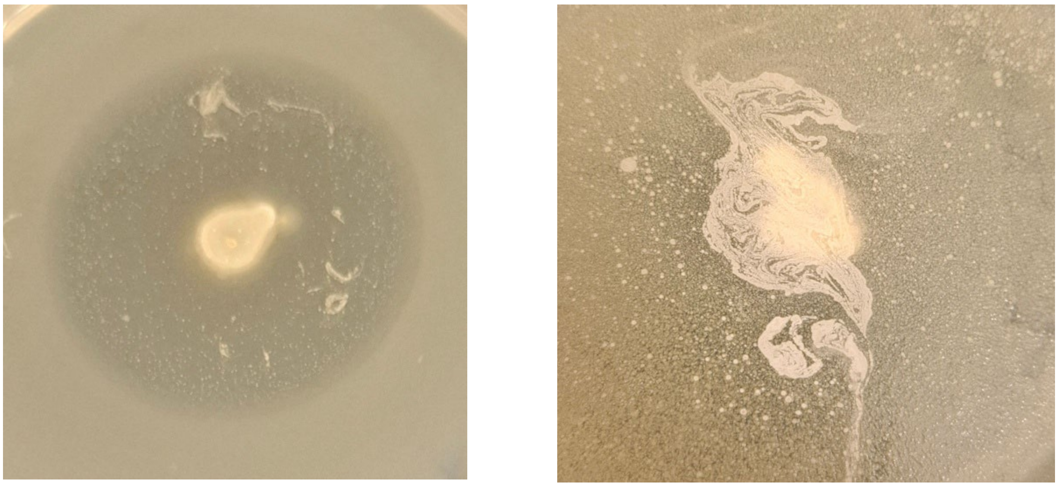
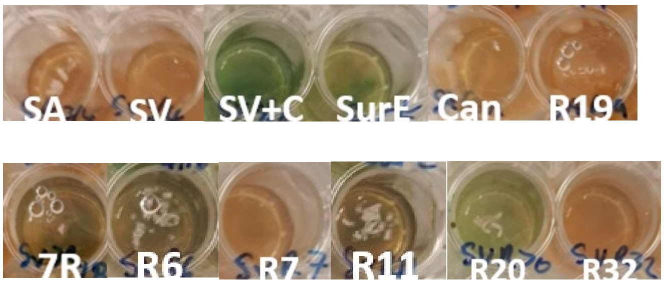


Figure 11. Chloramphenicol reporter strain "7R" overlayed with BW25113 (left) and ET12567 (pUZ8002) (right) in soft agar. *S. venezuelae* grown on NL5 is overlayed with soft agar (exact composition in the Appendix) containing one of two strains of *E. coli*. A zone of inhibition is produced in the BW25113 overlay, indicating antibiotic production by *S. venezuelae*. No such zone of inhibition is seen in the ET12567 (pUZ8002) overlay, indicating that the antibiotic produced by *S. venezuelae* is one that ET12567 (pUZ8002) is resistant to, supporting that it may be chloramphenicol that *S. venezuelae* produced.

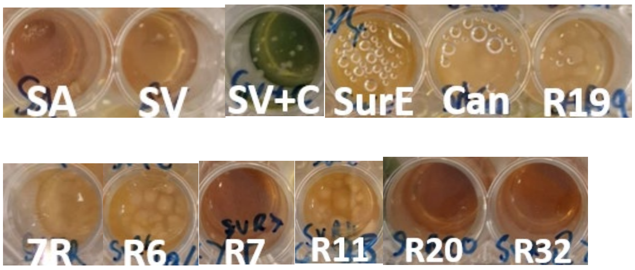
Liquid Media

Different types of liquid media were assessed in both shaking and static, as these conditions provide different stressors, such as mechanical stress (Schrader *et al*, 2023) and oxygen deprivation (van Keulen *et al*, 2003), respectively. Five reporter constructs out of the eight were activated within these conditions. Growth in liquid media was seen to be concentrated at the bottom of the well, and to the similar extent of growth as on solid media.

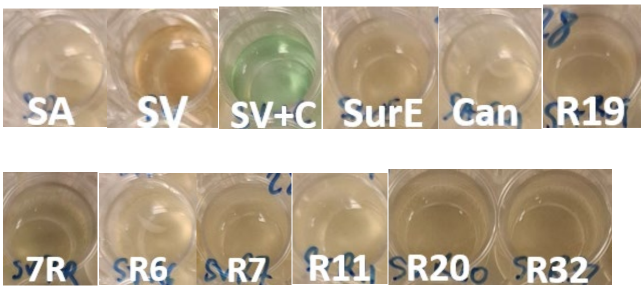
The three BGCs that showed frequent activation on solid media also showed activation in liquid media. These are 7R (activated in LB, 2X YT, and ISP1), SV R6 (activated in LB, 2X YT, and MHII), and SurE (activated in LB and 2X YT). In addition to this, liquid media saw the activation of two additional BGCs: cryptic regions 11 (activated in LB and ISP1) and 20 in *S. venezuelae* (activated in LB). This can be seen below in Figure 12 and Figure 13



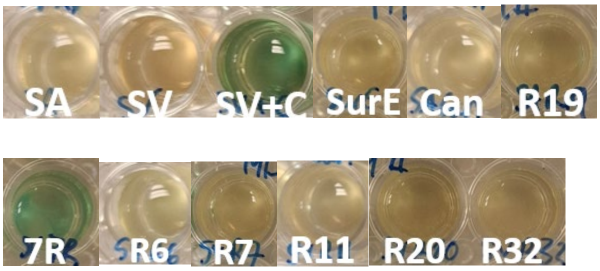
a. **LB 28°C**



b. **2XYT 28°C**



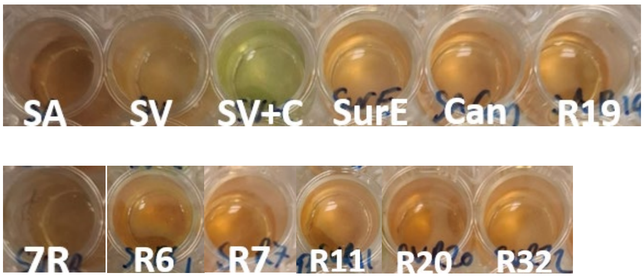
c. **MHII 28°C**



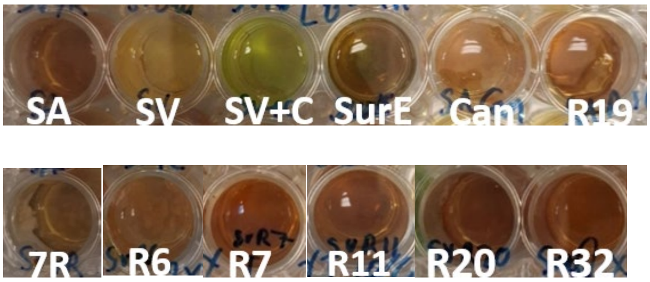
d. **ISP1 28°C**

Figure 12. Reporter strains grown in liquid media at 28°C. Images labelled underneath a to d with the media type.

