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Research Article

Novel Antibiotics from Actinomycetes targeting teichoic acid PG

Shilpa Chatterjee[1]*

1 Centre for Bacterial Cell Biology, Newcastle University, NE1- 7RU, UK.

ABSTRACT

WTAs (Wall teichoic acids) are cell surface anionic glycopolymers, present in the peptidoglycan (PG) layer of gram-positive bacteria. Tag T, U, V enzymes are a group of proteins, which ligate WTA polymers to the peptidoglycan. Previous research studies, have identified seven actinomycetes strains as Tag TUV inhibitors, that displayed differential activity against *B. subtilis* mutant strains *ΔtagTV* and *ΔtagUV*. To screen for differential activity and to extract and identify compounds whenever differential activity is observed. Actinomycetes strains were grown on a range of solid and liquid media, and tested for inhibitory activity against B. subtilis mutant strains *ΔtagTV* and *ΔtagUV*, using agar diffusion assays. Actinomycetes strains, exhibiting differential activity on optimized media, were chosen for large scale cultivation in bioreactors for active compound extraction. Actinomycetes strains 2161 and C17P2 displayed differential activity on optimized media. These strains, when grown in 15L working volume (wv) scale bioreactors produced compounds which were differentially active on the mutant strains *ΔtagTV* and *ΔtagUV*. Active compound extraction and purification of these strains produced fractions that were differentially active. Preliminary analysis of the mass spectrophotometry data of C17P2 identified a compound with mass 520.60. Actinomycetes strains exhibited differential activity against *B. subtilis* mutant strains *ΔtagTV* and *ΔtagUV* when grown on optimized media. After media optimization, upscale and compound purification, a semipure fraction from C17P2 strain was obtained. A compound with mass of 520.60 was identified.

KEY WORDS: Wall Teichoic Acid, Multidrug Resistance, Bioreactor, Chromatography, Mass spectrometry.

Introduction

Actinomycetes are one of the most diverse group of Gram-positive bacteria found in almost every environmental niche on the planet (1,2). Actinobacteria are predominantly inhabitants of the soil and marine ecosystem. They are generally noted for their filamentous branching growth pattern resulting in mycelium or extensive colony formation (3). Some of the species of the Actinomycetes are important pathogens *e.g. Nocardia asteroids*, the primary cause of nocardiasis, but mostly they are harmless to animals and higher plants and are beneficial sources of different antibiotics. *Streptomyces,* the largest genus of actinobacteria, deserves special mention as they are known to produce a wide range of secondary metabolites (4,5) such as antibiotics, antifungal, antiparasites, iron chelators that are not pivotal to growth but likely offers competitive advantages to the organism (6).

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***Address for Correspondence** Centre for Bacterial Cell Biology, Newcastle University, NE1 7RU, UK
E-mail: shilpachatterjee05@gmail.com

Actinobacteria are of significant economic importance to humans due to their ability to produce a wide range of specialised compounds. Actinomycetes have been for decades, one of the most important sources for the discovery of novel antibiotics. Waksman's extensive systematic screening of Actinomycetes, led to the discovery of the antibiotic *Streptomycin*, a protein synthesis inhibitor (1-3) produced by the Actinobacteria *Streptomyces griseus*, which was the first specific antibacterial agent showing efficacy not only against *M. tuberculosis* but also against gram negative pathogens like *V. cholarae* & *S. typhi* which were otherwise resistant to penicillin. This systematic screening of Actinobacteria eventually led to the discovery of other important and popular antibiotics like vancomycin and chloramphenicol (3).

To discover specialized metabolites of interest from Actinomycetes, different systematic screening methods are employed, the most commonly used of which is the Kirby

Bauer Disc Diffusion assay (7,8). In such an assay, a small filter paper disc containing liquid Actinomycetes extract is placed on an agar plate seeded with the bacterial strain to be tested. After overnight incubation of the plates, the zone of inhibition around the disks is used as a measure of determining susceptibility or resistance of the bacteria, with smaller or no zones indicating resistance of the organism to the particular compound/antimicrobial and clear zones indicating suspectibility of the organism to the compound and the presence of the antibiotic (9). Agar Plug Diffusion assay is also used for screening of secondary metabolites, often to highlight antagonism between microorganisms (10). It involves making an agar culture of the strain of interest (e.g Actinomycetes) on an appropriate culture media by tight streaking all over the plate. During growth, Actinomycetes secrete molecules which diffuse in the agar medium. Post incubation, plugs are created on the plates, cut aseptically & deposited on agar surface of another plate inoculated with the test microorganism (10,11). The zone of inhibition around the plugs is used as a measure to determine antibacterial efficacy of the active compounds produced by Actinomycetes.

This is followed by isolation of the active compound from the crude extract by several fractionation studies, its subsequent purification by TLC (Thin Layer Chromatography), HPLC (High Performance Liquid Chromatography) & its structural elucidation by using analytical methods like Mass Spectrophotometry (MS) and Nuclear Magnetic Resonance (NMR) spectroscopy (9,10). Structural elucidation is pivotal to avoid discovery of already known existing substances and is a pre requisite to develop novel antibiotics as there is an urgent need of it owing to the emergence, spread and rise of antimicrobial resistance (12).

(WTAs) Wall teichoic acids: A potential antibacterial target

Today with the advent of industrial system biology, most processes for secondary metabolite production are performed in Bioreactors (13,14). Bioreactors have been employed in the commercial production of antibiotics like streptomycin (Streptomyces griseus), Neomycin (Streptomyces marinensis), Rifamycin (Amycolatopsis mediterranei) (15). Bioreactors are particularly important as production strategies often carried out in shake flasks lack a mechanistic understanding of antibiotic production processes, offering poor prospects for successful scale up (16,17). Bioreactor is a fermentation vat used for growing living organsims like bacteria in industrial scale (18). The fermentation technique primarily used in the production of secondary metabolites is the submerged fermentation technique (SmF) (18,19). Development of submerged culture techniques has enhanced the cultivation of actinomycetes in large scale operations (19,20). In this method the substrates used for fermentation are always in the liquid state comprising of all the essential nutrients required for microbial growth. It is more beneficial, cost effective and less labor intensive than the solid-state fermentation process (21). Such processes demand the precise control of the physiological environment of the culture i.e the temperature, pH, O2 concentration, nutrient level, media flow rate etc which is attained by making use of bioreactors (20,21). The main purpose of bioreactors is to accomplish conditions where cell biomass can grow efficiently resulting in the production of secondary metabolites (21).

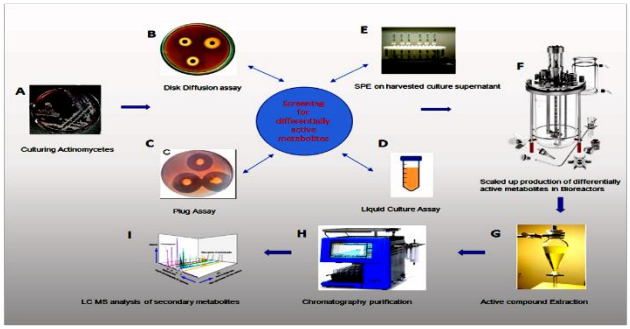


Figure 1: Schematic representation of the techniques employed in this project for screening and purification of secondary metabolites from actinomycetes.

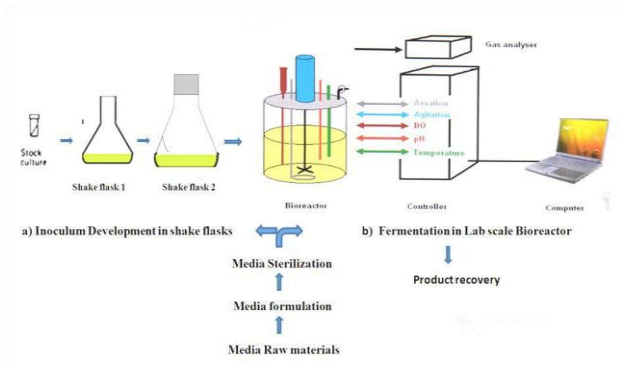


Figure 2: Schematic representation of the fermentation techniques employed in this project.

The emergence, spread and persistence of multi drug resistant (MDR) strains has rendered the conventional antibiotic therapy ineffective and futile & is thereby considered as one of the greatest global threats to human health (22, 23). This reiterates the urgent need for exploration and development of novel therapeutic strategies for combating/controlling the rise of MDR strains (32). The cell wall of bacteria has been a very important source of targets for development of antimicrobials so far (24,25).

The cell wall of gram-positive bacteria is composed of several peptidoglycan (PG) layers with alternative units of MurNAc (N-Acetyl Muramic acid) & GlnNAc (N-acetyl glucosamine) residues playing an important role in osmotic stability and cell shape maintenance (31). WTAs (Wall teichoic acids) are the most abundant PG linked cell surface anionic glycopolymers present in gram positive bacteria, almost as abundant as PG (22,24), playing a plethora of important functions including regulation of cell division, cell growth, morphology, ion homeostasis affecting cell wall rigidity and porosity (41), biofilm formation (29), host tissue adhesion (30) as well as conferring antibiotic resistance (39,40). Due to their crucial role in bacterial pathogenesis (26), WTAs are possible novel targets for development of novel therapeutics to overcome antimicrobial resistance (27,28). Most research studies so far with WTAs have focused on the non-pathogenic gram-positive model organism B. subtilis and the human pathogen Staphylococcus aureus (27,30).

Wall teichoic acids (WTA) biosynthesis:

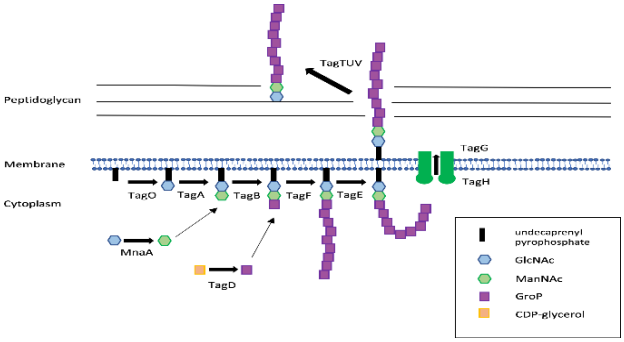


Figure 3: WTA assembly in B.subtilis 168CA. The WTA polymer is synthesised in the cytoplasm by Tag proteins O, A, B, F, E, before its export to the cell surface, through the Tag GH transporter, residing in the membrane. The process is finally completed with the ligation of the WTA polymer to PG by the Tag enzymes Tag T, U,V ligase. (GlnNAc – N- Acetly glucosamine), (ManNAc --N – Acetyl Mannosamine). Modified from (Brown et al. 2006)

WTA polymers are synthesized in a sequential fashion on an undecaprenyl phosphate carrier lipid by a series of Tag/Tar enzymes localized on the inner surface of the cytoplasmic membrane and then exported to the cell surface by an ABC transporter system (Tag GH). The WTA polymers are then covalently attached to the Muramic Acid residue on PG (36). In Bacillus subtilis 168, a family of enzymes, LCP(LytR-CpsA-Psr) proteins (27,31) namely Tag T/U/V ligase have been identified that catalyzes the ultimate step for attachment or ligation of the WTA polymer to PG. It was previously reported by Kawai et al (43) that knocking out of the corresponding genes tag T, U, and V is necessary to produce the lethal phenotype.