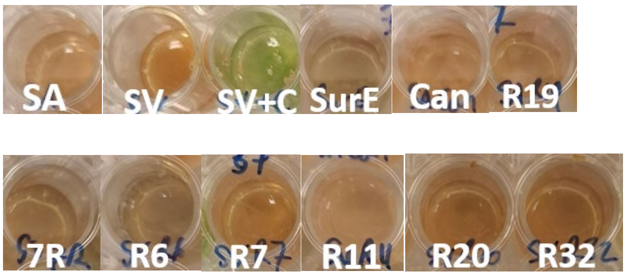
The reporter strains in the same types of liquid media but incubated at 37°C can be seen below in Figure 13.



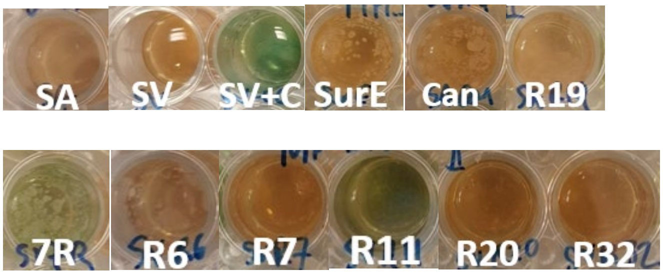
a. LB 37°C



b. 2XYT 37°C



c. MHII 37°C



d. ISP1 37°C

Figure 13. Reporter strains grown in liquid media at 37°C. Figures labelled underneath a to d with the media type. The images of the shaking plates are not included due to them being inadequate (condensation on the lid blocks clear sight of the wells).

The activation of all reporter strains in different types of liquid media can be seen below in Table 7.

Table 7. GUS activation of different reporter strains in liquid agar (static and shaking, as represented by "S" and "Sh", respectively). Both blue and green are interpreted as positive GUS signals, the colour of the signal produced is shown in the activation table. Names of *S. albus* BGCs are highlighted green, *S. venezuelae* BGCs are highlighted in red.

	LB			**2X YT**			**MHII**			**ISP1**		
	28	**37**		**28**	**37**		**28**	**37**		**28**	**37**	
	S	**S**	**Sh**	**S**	**S**	**Sh**	**S**	**S**	**Sh**	**S**	**S**	**Sh**
SurE	🟦				🟩							
Can												
R19												
7R	🟩				🟩						🟩	
R7												
R6	🟩					🟩			🟩			
R11	🟩										🟩	
R20	🟩											
R32												

DMSO, Ethanol and NaCl

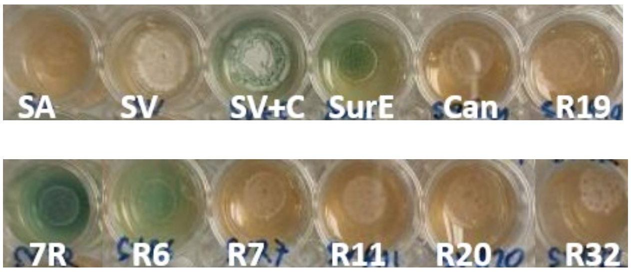
DMSO effect (Chen *et al*, 2000), ethanol shock (Sekurova *et al*, 2016) and osmotic stress by NaCl (Sun *et al*, 2017) were also tested due to providing stress conditions that have been seen to activate BGCs in the cited studies. While this study did not see these conditions activate new BGCs, they did show varying levels of signal intensity, indicating that they can up or downregulate these pathways.

SV 7R activated on all tested concentrations of DMSO and ethanol, but only on 2.5% NaCl. SV R6 activated on all tested concentrations of DMSO and ethanol, but only on 2.5% NaCl. SA SurE activated on all tested concentrations of DMSO and ethanol, but not on any concentration of NaCl.

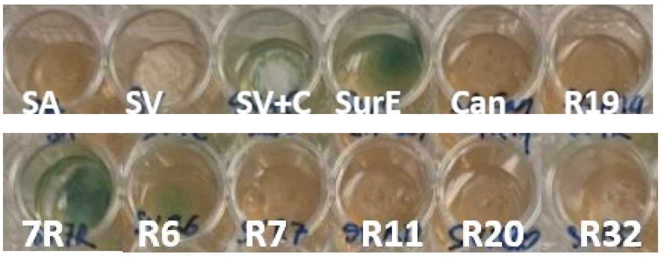
No activation at all was seen on NaCl 5% NL5, as supported by the Sun *et al* (2017) paper, that suggested that this level of osmotic stress was too high for *Streptomyces* to grow on this media.

Ethanol shock was more effective at increasing natural product expression compared to DMSO and osmotic stress parameters investigated. This can be seen in the image of Figure 14, Figure 15 and Figure 16 below.

a. **1% DMSO**

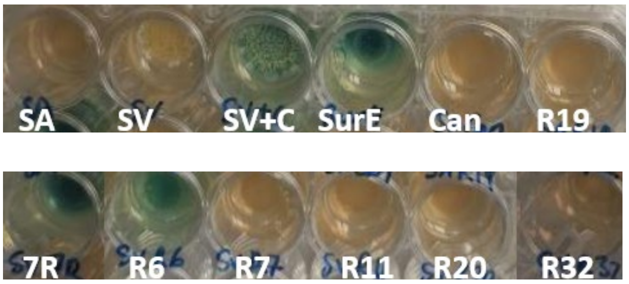


b. **2% DMSO**

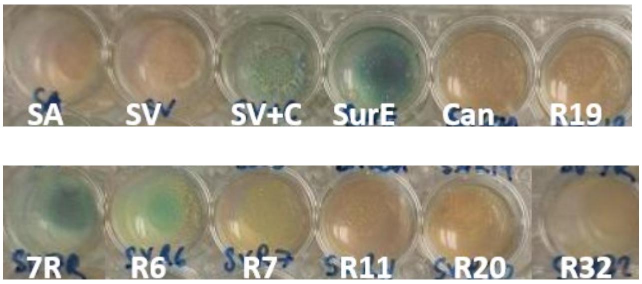


c. **3% DMSO**

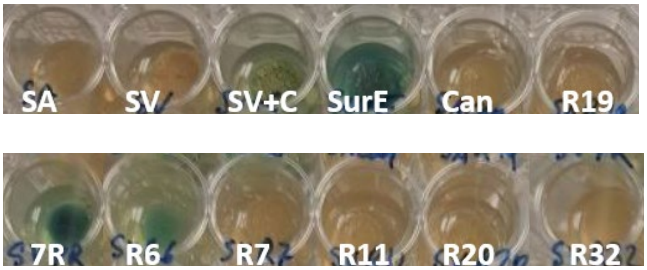
Figure 14. Reporter strains grown on NL5 media of multiple concentrations of DMSO, as stated in the subheading (a to c) underneath each image.