The pathway for WTA biosynthesis was first characterized in B. subtilis 168CA which makes polyglycerol phosphate WTA. WTA biosynthesis begins in the cytoplasm by a series of enzymes namely, Tag O, A, B, F, E before its export through the Tag GH transporter to the cell surface. Following formation of GlcNac-pp polymer by Tag O, an N acetyl Mannosaminyl transferase Tag A, transfers ManNac from UDP ManNac to the 4-OH of GlnNAc Residue to form a disaccharide. This dissacharide then becomes the substrate for Tag B, which is the glycerophosphate transferase, that transfers a single phospho glycerol unit from UDP glycerol to C4-OH of ManNac to complete synthesis of linkage unit (33,34,35), Next Tag F, a polymerase cytidyl transferase attaches 35 or more glycerol phosphate to the linkage unit to form WTA Anionic polymers. Once assembled the lipid linked WTA polymer is putatively modified by Tag E, the glycosyl transferase and then exported to the external surface from the bacterial membrane by the 2 component ATP binding cassette transporter (ABC transporter) Tag GH. WTA is then finally attached to PG by a series of enzymes which had remain unidentified so far, but was first reported by Kawai et al (53) as Tag T, U, V ligase which catalyzes the ultimate step in locating /attaching WTA polymer to the peptidoglycan.

It was by searching for Mre B interacting partners that led to the identification of these proteins by Kawai et al (43). Kawai et al (43) identified the 3 proteins namely, Tag T, U, V ligase of LCP (Lyt R-cps 2A-Psr) family in Bacillus subtilis 168CA of previously unknown function. As the single mutants of Tag T, U, V didn't have any discernible effect on cell growth and morphology, cell combination of double mutants namely Tag TU, Tag TV and Tag UV were

generated and tested for functional redundancy. The mutants Tag TU and Tag UV didn't differ noticeably and showed similar morphology and growth rate to that of the WT whereas the Tag TV mutant grew at a much slower rate than the WT exhibiting occasional bulges with wider cell morphology, implying an element of functional redundancy.

The triple mutant Tag TUV (mutant with all three ligases Tag T, U, V knocked out) although lethal rescued through the disruption of protein Tag O. By means of a PG WTA phosphate content assay, Kawai et al demonstrated that the cell wall of the double mutant Tag TU contained around 95% and that of the Tag T, U, V contained around 10% of the phosphate content as compared to that of the WT. As it's known that, WTA are the only source of phosphate in the cell wall, it can be inferred that WTA does have a predominant role to play in WTA PG attachment.

Tag T, U, V paralogues have a role in the ultimate step of WTA synthesis in B. subtilis have been shown by means of genetics studies as well as by measurement of WTA of mutants (36,37,38)

In addition to the afore mentioned studies, Kawai et al (43) also studied the co cystal structure of S. pneumoniae Cps 2A (an LCP homologue) identifying a polyisoprenoid binding pocket, observing a Mg 2+ in a location, indicating a role in phosphorolysis, suggesting CpsA's role as a phosphotransferase. It was demonstrated by Kawai et al (43) that Tag T & Cps 2A both produced a monophosphprylated lipid from a dephosphorylated substrate in Mg2+ dependent fashion. It was based on these findings, Kawai et al (43) concluded that Tag T, U, V are WTA ligases even though it doesn't constitute any direct evidence in support of the hypothesis. However, it was only recently reported & confirmed that S. auerus mutants lacking the Tag TUV homologues lcpA, lcpB,and lcpC displayed altered cell morphology compared to the WT(36,39,40). Not only this, by means of in vitro chemo enzymatic assays, it has been shown very recently that all 3 proteins can transfer WTA precursors to nascent PG establishing the fact that LCP proteins are nothing but PG linked polymer ligases in B. subtilis (41).

The WTA biosynthetic pathway has been proposed as a promising antimicrobial target, as its disruption could be lethal due to the toxic buildup of WTA intermediates (37,40,42). WTA inhibition in Methicillin resistant S. auerus has been reported to restore sensitivity to β lactam antibiotics (,3848). WTA inhibitors are useful as antibiotic targets. An antibiotic named targocil (40) inhibiting the WTA pathway have been identified in S. aureus which could be potentially used in combination with other β lactam antibiotics (42,44). WTA PG ligases are representative of potential antimicrobial targets owing to their presence outside the cell membrane (27,30).

Differential Screening to identify novel compounds targeting WTA-PG ligase.

In search of a novel Tag TUV inhibitor Demuris Ltd (Newcastle, Upon Tyne) devised a HTS (High Throughput Screen) using mycelia extract from 2820 Actinomycetes strains against B.subtilis WT 168CA (Wild type) and different mutant strains ΔtagTV and ΔtagUV. The hypothesis is that a double knock out would make the mutant strains hypersensitive to the inhibitors for the third ligase.

Demuris has collection of more than 6000 actinomycetes strains, isolated from diverse habitats (Table 1) that are capable of producing different natural products (48). In the previous project, B. subtilis 168CA mutants ΔtagTV and ΔtagUV were used for screening the actinomycetes extracts. Six candidate strains identified as Streptomyces sp showed differential inhibitory activity against the WT and the mutant strains (48). In this study the same actinomycetes were regrown, retested and studied for antimicrobial activity, for further isolation, extraction, characterization and purification of potential secondary metabolites.

The overall aim of this project is to screen the seven candidate actinomycetes strains for differential activity against B.subtilis 168CA mutants ΔtagTV and ΔtagUV. The project will also aim at carrying out scaled up production of the differentially active metabolites in lab scale bioreactors, with the eventual intention of extraction and purification of the differentially active metabolites.

Material and Methods

Bacterial Strains and Culture conditions:

Seven Actinomycetes strains namely T471, A263, B2121, NI095, 2161, C17P2 and S34(Table 1) identified from previous studies by Devi & Allenby 2013 as potential Tag inhibitors, by high throughput screening of 2820 Actinomycetes extracts were chosen for this study. These strains were cultured in GYM agar (Glucose 4g/L, Yeast

4g/L and Malt extract 10g/L, pH 7.0) media at 30◦C for 7 days.

Test strains:

Cultures of Bacillus strains (Table 2) were used as test strains for screening the Actinomycetes extracts. The Bacillus strains were maintained on Nutrient Agar media with appropriate supplements (10µg/mL chloramphenicol) for the mutants. B.subtilis overnight cultures were incubated on a shaker at 30◦C using 10mL Lysogeny broth(LB) and 0.125 mL of 40% glucose solution to block sporulation.

Phylogenetic Analysis:

Phylogenetic studies were based on the 16S rRNA sequence of the candidate actinomycetes strains, to identify the 16S rRNA. BLAST searches against database was conducted identifying nearest neighbor using the Ez taxon database. The16S rRNA sequences were aligned using Clustal W and a neighborhood joining tree was constructed using Mega X software package to determine the novelty of the candidate strains, using generalized time reversible substitution model and 1000 Bootstrap replicates with Bootstrap Testing. A similarity table was made with the nucleotide difference of all seven candidate strains and its nearest neighborhood/top hit taxon.

B. subtilis growth curve:

B. subtilis WT 168CA and mutant strains ΔtagTV and ΔtagUV were grown overnight in 10 mL of L.B with Chloramphenicol (10µg/mL) supplement for the mutant strains. The next day 400µL of overnight culture was transferred to 40mL of fresh LB. Optical density readings were taken every 1 hour over a 6 hours period by using a spectrophotometer (Thermo Scientific), set at an amplitude wavelength of 600nm.

Antimicrobial Assays:

Kirby Bauer's Disc Diffusion Assay:

Antibiotics were prepared for the disc diffusion assay by diluting from stock concentrations and then further sequentially diluted to give the desired concentrations. Sterile milliQ water was used as a solvent for making all the antibiotic concentrations, except for streptonigrin, where Di methyl sulphoxide (DMSO) was used.

A variation of the Kirby Bauer's test was used for the agar disc diffusion assays. The agar media was prepared by mixing 25mL Pen Assay Broth (PAB) with 25ml of molten nutrient agar (NA), the mixture was left to cool for 10-15 minutes in a 50◦C water bath. The medium was then inoculated with 50µl of overnight cultures of B.subtilis and poured into a 120mm×120mm square petri dish and left to set for at least 20 minutes. The antibiotics were spotted onto a 6mm Whattman grade 1 filter paper disc and air dried for 10 minutes. Discs were placed on the surface of the agar plate seeded with bacterial culture to be tested and incubated at 30◦C overnight. The zone of inhibition around the discs was measured the next day, to check for antibacterial activity of the antibiotics.

Agar Plug Assay

The antimicrobial activity of the Actinomycetes isolates was checked by using the Plug Assay. Test strains of B.subtilis WT 168CA and mutants ΔtagTV and ΔtagUV were prepared by growing their overnight cultures in 10mL of LB with chloramphenicol (10µg/mL) supplement for the mutant strains at 30°C.

Actinomycetes strains were grown on actinomycetes growth media, at 30◦C for 7 days. After 7 days, plugs were cut out of the 7 days old Actinomycetes plate, by using a 1mL pipette tip. The plugs were levered out using two sterile 5 µL loops and placed carefully on a 120mm×120mm petri dish for activity assay. The agar medium was prepared and inoculated following the same protocol as the disc diffusion assays and poured around the plugs gently without disturbing the plugs and left to set. The plates were incubated at 30◦ C overnight. The diameter of the zone of inhibition around the plugs was measured the next day to determine the antibacterial activity of the actinomycetes plugs.

Liquid Culture Assay

A 5µL loop full of Actinomycetes culture was used to inoculate 40mL of Actinomycetes growth media (Table 4) in 250mL (wv) Erlenmeyer flasks and placed on a shaker at 30°C. After growth, 20 mL of Actinomycetes culture was pipetted into a 50 mL falcon tube & centrifuged at 9000 rpm for 12 minutes. The supernatant was poured into a fresh 50 mL falcon, and tested for activity against the Bacillus test strains using the disc diffusion assay.