a. 2% Ethanol

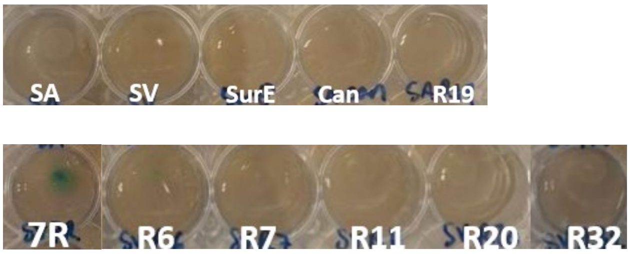


b. 4% Ethanol

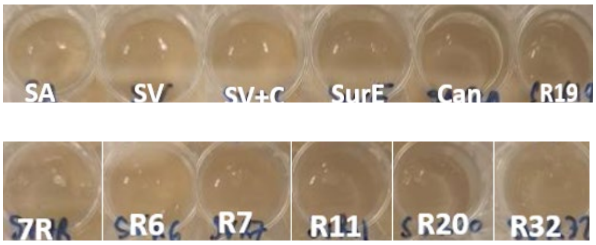


c. 6% Ethanol

Figure 15. Reporter strains grown on NL5 media of multiple concentrations ethanol, as stated in the subheading (a to c) underneath each image.



a. 2.5% NaCl (image of positive control unavailable).



b. 5% NaCl

Figure 16. Reporter strains grown on NL5 containing 2.5% NaCl (a) and 5% NaCl (b).

The activation of all reporter strains on different concentrations of DMSO, ethanol and NaCl can be seen below in Table 8.

Table 8. Concentrations of DMSO, Ethanol, and NaCl. Names of *S. albus* BGCs are highlighted in green and names of *S. venezuelae* BGCs are highlighted in red.

	DMSO			Ethanol			NaCl	
	1%	2%	3%	2%	4%	6%	2.5%	5%
SurE	▨	▨	■	■	■	■		
Can								
R19								
7R	■	■	■	■	■	■	▨	
R7								
R6	▨	▨	▨	■	▨	▨	▨	
R11								
R20								
R32								

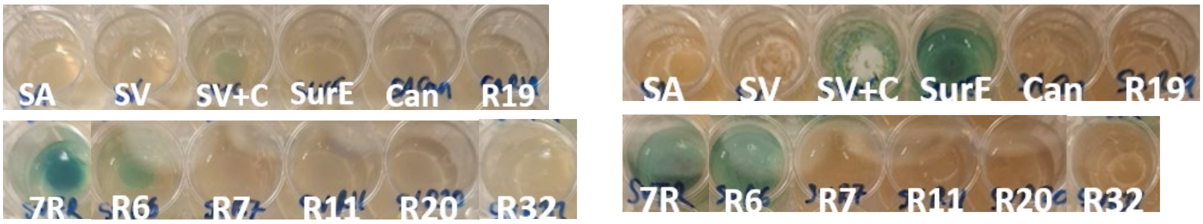
Antibiotics

Antibiotics were used as elicitors due to many studies, including some on the *Streptomyces* species used in this study (Tanaka *et al*, 2017), utilising them to activate natural product production. Ten elicitors at concentrations 10 µM and 100 µM (both subinhibitory concentrations for all included elicitors) were tested. Some antibiotics at some concentrations activated BGCs that others did not (most specifically seen with the *S. albus* surugamide BGC), although nothing activated was not previously activated by changing media composition. This activation profile can be seen in Figure 15, Figure 16 and Table 9 below.

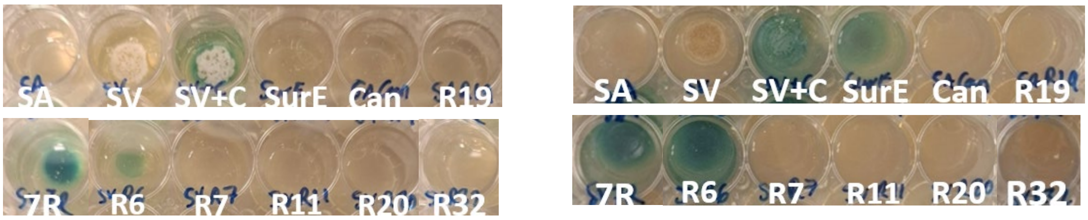
These conditions did not activate any new BGCs in this study but saw different levels of activation of the previously activated BGCs, as seen by different intensities of reporter signal. The SurE BGC in *S. albus* is not expressed on plain NL5 but was seen to activate on NL5 in the presence of antibiotics: triclosan, erythromycin, bacitracin, lincomycin, amphotericin, sulfadiazine, and cefadroxil (as shown in Table 8). These results indicate that the signals seen are due to the presence of the antibiotics, suggesting that these elicitors activate this biosynthetic pathway.

SV 7R and SV R6 activated on all tested antibiotics, but SV R6 showed a weaker signal (as seen by the naked eye, in comparison to each other) on rifampicin, tetracycline, lincomycin, fluconazole, and cefadroxil. This indicates that SV 7R is more strongly upregulated than SV R6.

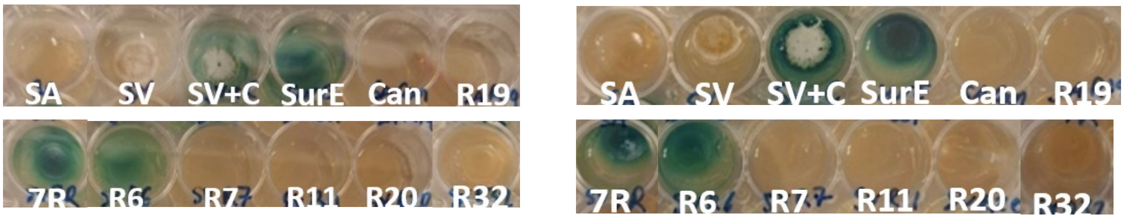
Overall, more intense signals were seen at 10 µM than 100 µM for all antibiotics, suggesting that the higher concentration may be exhibiting have some inhibitory effect on these metabolic pathways. This can be seen in Figure 17 and Figure 18 below.