Solid Phase Extraction (SPE) Assay

Actinomycetes extracts which showed no activity or very weak activity in the liquid culture assays were concentrated using SPE. For SPE, Actinomycetes extracts were prepared as above. SPE was performed using Hyper Sep

C18 cartridges (ThermonScientific) and a vaccum manifold. The cartridge was equilibrated with 8mL methanol (MeOH) and washed with 8mL of milli Q water before addition of actinomycetes extract which was eluted. A further 8ml of MeOH was loaded and eluted. This elute was evaporated using a GeneVac centrifuge evaporator, suspended in (200µL) of DMSO and used for Activity as previously described.

Freeze Dried Extract Assay

Actinomycetes extract which showed no activity in liquid culture assays, were filter sterilized using MILLEX-HA filter Unit 0.22um & 20 ml BD Plastipak Syringe. The filtered extracts were freeze dried to concentrate the samples. Extracts were then suspended in DMSO and 10 µl of the extracts were tested against Bacillus Subtilis (WT 168CA) and mutants ΔtagTV and ΔtagUV strains using Disc Diffusion method.

Growth Curve study of Actinomycetes

A 500 mL working volume (wv) flask was inoculated with 5% exponentially growing actinomycetes culture. Samples(20mL) were taken every 24h. The optical density was measured at 450nm in triplicates due to the mycelial nature of actinomycetes. The residual sample was centrifuged at 13000 rpm for 10 minutes and the supernatent was either concentrated using SPE or directly used in activity assays.

Large Scale cultivation in Bioreactors

Bioreactor cultivations were performed in 20L scale autoclavable bioreactors (Applikon Biotechnology). An initial working volume of 15L at 30°C and aeration of 15L/min were used. Dissolved O2 tension (DOT) was set to a minimum of 40% saturation which was controlled via a cascade control over the stirrer speed and an oxygen valve. The stirrer speed was set to a minimum of 250 rpm and a maximum of 750 rpm. Once the maximum agitation was reached pure oxygen(2L/min) was sparged in addition to compressed air. The pH was not controlled throughout the fermentation but set at the onset to pH 7 using 1M NaOH. The growth medium was supplemented with 0.05% of PPG with an automated addition if more was required. The bioreactor was inoculated with 500mL of exponentially growing actinomycetes culture. Throughout the fermentation, 30mL of aliquots were retreived daily for activity test and OD measurements.

Amberlite Extraction

For Amberlite extraction, Amberlite resins (XAD-16, 20g/L) were added. The resin was separated from the broth by filtering through sieve and washed with deionized water before treatment with methanol. 300g of resin was then washed with 2500mL of methanol, filtered and dried under pressure using a rotary evaporator. The crude extract obtained after methanol evaporation was tested for activity against Bacillus strains. The pH of the elute was adjusted to 7.0 and treated with ethyl acetate, mixed thoroughly for 10-15 minutes & allowed to separate organic-aqueous phase using separating funnel. Organic phase was dried to obtain weight of the crude organic fraction and resultant compound was re suspended in methanol for activity test against Bacillus strains.

Normal Phase Chromatography (NPC)

The extract was dry loaded onto 10mL of silica gel. Sample was loaded onto a 100g SNAP ultra biotage cartridge. A linear gradient from Dichloromethnae(DCM) to Methanol(MeOH) was performed using a flow rate of 100mL/min on a Isolera Prime(Biotage) solvent handling machine. The resulting fractions were tested using a disc diffusion assay.

Reverse Phase Chromatography

The extract was filtered through a 0.22µm filter prior to chromatography analysis. A linear gradient from MeOH to water was performed using a flow rate of 50mL/min on a Isolera Prime (Biotage)solvent handling machine. The eluted fractions were tested for activity using disk diffusion assay.

LC MS analysis

LC MS analysis was carried out using Agilent Technologies 1260 infinity system The compound separation was attained by an Agilent Eclipse C18 Plus coulmn(4.6× 150mm×3.5µm) with an integrated diode array detector and for injecting sample, 100µl of the sample was prepared by dissolving it in 900µl of milli Q sterile water.

Table 1: Source of actinomycetes strains:

Strains	Lab Code	Source of the putative Actinomycetes strains for Tag Inhibitor as per the Demuris report (54)
N1095	DEM 10965	NA
C17P2	DEM 20570	Root system of Paraserianthes falactaria, Indonesia
B2121	DEM 20572	Root system of Paraserianthes falactaria, Indonesia.
S34	DEM 20808	Carmarthen Bay, UK, 4.5 miles offshore. <46 metres deep
T471	DEM 21088	Garden Soil, Yogyakarta, Indonesia
2161	DEM 30270	Rhizosphere soil of wild tea plants (Camellia oleifera), China
A263	DEM 30132	Barley grain.

Table 2: Relevant genotype of *Bacillus*

Test Strains	Relevant genotype
WT 168CA	trp C2
168CA TagUV	trpC2Δ tag U(cat) ΩtagV::pMutin4
168CA TagTV	trpC2Δtag T (cat) ΩtagV::pMutin4

Results

Subtilis growth curve study

The B.subtilis double mutant ΔtagUV displayed a more or less similar growth pattern to that of the B.subtilis WT 168CA(Wild Type). B. subtilis double mutant ΔtagTV exhibited a much slower rate of growth(Figure 4) compared to the WT 168CA. This was consistent with the previous findings of Kawai et al (43)

16S rRNA Phylogenetic Analysis

Six Actinomycetes strains were identified as Streptomyces based on their 16S rRNA gene. Five of these matched the same strain or nearest type strain previously identified by Devi and Allenby, 2013. Strain T471 was previously identified as S.rangoonensis while this analysis identified it as S. flocculus, the strain from which streptonigrin was originally isolated(44). Strain A263 was identified as Streptomyces cacaoi which was previously identified as Streptomyces hydrogenans. Strain N1095 previously identified as Nocardia sp, was identified as Gordonia jacobaea.

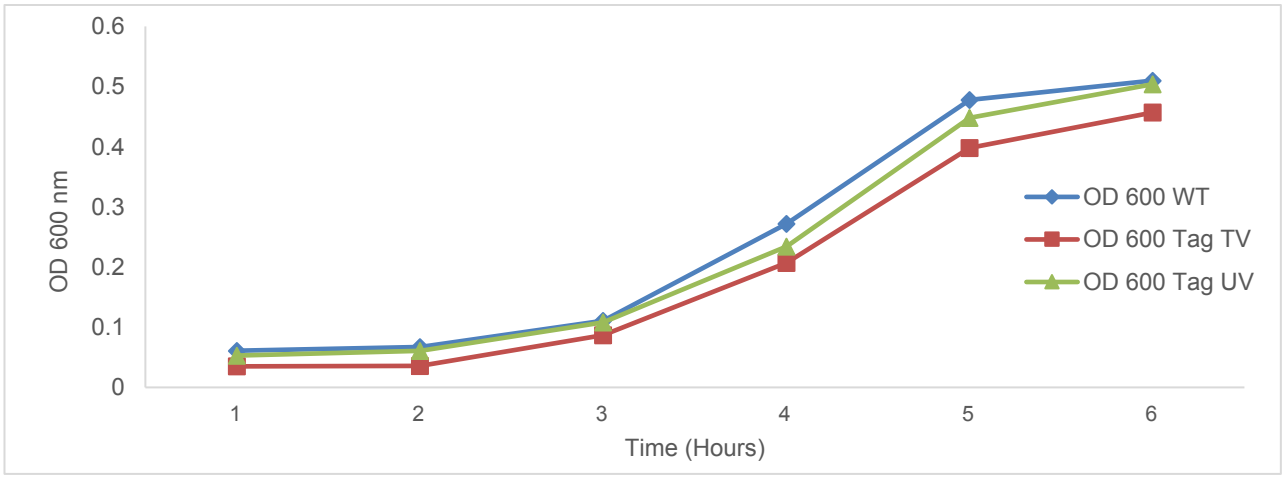


Figure 4: *B.subtilis 168CA mutant growth curves: Curves showing differential growth pattern of B.subtilis WT 168CA, Δ*tagUV *and Δ*tagTV *double mutant strains, OD were taken at 600nm every one hour over a 6 hour period.*

Table 5: *Similarity Table of the Actinomycetes strains with their closest match in the EzTaxon database*

Strain	Nearest strain based on 16s rRNA gene sequence	Nucleotide Differences	Similarity %	Previous identification
T471	*Streptomyces flocculus*	12/1493	99.10%	*Streptomyces rangoonensis*
A263	*Streptomyces cacaoi*	0/1485	100%	*Streptomyces hydrogenans*
B21R1	*Streptomyces bingchenggensis*	9/1490	99.38%	*Streptomyces bingchenggensis*
C17P2	*Streptomyces seoulensis*	7/1488	99.52%	*Streptomyces recifensis*
2161	*Streptomyces sanglieri*	0/1407	100%	*Streptomyces sanglieri*
S34	*Streptomyces hydrogenans*	8/1483	99.51%	*Streptomyces hydrogenans*
N1095	*Gordonia jacobaea*	0/1484	100%	*Nocardia nova*

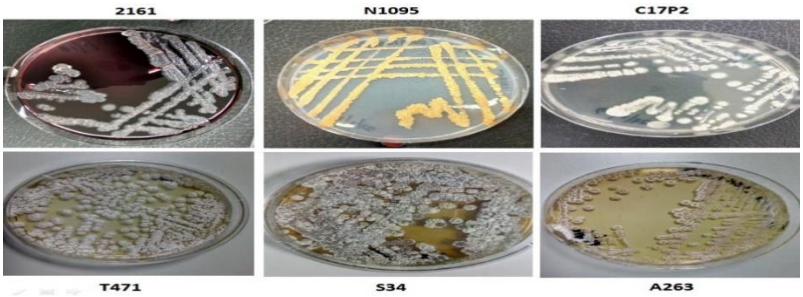


Figure 5: *Colony morphology of actinomycetes strains: Colonies of actinomycetes on GYM agar plate after 7 days of growth.*

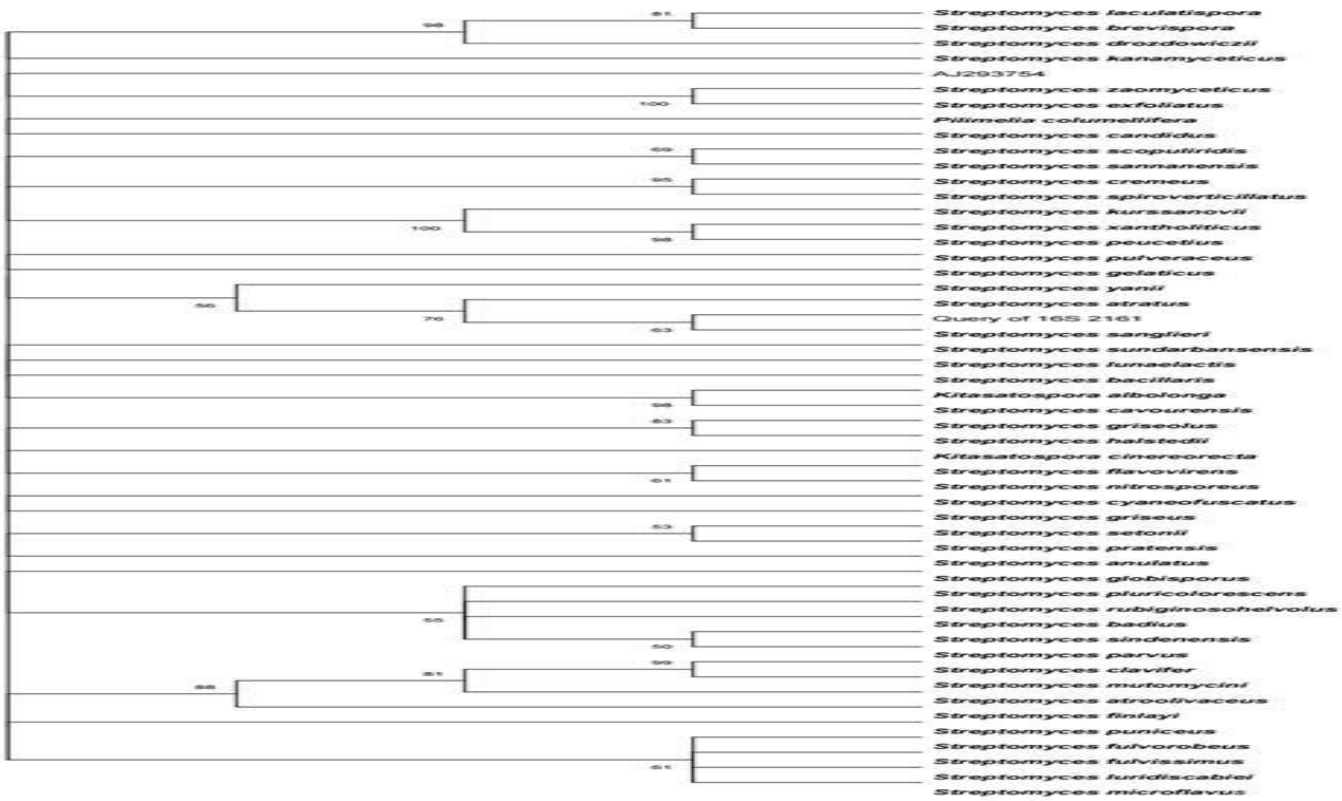


Figure 6: *Phylogenetic tree of strain 2161: Tree based on a 1407 bp region of the 16srRNA gene showing its nearest neighbour. Tree rooted with Amycolatopsis mediterranei (i.e. AJ293754 as mentioned in the tree.*

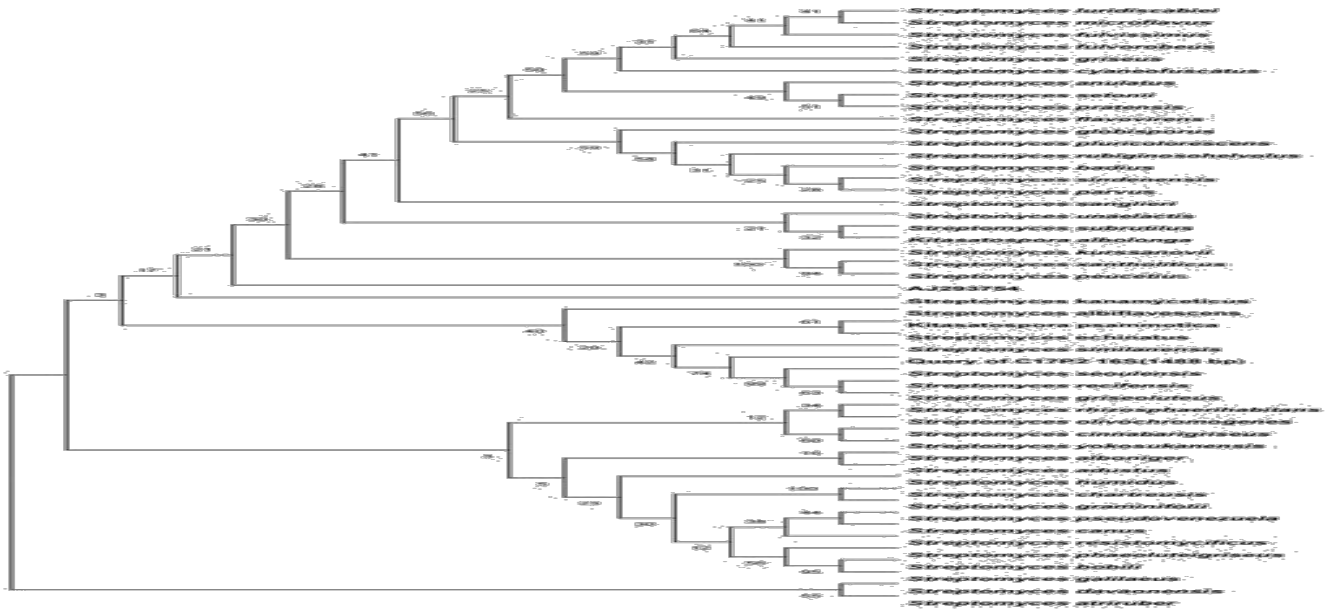


Figure 7: *Phylogenetic tree of strain C17P2 based on a 1488 bp region of the 16srRNA gene showing its nearest neighbour. Tree rooted with Amycolatopsis mediterranei.*

Disk Diffusion Assay:

Test for positive and negetive controls:

To validate the theory that B.subtilis mutants were only more sensitive to a specific antibiotic produced by actinomycetes, disc diffusion assays were conducted using a comprehensive panel of 12 antibiotics. Two fold dilutions of the antibiotics were made, using a concentration gradient of 12.5-200µg/mL. In general, the antibiotics showed similar patterns of inhibitory activity between the WT(Wild Type) and the mutant strains, Δtag TV and Δtag UV with no zone of inhibition >1mm for the mutant strains. Only streptonigrin(an antibiotic produced by the actinomycetes strain T471) as per the Demuris report(48)showed differential activity against both the mutant strains, with substantial differential activity against the ΔtagTV mutant strain producing zone of inhibition >4mm for the Δtag TV (Figure 13) mutant strain compared to the WT. This was expected and was consistent with the previous findings of the Demuris report(48).

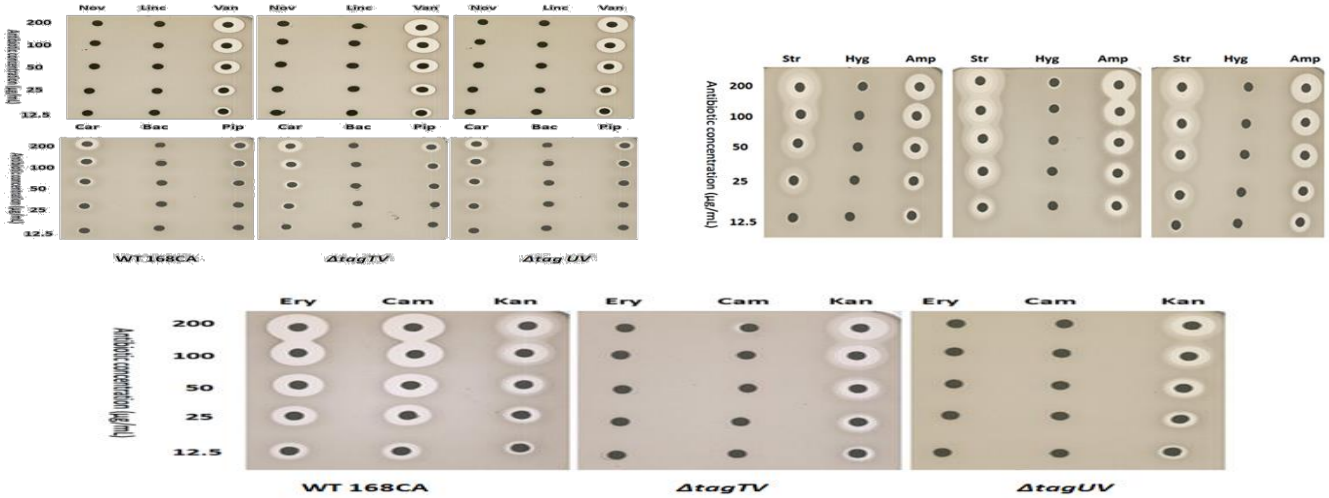


Figure 13*: Representative antibiotic disk diffusion assays. Antibiotics represented with their abbreviations. Ery-erythromycin, Cam-cholramphenicol, Kan- kanamycin, Str- streptonigrin, Hyg - hygromycin, Amp- ampicillin. An antibiotic concentration gradient from 200µg/mL was used for the assay.*

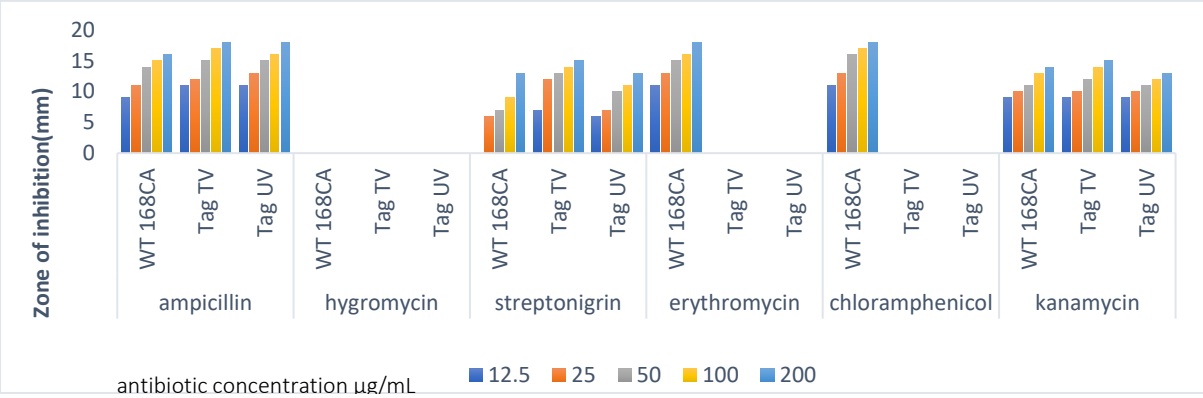


***Figure 14**: Representative antibiotic disk diffusion assay: Antibiotics represented with their abbreviations. Nov- novobiocin, Linc- lincomycin, Van- vancomycin, Car- carbenicillin, Bac-bacteriocin Pip- piperacillin. An antibiotic concentration gradient from 200µg/mL was used.*