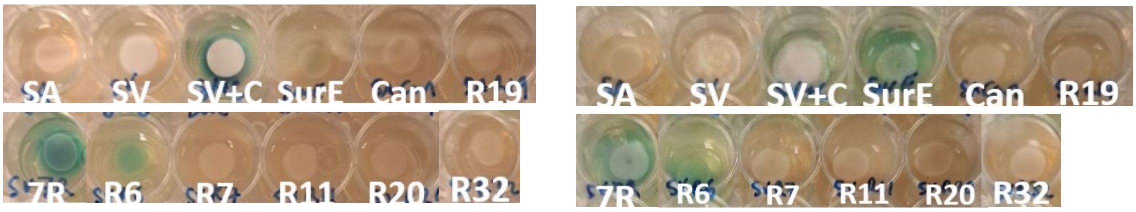
a. **Rifampicin (left) and Triclosan (right)**



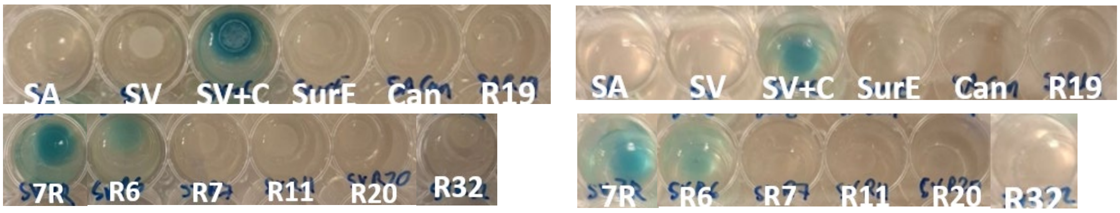
b. **Tetracycline (left) and Erythromycin (right)**



c. **Bacitracin (left) and Lincomycin (right)**



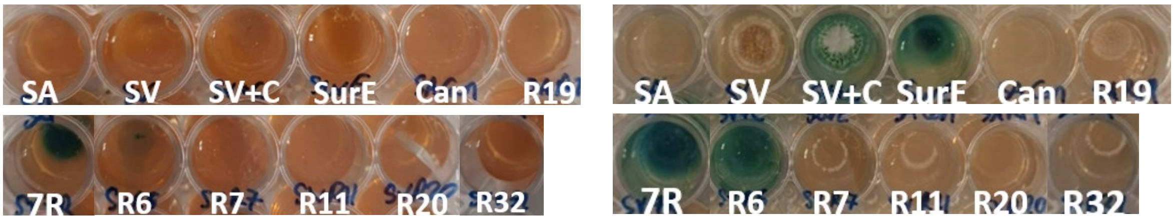
d. **Amphotericin (left) and Sulfadiazine (right)**



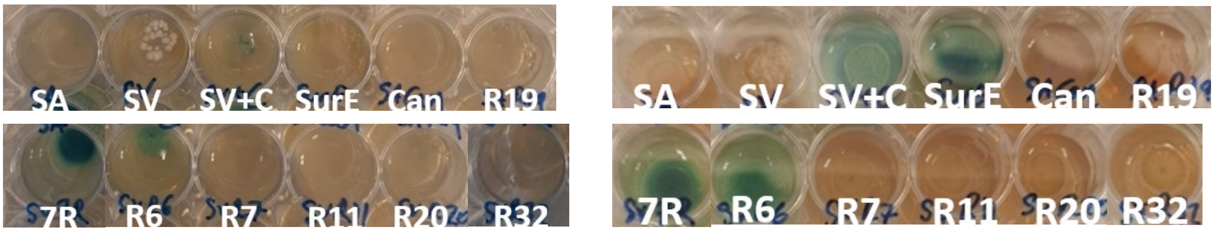
e. **Fluconazole (left) and Cefadroxil (right)**

Figure 17. Reporter strains on NL5 elicitor plates of 10 µM concentration. Each elicitor is labelled beneath the images (a to j).

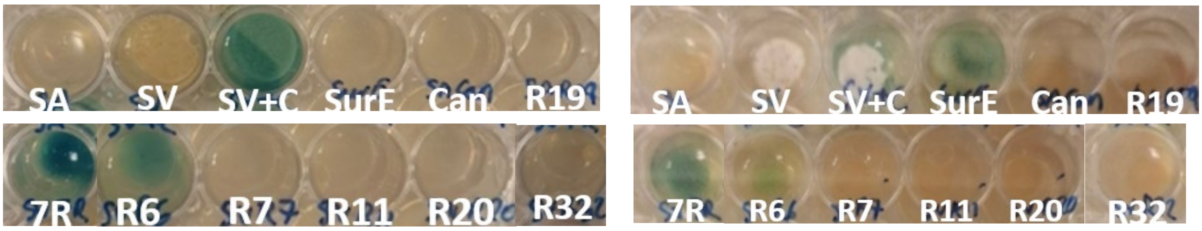
Images of the reporter strains of plates of the same elicitors at 100 µM can be seen in Figure 18 below.



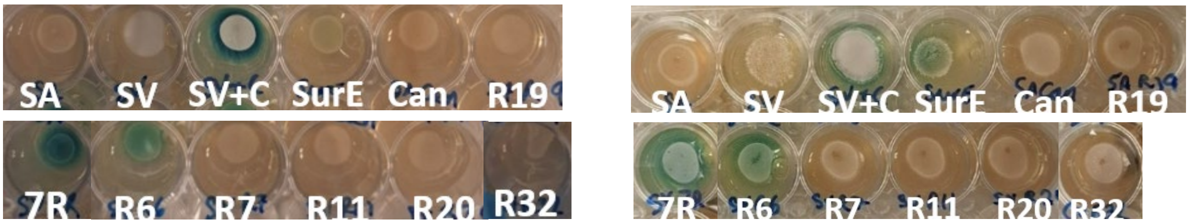
a. **Rifampicin (left) and Triclosan (right)**



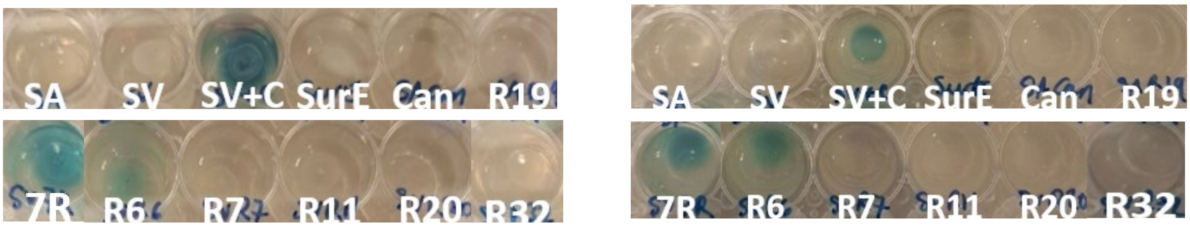
b. **Tetracycline (left) and Erythromycin (right)**



c. **Bacitracin (left) and Lincomycin (right)**



d. **Amphotericin (left) and Sulfadiazine (right)**



e. **Fluconazole (left) and Cefadroxil (right)**

Figure 18. Reporter strains on NL5 elicitor plates of 100 μM concentration. Each elicitor is labelled beneath the images (a to j).