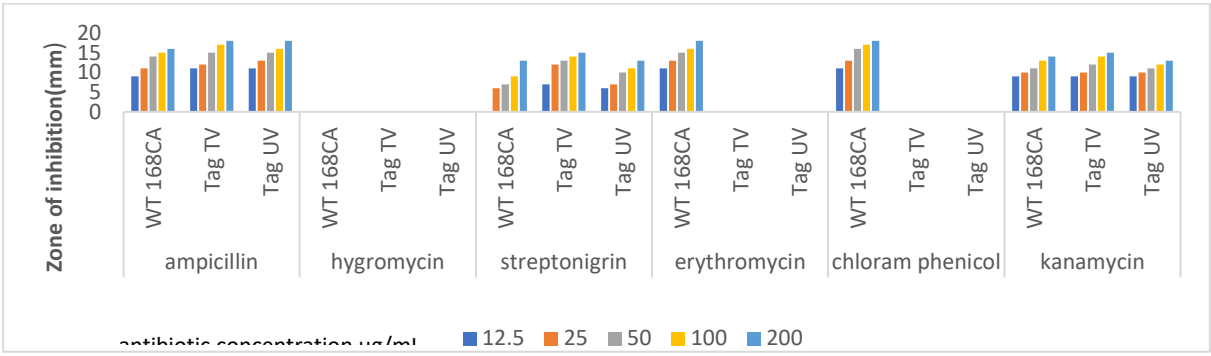


Figure 15: Antibiotics inhibitory activity at various concentrations against B.subtilis 168CA WT and mutant strains.

Exploratory Agar Plug Diffusion Assays:

Screening for differential activity of actinomycetes strains:

An initial agar plug diffusion assay (Figure 16) was conducted from GYM plate, using 6 of the 7 Actinomycetes strains namely A263, T471, C17P2, S34, 2161 & N1095, grown on GYM agar media for 7 days. Strain B2121 was not a part of this assay as it did not grow on GYM media.

Only strain T471 showed differential inhibitory activity against the ΔtagTV strain with zone of inhibition 3 mm larger than the WT(Figure 16A and B). Strain S34 showed minimal zone of inhibition against all 3 strains. Strain 2161 produced a heavy pigment making it difficult to interpret if a genuine zone of inhibition was present or not.

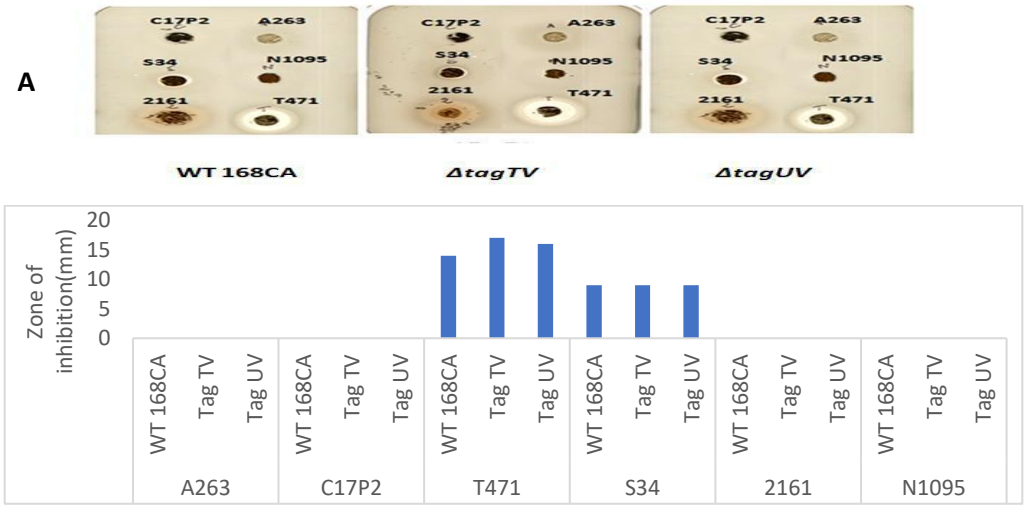


***Figure 16:** Preliminary agar plug assay from GYM media: A: Plate photograph showing activity of different actinomycetes plugs against B.subtilis WT 168CA & mutant strains. B: Bars representing zone of inhibition(diameter) of actinomycetes plugs against B.subtilis WT 168CA & mutant strains.*

Liquid Culture Assay

For purification of compounds, liquid culture assays were conducted alongside plug assays, to establish reproducible production in liquid media. Disk diffusion assay was used to test activity of actinomycetes extracts(10µL of each extract) using the same six Actinomycetes strains (as mentioned above) grown in liquid GYM broth for 7 days at 30°C in the shaker. Only strain T471 displayed differential inhibitory activity with zones of inhibition 3 mm and 5 mm larger for ΔtagTV and ΔtagUV respectively compared to the WT(Figure 17A and B). This was expected as T471 is known to be a streptonigrin producer as previously mentioned. No other strains showed any activity.

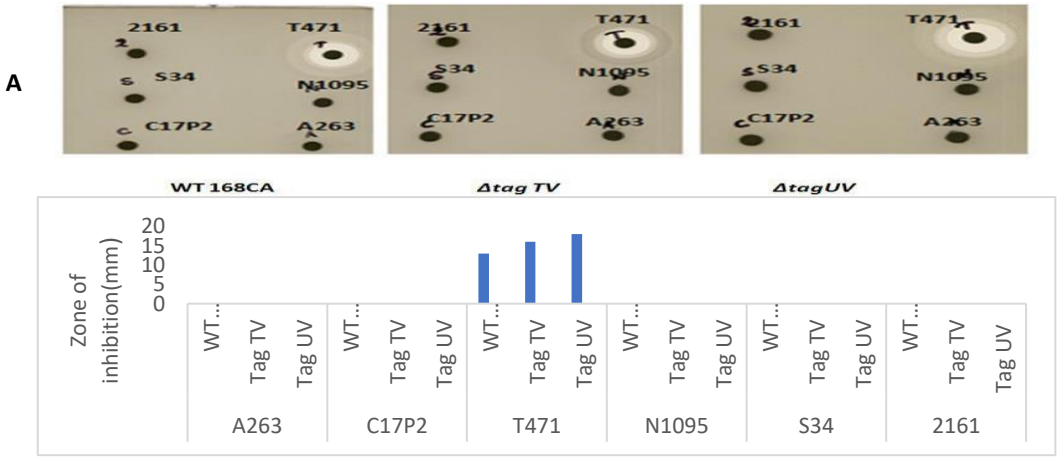


Figure 17: *Preliminary agar disk diffusion assays from GYM media: A: Plate photographs showing activity of actinomycetes (T471) liquid culture extract against B.subtilis WT and mutant strains. B: Bars representing zone of inhibition(diameter) of actinomycetes(T471) extract against B.subtilis WT and mutant strains.*

Media Optimization study

As most of the strains showed very little or no activity in the initial Agar Plug Diffusion Assay from GYM media, media optimization study was considered by growing the strains using a panel of six different optimized media namely RA3, GLM, V6, AF/MS, INA5 & M8 agar media, to determine if the differential inhibitory activity of the strains could be enhanced by altering the nutritional composition of the growth medium.

Actinomycetes strains exhibiting slight or noticeable differential activity activity when grown on solid optimized media were regrown in liquid culture using that same medium. As the liquid culture extracts exhibited no activity like the preliminary disk diffusion assay, SPE(Solid Phase extraction) on those extracts were performed to concentrate them and used alongside to screen for differential activity.

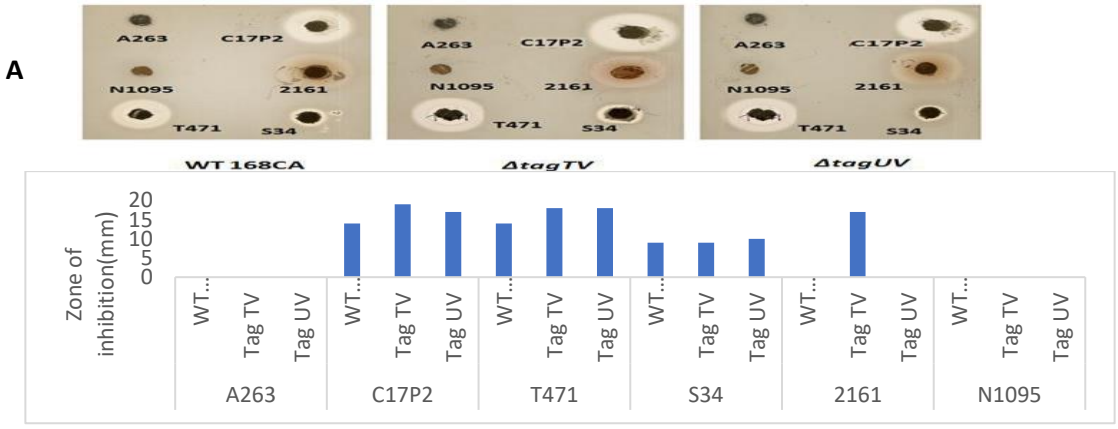


Figure 18: Plug assay from optimized media RA3. A: Plate photograph showing activity of different actinomycetes plugs against B.subtilis WT and mutant strains. B: Bars depicting zone of inhibition(diameter) of actinomycetes plugs against B.subtilis WT and mutants.

Strain C17P2 showed differential activity against the Bacillus mutant strains with zone of inhibition 5 mm and 2 mm larger than the WT for ΔtagTV and ΔtagUV mutant respectively(Figure 18A and B). Strain 2161 was only active against ΔtagTV produing a clear halo. Strain T471 was also differentially active against both the mutant strains ΔtagTV and ΔtagUV. Strain S34 displayed very slight differential activity against ΔtagTV. As strains C17P2, 2161 and S34 were differentially active when grown on solid RA3 optimized media, they were regrown in liquid culture using the same media as shown below to screen for reproducible production in liquid optimized media for subsequent upscaling(Figure 19A).

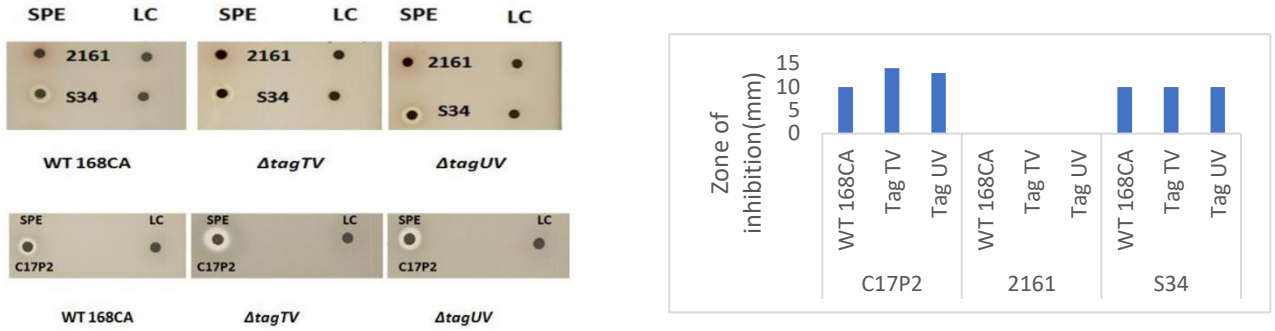


Figure 19: *Disk diffusion assays from optimized media RA3. A: Left lane showing activity of SPE (Solid phase extracts of different actinomycetes) & right lane corresponds to the LC (Liquid culture extracts) of actinomycetes. Bars depicting zone of inhibition of SPE extracts of actinomycetes against B. subtilis WT and mutant strains.*

The solid phase culture extracts (SPE) of C17P2 only showed differential activity against the mutant strains, with zone of inhibition 4mm larger than the WT for ΔtagTV mutant (Figure 19A and B). The solid phase culture extracts of S34 although produced tiny halos against all three strains were not differentially active. The solid phase culture extract of 2161 did not show any activity at all. The liquid culture extracts on the other hand were completely inactive for all three strains.

Liquid culture assay was not carried out for the T471 strain, instead of it being differentially active in the plug assay from optimized media RA3, as it produced differential activity in liquid culture assays from GYM media as previously shown. Moreover T471 strain being a known producer of streptonigrin was a false positive in the assay and hence was deprioritized for further media optimization studies.

To screen for differential activity of the other actinomycetes strains media optimization assays were continued. The actinomycetes strains were next grown on V6 media to screen for differential activity as shown below.

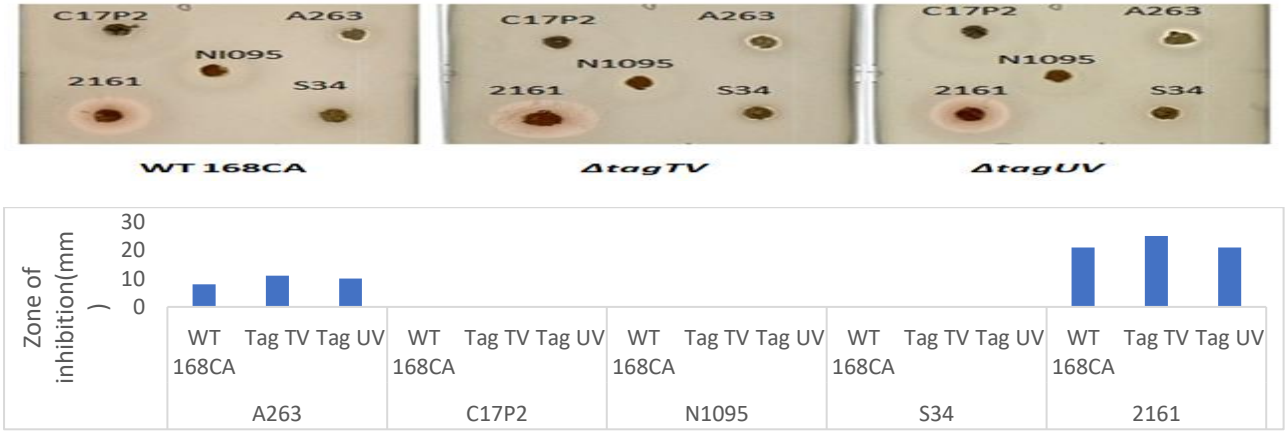


Figure 20: Plug assay from optimized media V6. A: Plate photograph showing differential activity of actinomycetes plugs against B.subtilis WT and mutant strains. B: Bars depicting zone of inhibition(diameter) of actinomycetes plugs against B.subtilis WT and mutants.

Strain 2161 exhibited differential activity against the mutant strain ΔtagTV with a zone of inhibition 4mm larger than the WT, when grown on V6 agar media (Figure 20A and B). Strain A263 displayed very slight differential activity. Liquid culture assays were hence conducted with the culture extracts of 2161 and A263. Only solid phase culture extract (SPE) of strain 2161 produced clear halos against both the mutants with no noticable activity against the WT (Figure 21A).

Secondary metabolite fermentation:

As strains C17P2 and 2161 exhibited differential inhibitory activity, in optimized liquid media (Table 7), growth curve study of both these strains in their respective optimized media was performed. The purpose of the growth curve study was a) to find the relationship between growth and antibacterial agent production in optimized liquid media and b) to determine the onset of secondary metabolite production in submerged culture. RA3 media was used for scaled up production of C17P2 as this strain exhibited noticeable differential activity when grown in both solid and liquid RA3 media (Table 6 and 7), whereas V6 media was used for scaled up production of 2161 for the same reason. (Table 6 and 7).

Growth study of strain 2161: As strain 2161 showed reproducible activity in optimized liquid media V6, we chose it for growth curve studies in both shake flask and bioreactor. The strain was at first grown in 500 mL(wv) shake flasks to screen and validate results of the media optimization assays i.e secondary metabolite production prior to its cultivation & scaled up production in 15L (wv) bioreactors.

An optical density of 3.9 was reached after 96 hours of incubation (Day 5) when strains were grown in bioreactor compared to 0.45 when grown in shake flasks (Figure 26A and C). The supernatant harvested on Day 4 and 5 from bioreactor produced larger zones of inhibition compared to that of the shake flask (Figure 26 D). Clear halos were only observed against the two mutant strains ΔtagTV and ΔtagUV on both occasions (Fig 26 B and D). Hence the supernatant/extract was differentially active.

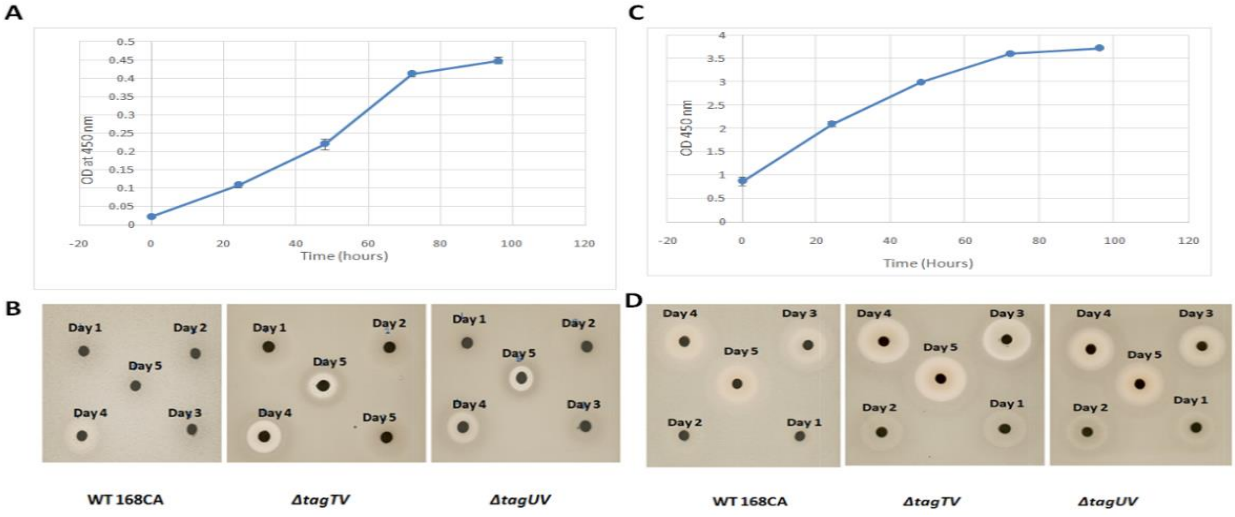


Figure 26: *Secondary metabolite fermentation of strain 2161: A: Growth Curve of 2161 strain when grown in shake flasks. B: Disk diffusion assay of extract harvested from shake flask C: Growth Curve study of 2161 strain when grown in bioreactor. D: Disk diffusion assay of harvested extract from bioreactor.*

No activity was observed for the supernatant harvested on day 1 and 2 from both shake flask and bioreactor (Figure 26 B and D). The supernatant harvested on day 3 from bioreactor also exhibited differential activity against both the mutant strains (Figure 26D).

Growth Curve study of C17P2

The C17P2 strain was at first grown in 500 mL (wv) shake flasks to screen for secondary metabolite production, prior to its cultivation & scaled up production in 15L (wv) bioreactors. An optical density of 3.7 was reached after 96 hours of incubation (Day 5) when strains were grown in bioreactors (Figure 27B) compared to 0.9 when grown in shake flasks (Figure 27A). The supernatant retreived on day 4 and Day 5 from the bioreactor was found to be differentially active on both the mutant strains (Figure 27C).