The activation of all reporter strains on different types and concentrations of antibiotics can be seen below in Table 9.

Table 9. Activation Table on 10 different antibiotics at concentrations of 10 µM and 100 µM. Names of *S. albus* BGCs are highlighted in green and names of *S. venezuelae* BGCs are highlighted in red.

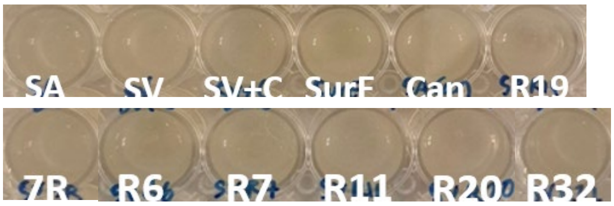
	Rifamp		**Triclos**		**Tetracy**		**Erythro**		**Bacitra**		**Lincom**		**Ampho**		**Sulfadi**		**Flucon**		**Cefadr**	
	10 µM	100 µM	10 µM	100 µM	10 µM	100 µM	10 µM	100 µM	10 µM	100 µM	10 µM	100 µM	10 µM	100 µM	10 µM	100 µM	10 µM	100 µM	10 µM	100 µM
SurE			■	■			■	■	■		■	▨	▨	▨	■	▨			▨	
Can																				
R19																				
7R	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
R7																				
R6	▨	▨	■	■	▨	▨	■	■	■	■	■	▨	■	■	■	■	▨	■	▨	■
R11																				
R20																				
R32																				

pH

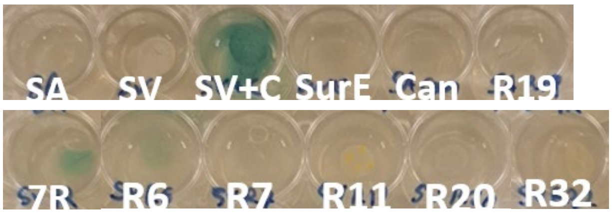
Media of varying pH was tested in an attempt to replicate the success of previous studies (Berendsen *et al*, 2013). No new BGCs were activated, but the varying intensities of the GUS signals allow the identification of an optimum pH for the upregulation of these pathways.

No new activations were seen, and SurE did not activate at all (as it does not activate on plain NL5) indicating that pH changes are not sufficient to trigger this metabolic pathway.

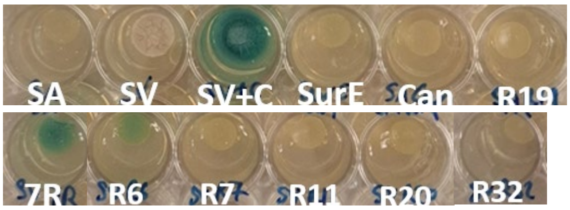
SV 7R and SV R6 activated on pH values 4.5 to 8.5. SV 7R (for chloramphenicol) and SV R6 show a variation of signal intensities, allowing an optimum pH to be identified. No growth was seen at pH 3.5 and hence no activation, indicating that it is too acidic for spore germination or mycelia formation. Plain NL5 has a pH of approximately 7.5, but this agar showed as strong of a signal for SV 7R as pH values 5.5, 6.5 and 8.5, indicating that pH within the range of 5.5 to 8.5 is sufficient for secondary metabolite production. The results of pH testing are displayed in Figure 19 and Table 10.



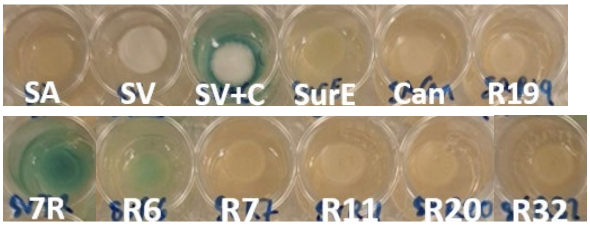
a. pH 3.5



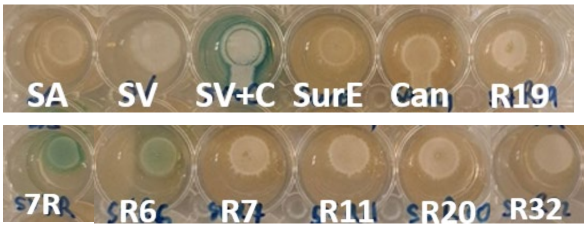
b. pH 4.5



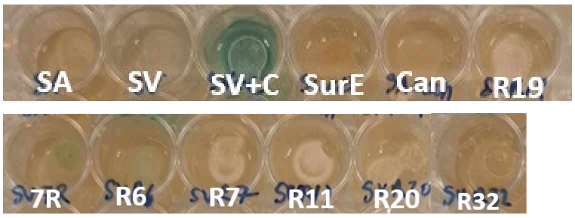
c. pH 5.5



d. pH 6.5



e. pH 7.5



f. pH 8.5

Figure 19. Reporter strains on plates of NL5 with ascending pH values (a to f).

The activation of all reporter strains at different pH values can be seen below in Table 10.

Table 10. Activation table of NL5 media at various pH values. Names of *S. albus* BGCs are highlighted in green and names of *S. venezuelae* BGCs are highlighted in red.

	3.5	**4.5**	**5.5**	**6.5**	**7.5**	**8.5**
SurE						
Can						
R19						
7R		▨	■	■	■	▨
R7						
R6		▨	▨	▨	▨	▨
R11						
R20						
R32						

Temperature

As seen in Table 6 and Table 7, there was a small difference in activation between cultivation at 28°C and 37°C. 28°C was seen to have a slightly higher activation profile, which aligns with the *S. coelicolor* study (Elimam, personal communication). This finding in the *S. coelicolor* study was the reason that the elicitor and pH studies were run at 28°C instead of 37°C.

For example, SA SurE produced a signal on GYM at 28°C but not 37°C, SV 7R and SV R6 produced a signal on MHA-II at 28°C but not 37°C, and SV R6 produced a signal on PDA at 28°C but not 37°C.

Overall Activation

Table 11. Overall Summary of Activation. Detailing the BGCs investigated, their activation status in this study, and under which conditions they activate if applicable.