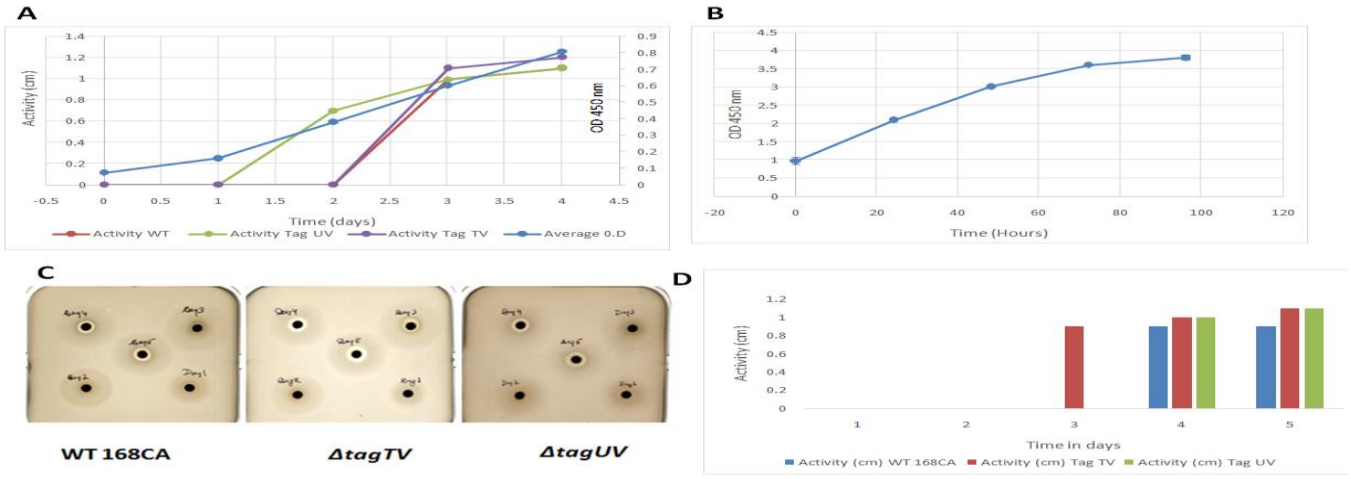


Figure 27: *Secondary metabolite fermentation of strain C17P2: A: Activity vs OD graph of C17P2 strain when grown in shake flask B: Growth curve of C17P2 when grown in bioreactor. C: Disk diffusion assay of C17P2 extract harvested from bioreactor D: Bar chart showing zone of inhibition of C17P2 extract harvested from bioreactor against B.subtilis WT and mutant strains.*

Bioreactor monitoring and control: The effects of agitation and aeration rates on the production of secondary metabolites in the 15L(wv) bioreactors (Figure 29) were systematically investigated thoughout the fermentation runs. An example is cited for cultivation of strain 2161. In the (figure 29) the variation of the dO2 (dissolved oxygen) and stirring speed during bioreactor cultivation of 2161 is shown. No decrease in dO2 level was observed upto 10 hours post inoculation of actinomycetes, indicating low metabolism of actinomycetes, that is characteristic of adaptation of the microorganism to growth conditions (i.e lag phase). From this time point, level of dO2 dropped significantly which can be attributed to microbial growth and metabolism, until about 40% saturation was reached and remained at this value for about 80 hours, during which the stirring speed was automatically increased to near the operational limit (750 rpm).

Figure 28: *Image of 15L (wv) Bioreactors (Applikon Biotechnology) used in this project*

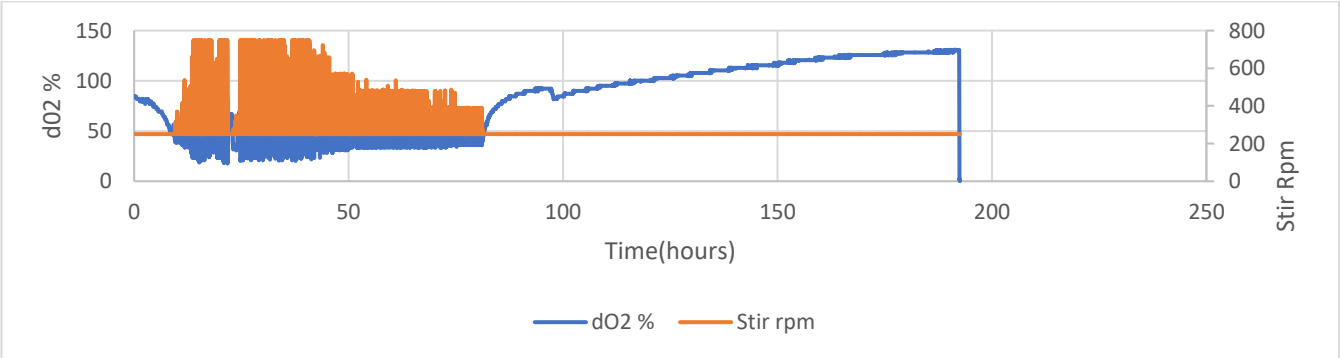


Figure 29: *Variation of dO2 and stirring speed in bioreactor cultivation of 2161 strain.*

Extraction and purification of differentially active compounds

Based on differential activity observed from media optimization assays and bioreactors, strains 2161 and C17P2 were prioritized for active compound extraction.

Active compound extraction of strain 2161

As substantial differential activity was observed for the 2161 extract retreived from the bioreactor, on day 4 and 5 (Figure 26D). The product from bioreactor was harvested on Day 6 and amberlite extraction was performed, as amberlites beeds are crucial for adsorbtion of secondary metabolites. The crude extract obtained post amberlite extraction exhibited differential activity against the mutant strains producing clear halos only against the mutant strains (Figure 30A). A smallscale trial was conducted to check at which pH the solvent extraction using ethyl acetate is best. The pH of the crude extract was adjusted to pH 4,7,10 and tested for activity, as the aqueous fraction showed differential activity in all three pH(Figure 30B), pH 7 was chosen at random and ethyl acetate extraction was performed using a separating funnel. Only the aqueous fraction was found to be differentially active (Figure 30C).

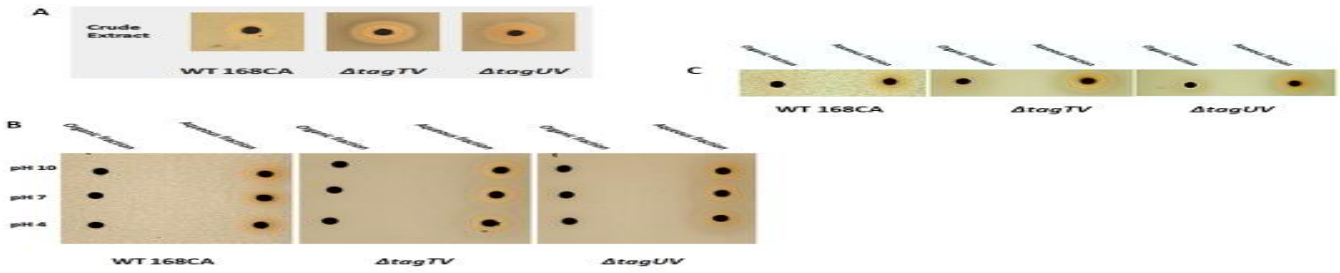


Figure 30: *Strain 2161 active compound extraction. A: Disk diffusion assay of crude extract. B : Disk diffusion assay of crude extract at different pH(Left Lane-Organic fraction, Right lane-Aqueous fraction). C: Disk diffusion assay of crude extract post ethyl acetate extraction (Left Lane-Organic fraction Right lane- Aqueous fraction).*

As the aqueous fraction was differentialy active against the mutant strains ΔtagTV and ΔtagUV, we assumed that our compound of interest stayed in the aqueous fraction and subsequent chromatography assays were done with the aqueous fraction. The dried organic fraction weighed had a crude weight of 1.56g. Reverse phase chromatography was performed on the active aqueous fraction using a MeOH to water gradient.

Post Reverse phase chromatography, 69 eluted fractions were collected (Figure 31A: Disc assay result of first 46 fractions are shown) and tested only against ΔtagTV , which was most sensitive to the 2161 strain as per the agar diffusion assay results. Fractions 2,3,25 were found to be active against the mutant strain ΔtagTV (Figure 31A). These 3 active fractions were then tested against the WT and ΔtagUV to screen for differential activity. Fraction 2 was found to be active only against the mutant ΔtagUV (Figure 31B) & hence was differentially active. HPLC analysis on these active fractions suggested that the fractions were not clean enough (Figure 32), so it was not progressed further for mass spectrophometry analysis.

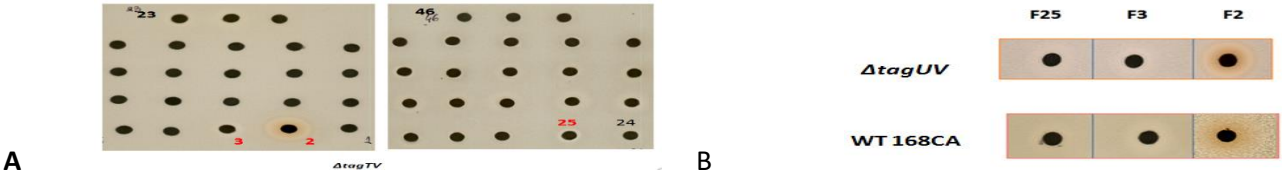


Figure 31: *A: Reverse phase chromatography eluted fractions disk diffusion assay of strain 2161 . Active fractions highlighted in red. Agar seeded with B.subtilis* ΔtagTV *strain. Numbers signify eluted fraction number. B: Representative disk diffusion assay with Reverse phase chromatography active eluted fractions of strain 2161 against WT 168CA and* ΔtagUV.

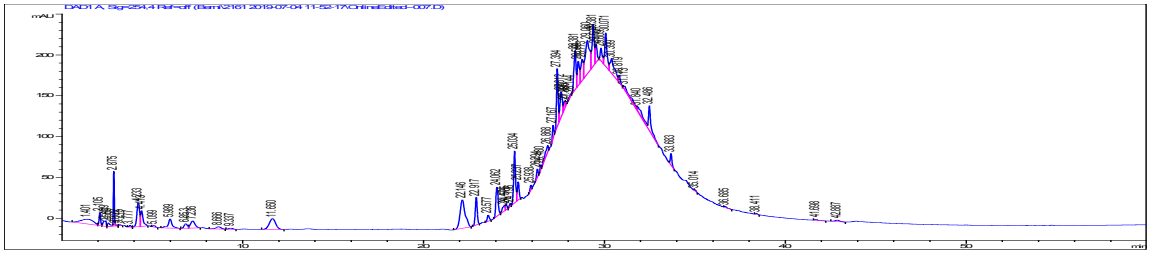


Figure 32: *HPLC chromatogram of eluted differentially active fraction(Fraction 2) of strain 2161.*

Extraction and purification of differentially active metabolites from C17P2:

As differential activity was observed for the C17P2 extract retreived on day 4 and 5 from the bioreactor as previously described (Figure 27C), the product from bioreactors was harvested on Day 6 and amberlite extraction was performed. The crude extract obtained post amberlite extraction exhibited differential activity against the mutant strains (Figure 33A) with a larger zone of inhibition against ΔtagTV mutant. Petroleum ether extraction was carried out at first to get rid of the lipids followed by ethyl acetate extraction. A smallscale trial was conducted to check at which pH the solvent extraction using ethyl acetate is best. The pH of the crude extract was adjusted to pH 4, 7 and 10 and tested for activity, as the extract showed the best differential activity at pH 7(Figure 33B), the pH of the entire extract was adjusted to 7 and ethyl acetate extraction was carried out with this extract.