BGC	Activation	Conditions
SA SurE	**Yes**	**Some solid agar (ISP3, ISP4, GYM, and SFM), some liquid media (LB and 2X YT), all tested concentrations of DMSO (1%, 2%, 3%) and ethanol (2%, 4%, 6%), and many antibiotics (triclosan, erythromycin, bacitracin, lincomycin, amphotericin, sulfadiazine, and cefadroxil)**
SA Can	**No**	**N/A**
SA R19	**No**	**N/A**
SV 7R	**Yes**	**Near all. All solid media, liquid media LB, 2X YT, and ISP1. Activates on all concentrations of DMSO (1%, 2%, 3%) and ethanol (2%, 4%, 6%), and 2.5% NaCl. Activates on all tested antibiotics and pH values of 4.5 to 8.5.**
SV R7	**No**	**N/A**
SV R6	**Yes**	**Activates on all solid media except LBA, and all liquid media except ISP1. Activates on all concentrations of DMSO (1%, 2%, 3%) and ethanol (2%, 4%, 6%), and 2.5% NaCl. Activates on all tested antibiotics and pH values of 4.5 to 8.5.**

SV R11	**Yes**	**Liquid media LB (static) at 28°C and ISP1 (static) at 37°C.**
SV R20	**Yes**	**Liquid media LB (static) at 28°C.**
SV R32	**No**	**N/A**

Discussion

This work saw five out of eight reporters for different BGCs in *Streptomyces* activate in different conditions. This total activation profile could have been achieved by the minimum conditions of testing a small number of media (three solid and two liquid) at 28°C. This small number of conditions would make this approach useful for antibiotic discovery as it could greatly simplify activating silent BGCs, allowing the potential production of secondary metabolites to be identified quickly for further investigation. This study supports the preceding research of this method in *S. coelicolor,* which saw the vast majority of BGCs activate by simply cultivating on a handful of different types of agar (Elimam, personal communication). However, this study saw a slightly lower activation rate than that in *S. coelicolor*, ~62% compared to 80%, despite the *S. coelicolor* study not including liquid media, a condition responsible for the activation of two of the five seen to give a signal in this research. Even so, these combined results support that the majority of BGCs investigated can be activated with simple cultivation condition changes, indicating that reporter-informed OSMAC may be a worthwhile approach across the genus (Elimam, personal communication).

However, the presence of a GUS signal is not entirely reflective of the production of a secondary metabolite, as the GUS can be produced from only the translation of its gene. However, post-translational processes may be required to produce a secondary metabolite (García-Estrada *et al*, 2008). This would not be able to be represented by the GUS activation, and so the reporter signal always requires further investigation. The subsequent paragraphs will go into the specifics of GUS activation of the promoter fusion strains in certain conditions.

The reporter construct “7R” for chloramphenicol in *S. venezuelae* was seen to activate in nearly all conditions, which aligns with the Fernández-Martínez *et al* 2014 paper, which identified this promoter region that was used in this construct as a regulatory gene that was essential for chloramphenicol production and active in many conditions. Another reporter construct was made for the chloramphenicol BGC, one with the promoter region designed in this study following antiSMASH predictions (“SV R7”). This designed reporter strain did not show any signal in any conditions. The activation of the other promoter construct (7R) for this region indicates that in the designed strain SV R7, secondary metabolite production may still be occurring despite the lack of a signal, as the promoter region is indicated to not be accurately captured in the design. The production of chloramphenicol by this strain was supported by *S. venezuelae* producing a zone of inhibition in antibiotic susceptible *E. coli* overlay but not inhibiting growth of a chloramphenicol resistant *E. coli* overlay. This supports the activation of GUS being indicative of the transcription and translation of the targeted BGC promoter, and hence possibly indicating the production of a secondary metabolite. However, this approach does not take into account any necessary post-translational modifications which may be crucial for the production of a natural product, and so GUS signal cannot be used as definitive proof of secondary metabolite production. The evidence of chloramphenicol production in this case, and the presence of a GUS signal for one promoter for a BGC but not another designed promoter for the same BGC illustrates the importance of promoter design.

While undertaking this study, the Lai *et al* paper (2024) was published, which also investigated region 6 of *S. venezuelae* ATCC 10712, the cryptic region that frequently produced positive GUS signals. This study found region 6 to encode natural product arcyriaflavin A and used heterologous expression to produce it, but did not find evidence of wildtype *S. venezuelae* producing arcyriaflavin A on “a range of routine *Streptomyces* growth media”, despite the use of multiple methods of detection (including high-performance liquid chromatography and mass spectrometry). This contrasts the findings of this GUS OSMAC study, as a reporter signal for this region was given in many conditions, even relatively plain/common growth media, such as NL5 and PDA, as seen in Table 6. However, the paper gave no information on what these conditions were and how many were tested. Informed by Lai *et al* (2024), it is likely that the GUS signal may be a false positive (the promoter is transcribed and translated, but the post-translational processes necessary for secondary metabolite production were not completed), and the secondary metabolite not produced. Further testing on the region 6 strain (such as mass spectrometry) would be needed to confirm or deny the presence of this secondary metabolite.

Surugamide BGC in *S. albus* was seen to be activated under some conditions, particularly those utilising elicitors in comparison to plain media, as seen in Table 9, as this BGC did not activate on plain NL5, but did on NL5 with added antibiotics.

These results align with Xu *et al* (2017), the paper identifying surugamide as the natural product encoded by the BGC, as well as its promoter region SurE which was used in this research. This paper saw surugamide activation when using high-throughput elicitor screens, specifically ivermectin and etoposide, which were unable to be tested in this study due to the lack of their availability and time constraints. This study also tested ~500 more elicitors, but this library was not listed and so the overlap of any results between this study and that by Xu *et al* cannot be assessed.

Two BGCs that did not produce a GUS signal under the other conditions were seen to activate GUS production in static liquid media (shown in Table 7). Both of these BGCs: R11 and R20 were from *S. venezuelae*, which can sporulate in liquid media, which may trigger different metabolic pathways (Gomez-Escribano *et al*, 2021). However, these reporter strains would also be sporulating on the solid media that it gave no signal on, and so sporulation alone cannot be responsible for this signal. Static media creates a group of unique conditions that may not be present in shaking liquid media or solid media. Static media creates an oxygen gradient with the bottom of the media having low oxygen (van Keulen *et al*, 2003), which may create hypoxic conditions that could trigger BGC activation (Clark and Bushell, 1995). Out of all the conditions tested, static liquid media was the only condition to supply this level of oxygen deprivation. Static growth can impose physiological stress due to local nutrient deprivation, waste buildup and pH shifts, all factors which may trigger a survival response of secondary metabolite activation (Kleyer *et al*, 2019). In addition to these stressors, liquid media also provides certain advantages that may lead to BGC activation. The production of some secondary metabolites is regulated by density-dependent signalling molecules such as γ-butyrolactones and autoinducers. Static media allows the formation high local cell densities and thus allow for the buildup of these molecules to a point that may allow BGC activation (Mukherjee and Bassler, 2019). Also, shaking provides mechanical stress (Schrader *et al*, 2023), which can disrupt the formation of different structures, such as biofilm-like pellets. Due to this, static conditions are better for allowing the formation of different morphologies, which have been seen to show different secondary metabolisms (Matsumoto *et al*, 2024). Any of these specific conditions, or perhaps a combination of them, may be the cause for the activation of these silent BGCs.

S. venezuelae’s BGC region 20 was identified by antiSMASH as having sequence similarity to the auricin cluster, however, Lee *et al* (2021) identified this as a misannotation and found that this BGC is more likely to be responsible for jadomycin biosynthesis. Jadomycin was previously seen to be produced under ethanol shock (Sekurova *et al*, 2016), but there was no GUS signal seen from the corresponding reporter strain under these conditions in this study. Jadomycin production is believed to be tightly controlled by specific promoters (Sekurova *et al*, 2016). Also, jadomycin production in *S. venezuelae* is under the control of the repressor gene *jadR2*, which requires a sufficient stressor to activate (Yang *et al*, 1995). In this research, ethanol was added to the media pre-inoculation. However, Doull *et al* (1994) found that introducing ethanol between six and thirteen hours after inoculation was most effective for triggering jadomycin production. This is theorised due to the applied stress being most effective when applied during active growth phases (Doull *et al*, 1994), meaning that adding ethanol before inoculation may not have been efficient to spur activation of this BGC. Unfortunately, time constraints on this project prevented ethanol stress testing during the growth phases, so this effect on activation could not be verified within this project. However, R20 was seen to activate in liquid LB at 28°C. This result may be because of the stress conditions that are produced by cultivating in static liquid long term, as the signal arose after a much longer incubation period than others (approximately ten days, as opposed to the usual three days).

R11 of *S. venezuelae* was seen to activate GUS production in two different types of liquid media (shown in Table 7). This BGC is cryptic (has a very low similarity to its most similar predicted product), and so its activation may provide promise for the use of OSMAC to activate silent BGCs which may encode antibiotics, although it requires further investigation (such as mass spectrometry) to confirm and characterise the presence of any secondary metabolite, as well as antimicrobial assays to assess any antimicrobial potential.

Candicidin (SA Can) and minimycin (SA R19) of *S. albus* were not seen to activate in this study. Candicidin has been seen to be always activated in *S. albus* S4 (McLean *et al*, 2016), and so the lack of a GUS signal in this study is surprising. This difference in activation could potentially be due to differences in the regulation of this secondary metabolite between the *S. albus* S4 strain and the strain of *S. albus* used in this study (*S. albus* J1074). Activation has been seen within the minimycin BGC in *S. albus*, but this activation was seen to yield indigoidine, a compound which shares the same BGC as minimycin (Olano *et al*, 2014). Activation of minimycin itself has not been seen in the literature, and the promoter designed for this region was designed focused on the likely minimycin promoter within this cluster. Therefore, the lack of minimycin activation in this study aligns with the literature.

R32 of *S. venezuelae* was never seen to produce a GUS signal. This BGC is entirely cryptic, and so its lack of activation may be due to this BGC simply not being activated under any of these conditions, or the promoter region being chosen inefficiently.

The conditions of activation of all these BGCs helps to define a framework of the minimum number of conditions needed for an effective antibiotic discovery platform, as the stressors needed for majority activation can be identified, as well as the optimum pH and osmotic stress for the strongest activation. Furthermore, this research has a secondary use of providing insight into *Streptomyces* biology that may help to elucidate the metabolic pathways taken under certain conditions and define the promoters involved in this.

Some conditions of activation were not investigated in this study. The co-culture approach of cultivating the *Streptomyces* species of interest with other microorganisms was not taken in this experiment due to the complexities not aligning with time constraints of the research. For example, culture compositions are difficult to replicate and co-culture induced activation requires further investigation to determine the specific cause(s) of the secondary metabolite production. Also, some of the impacts of co-culture can be mimicked by using elicitors, as the cause of activation within co-culture can be molecules that can be used without the need for the living organism, such as antibiotics that may be produced by microorganisms in the co-culture (Romano *et al*, 2018).

Furthermore, this research was limited, as the condition and strain combinations were tested only once due to the large number of combinations and limited time. The results of this study must be verified by repetition to support the conclusions drawn.

This study found that the major downfall of the OSMAC approach relying on GUS activation is that the promoter for the BGC must be meticulously chosen, and the entirety of this promoter must be captured in the construct for the reporter to be capable of producing a signal. If the promoter region is incorrectly selected, no GUS signal would be present even if the biosynthetic genes are activated and the natural product is produced. Therefore, some of the regions that never showed a signal might still be producing the natural product, but the promoter region was not selected correctly. The pathway may simply never be activated, but unless the promoter region is confirmed, there is a possibility that it could be a false negative due to incorrect promoter selection. Selecting promoters in some of these regions of these species is rather empirical as they are largely uncharacterised and ill-defined, relying on the utilisation of software to recognise sequences similar to known promoters. Also, the presence of multiple possible promoters, as often predicted by these softwares, lessen the chances of selecting a suitable region to adjoin to *gusA* for this investigation. This idea was shown by one of the two promoters investigated for the chloramphenicol BGC of *S. venezuelae* showing a signal, especially in nearly all conditions, but the other promoter never producing a reporter signal at all. This promoter, SV R7, was one made by the same rationale as applied to the BGCs without previously characterised promoters, and so its lack of success is indicative that this rationale does not always catch promoters, and so these BGCs, particularly those not giving signal, could be active but not identified as such due to the shortcoming of the reporter system. This issue could potentially be amended in part by creating additional reporter constructs, capturing different potential promoters, for any BGCs that did not initially show any activation. BGCs that are known to be activated in some conditions could have their promoters more accurately captured by using capped RNA sequencing to experimentally capture transcriptional start sites (Duttke *et al*, 2019). However, as this method requires the production of RNA, it would not be of use for silent BGCs and hence would not be able to be used at large when employing the GUS-OSMAC approach for antibiotic discovery.

Reporter strains for three different BGCs were not seen to give a signal in any conditions. In the *S. coelicolor* investigation by Elimam *et al* (personal communication), it was seen that some of the silent BGCs only activated under very specific conditions such as under blue light or in the presence of arsenic. These specific conditions were tested due to literature and previous research indicating the impact of these factors. As *S. albus* and *S. venezuelae* BGCs are less characterised, any potential specific conditions necessary for activation are not reported. However, as this study is in the context of assessing this method for antibiotic discovery, the focus on these conditions is those that produce a global effect: conditions that activate many separate BGCs, and so these specific requirements are not a useful consideration for a high throughput antibiotic discovery platform.

The use of the reporter system OSMAC approach in *S. albus* and *S. venezuelae* saw a lower activation profile than the use of this approach in investigating *S. coelicolor*, the project preceding this research, completed by Elimam *et al* in the same laboratory group. In *S. coelicolor*, the OSMAC approach saw a reporter signal in 80% of BGCs across different conditions (Elimam *et al*, personal communication), whereas this study only saw ~62% of BGCs tested giving a GUS signal in this study. This difference may be because more time and research were able to go into the design of the promoters for this species, and hence it could be more reflective of the true activation profile in *Streptomyces*. In addition to this, *S. coelicolor* is more extensively researched and so more promoters had previously been identified and were able to be implemented in this approach. The *S. coelicolor* investigation included a larger number of reporter constructs, and this study does not include enough strains to be seen as an overall reflection of this approach.

However, the positive signals themselves are not representative of secondary metabolite production as the necessary post-translational modifications may not occur despite the GUS signal. Therefore, the positive signals seen in this study require further investigation, such as extracting any natural products and identifying them by mass spectrometry, to note anything novel, as the BGCs can only be predicted to encode antimicrobials with limited accuracy, and so these clusters may encode unexpected and unpredicted novel secondary metabolites.

Conclusion

This study saw five of the eight BGCs investigated produce a GUS signal indicating secondary metabolite production by manipulating simple parameters. This is a lower activation rate than seen in the preceding *S. coelicolor* study of the same nature by Elimam *et al*, but still shows some promise, especially when both studies are considered together to assess the use of this approach in *Streptomyces* as a whole.

The reporter OSMAC approach taken has some potential in antibiotic discovery as it allows potential activation of cryptic or silent BGCs (as seen with *S. venezuelae*'s region 11 in this study) to be quickly identified and thus quickly investigated further. However, this approach is not to be taken as entirely definitive as the presence of a GUS signal does not confirm the presence of a natural product, as the promoter could be transcribed and translated (causing a GUS signal) but the necessary post-translational processes for secondary metabolite production may not occur. Due to this, the reporter signal can only be taken as a hint that the pathway is activated, and further investigation is still required to confirm the presence of a secondary metabolite. Similarly, the lack of a signal does not necessarily mean the BGC is not expressed, as it could be an issue in selecting the promoter, especially in poorly characterised regions. Therefore, it is likely that further investigation, such as mass spectrometry, may be needed for regions that are not producing signal as well as those that are not in order to fully investigate the secondary metabolism of a microorganism. As a result, this approach is overall useful but might not catch all BGCs, and so some of these inactivated BGCs may require further study, or additional research could be implemented to more reliably identify promoters.

Due to the majority activation seen in this study and the *S. coelicolor* study, this approach could be useful for antibiotic discovery due to the large number of BGCs activated with a low workload. Furthermore, this approach could be very useful in adding to the knowledge of metabolic pathways and promoters in *Streptomyces* due to the fast identification of production in different conditions. Adding to this knowledge could eventually loop back to aid antibiotic discovery, as the promoters being better understood may aid in accurately producing reporter constructs that truly reflect BGC activation. Also, this knowledge may link the activation of certain types of BGC to specific conditions, allowing other BGCs of this type to be activated more efficiently in future research.

Despite its uncertainties, utilising the GUS-informed OSMAC approach could potentially lead to the discovery and characterisation of novel compounds due to the GUS signals allowing BGCs with potential secondary metabolite production to be easily identified, focusing downstream efforts on the more promising pathways. Reporter-utilising OSMAC may be a worthwhile approach as it has a decent success rate in triggering the transcription and translation responsible for a GUS signal, which in some cases could represent the production of the pathway’s metabolite. This success rate is high comparable to the relatively low workload and complexity of this approach, especially as these studies have helped to define the conditions that show the highest rates of activation. This data could allow the implementation of a framework of a few conditions, such as three types of solid media and two types of liquid, that this study indicates has the potential to activate transcription and translation in the majority of *Streptomyces* BGCs, which may lead to the production of a number of natural products.

To fully assess this approach for antibiotic discovery in *Streptomyces* and bacteria as a whole, more species should be studied this way. Assessing the overall success of this method across multiple species would aid in producing an accurate rate of success in BGC activation and thus evaluating the use of this method in identifying novel antibiotics to potentially tackle the rising AMR problem. Furthermore, this testing may result in the characterisation of novel secondary metabolites along the way.

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Appendix

Solid Agar Compositions

0.5% Soft Agar	
Difco Nutrient Broth Powder	4 g
Agar	5 g
Distilled water	1000 ml

GYM (ISP2 + $CaCO_3$)	
Yeast Extract	4 g
Malt Extract	10 g
Dextrose	4 g
Agar	12 g
$CaCO_3$	2 g
Distilled water (pH to 7.2 before agar addition)	1000 ml

ISP-1 Agar	
Readymade	38 g/l

ISP-2 Medium	
Readymade	38 g/l

ISP-3 Medium	
Readymade	38 g/l

ISP-4 Medium	
Readymade	38 g/l

LB Agar	
Readymade	40 g/l

MHA-II	
Beef Extract	2 g
Acid Hydrolysate of Casein	17.5 g
Starch	1.5 g
Agar	17 g
Distilled Water	1000 ml

NL5 Medium	
L-Glutamine	5.8 g
NaCl	1 g
K_2HPO_4	1 g
$MgSO_4.7H_2O$	0.5 g
Agar	10 g
50% Glycerol (separately autoclaved, added after autoclaving)	48 ml
Trace elements solution-2 (separately autoclaved, added after autoclaving)	2 ml
Distilled water (pH to 7.3)	950 ml (to 1000 ml)

PDA	
Potato infusion	200 g
Dextrose	20 g
Agar	20 g
Distilled water	1000 ml

SDA	
Dextrose	40 g
Peptone	10 g
Agar	15 g
Distilled water	1000 ml

SFM (also known as MS)	
D-Mannitol	20 g
Soya Flour (Holland and Barret)	20 g
Agar	20 g
Tap water (warm)	1000 ml

Liquid Media Compositions

2X YT	
Tryptone	16 g
Yeast	10 g
Sodium chloride	5 g
Distilled water	1000 ml

ISP-1 Broth	
Readymade	8 g/l

LB Broth	
Readymade	20 g/l

MH-II	
Readymade	22 g/l

SOC Recovery Medium	
LB Broth	10ml
1M $MgCl_2$	100 µl
2M Glucose	220 µl

Trace element solution-1 (ActinoBase)	
$FeSO_4 . 7H_2O$	1 g
$MnCl_2 . 4H_2O$	1 g
$ZnSO_4 . 7H_2O$	1 g
Distilled water	1000 ml

Trace element solution-2 (ActinoBase)	
$ZnCl_2$	40 mg
$FeCl_3 . 6H_2O$	200 mg
$CuCl_2 . 2H_2O$	10 mg
$MnCl_2 . 4H_2O$	10 mg
$Na_2B_4O_7 . 10H_2O$	10 mg
$(NH_4)_6Mo_7O_{24} . 4H_2O$	10 mg
Distilled water	1000 ml