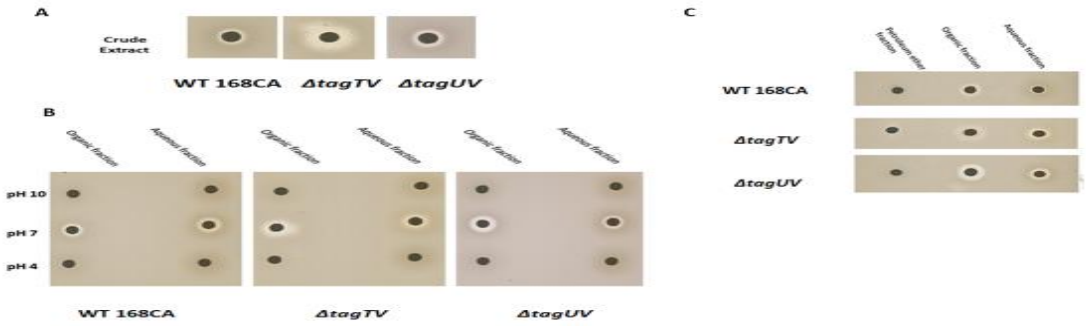


Figure 33*: Strain C17P2 active compound extraction: A: Disk diffusion assay of Crude extract B: Ethyl acetate extraction using B.subtilis 168CA ΔtagUV and ΔtagTV. C. Disk Diffusion assay post petroleum ether and ethylacetate extraction(Left lane – Petroleum ether fraction(inactive) Middle lane- Organic fraction Right lane- Aqueous fraction)*

Post ethyl acetate extraction both the organic phase and aqueous phase were tested for activity, as the organic fraction exhibited differential activity(figure 33C) producing larger halos against ΔtagUV strain, it was assumed that our compound of interest stayed in the organic fraction. The organic fraction had a dry weight of 1.02 g.

The activity observed for the aqueous fraction could be attributed to remains of our compound of interest in the aqueous phase, owing to improper separation.

59 eluted fractions were collected post chromatography and tested for differential activity against the Bacillus strains. Fractions 16,17,21,23,27,29 were active against the WT(Data not shown). Only fraction 16 was differentially active against ΔtagTV and ΔtagUV producing clear halos against both the mutant strains, with a much larger halo for ΔtagTV mutant strain compared to the WT (Figure 34). A mass spectrophotometry analysis was done on this differentially active fraction 16.

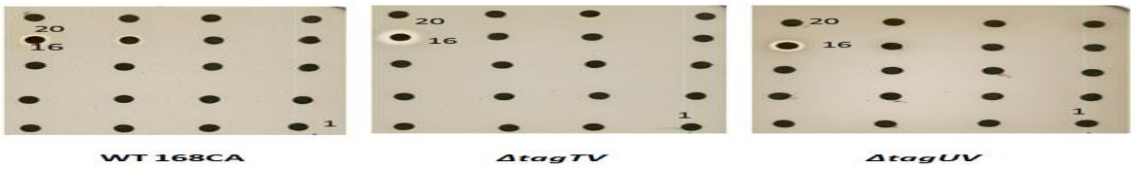


Figure 34: *A Normal phase chromatography eluted fractions disk diffusion assay of strain C17P2 . Agar seeded with B.subtilis* ΔtagTV *strain. Numbers signify eluted fraction number.*

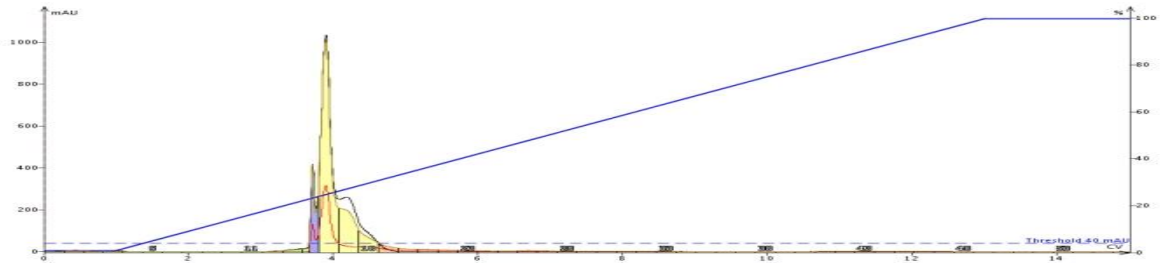


Figure 35 : *Gradient used for chromatography analysis : Normal phase chromatography performed with an Isolera Prime (Biotage), with a DCM (Solvent A) to MeOH (Solvent B)gradient and a flowrate of 100 mL/min over 50 minutes.*

4.9 Strain C17P2 putative compound identification:

Analysis of MS data for the differentially active fraction 16 revealed a peak of interest (Figure 36). This peak corresponded to a compound with accurate mass of 520.6026. The Dictionary of Natural Products (DNP) database was searched for compounds with same accurate mass in a range of +/ - 1. The peak matched to several putative compounds. Alisamycin, Albofungin, Ainsliadimer B were identified as the three top hits.

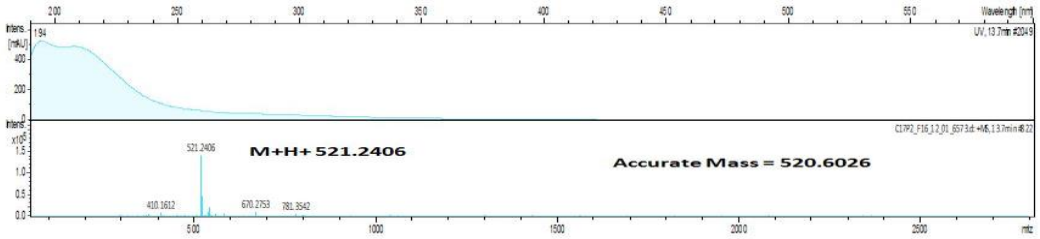
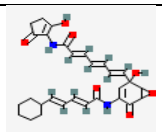
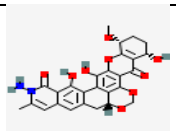
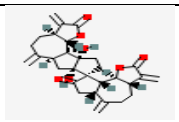


Figure 36: *MS spectrum of C17P2 fraction plotted against Intensity of compound Vs m/z ratio.*

Table 8 : Nearest match of compounds identified from DNP database:

Name of top hits identified	**Molecular formula**	**Biological Source**	**Chemical Structure**	**Accurate mass**	**Biological Importance**	**Spectrum of Activity**
Alisamycin	$C_{29}H_{32}N_2O_7$	*Streptomyces Actuosus* (Actinomycetes)	 Source: Pubchem	520.60	Weak Antitumor Activity	Primarily Against Gram positive bacteria including *B.subtilis*
Albofungin	$C_{27}H_{24}N_2O_9$	*Streptomyces Tumemacerans* (Actinomycetes)	 Source: PubChem	520.49	Potent Antitumor Activity	Antifungal & antibacterial activity
Ainsliadimer B	$C_{30}H_{32}H_8$	*Ainsliaea Macrocephala* (Flowering Plant)	 Source: PubChem	520.69	Anticancer & Anti Inflammatory Activity	Antihelminthic & antibacterial activity

Discussion

Actinomycetes strains display differential inhibitory activity

This project validated the previous work of the Demuris team (48) in demonstrating that, the actinomycetes strains produced antibiotics that exhibited differential activity against B.subtilis 168CA ΔtagTV and B.subtilis 168CA ΔtagUV strain. In the preliminary disc diffusion assay, of all the antibiotics used, only streptonigrin, isolated from strain T471 (as per the Demuris report) (48), showed differential inhibitory activity against the mutant strains ΔTagTV and ΔTagUV , which points to the fact that the differential inhibitory activity was the result of an uncommon antibiotic produced by the actinomycetes strains. One possible reason, why some antibiotics like streptongrin exhibited differential activity against the mutant strains can be attributed to the reduced wall teichoic acid concentration in the bacterial cell wall of the mutants. It was reported previously by Kawai et al (43) that ΔtagTU double mutants of B.subtilis displayed a reduced WTA concentration, the fact that the other mutants ΔtagTV and ΔtagUV also display similar WTA concentration can be inferred from this. Therefore it can be hypothesised that the reduced WTA concentration in the bacterial cell wall of the mutant strains, makes them less robust and more accessible to the antibiotics acting on the third wall teichoic acid ligase.

Five strains A263, T471, C17P2, B2121 and 2161 exhibited differential inhibitory activity when grown in solid optimized media & three strains namely A263, C17P2 and 2161 exhibited differential activity in both solid and liquid optimized media and were obvious choices for upscale in bioreactors for compound extraction and purification as reproducible activity in liquid optimized media makes upscaling significantly easier.

Strain N1095 failed to display any inhibitory activity, which was previously reported as the least active strain according to the Demuris report (48). However, with further media optimization differential activity may be observed. As we received desired results for the other strains, a further optimization was not attempted for this strain.

Strain B2121 showed substantial differential activity in solid optimized media but didn't grow at all in the liquid media, which can be attributed to the fact that Streptomyces often require a solid surface to grow on. However due to time limitations in the upscale for compound purification this strain was also deprioritized.

Strain T471 was not considered for active compound extraction in spite of exhibiting differential activity in the preliminary agar plug assay from GYM media as well as in the media optimization assay from RA3 media, as it was a known producer of streptonigrin as per the Demuris report(48) and hence a false positive in the assay. It is currently not known why streptonigrin which forms a complex with DNA and topisomerase II resulting in DNA cleavage(44) would show up positive in this screen. Further information about this and the validation of the screen might be obtained when further molecules are successfully identified.

Strain S34 displayed very slight activity in the preliminary agar plug assay from GYM plate but didn't produce any noticeable differential activity in the subsequent media optimization assays and hence was deprioritized.Strain A263 displayed good differential activity in both solid and liquid media but due to time constraints in this thesis, was not taken further and remains the subject for further studies.

Differentially active compounds extracted from strains C17P2 and 2161

Strains C17P2 and 2161 were prioritized for active compound extraction, as both these strains exhibited substantial differential activity in both solid and liquid media in the media optimization assays (Table 6 and 7) Strains 2161 and C17P2 were cultivated in lab scale bioreactor to upscale and maximise the production of the active compound. These strains, when grown in 15L working volume(wv) scale bioreactors produced compounds which were differentially active on the mutant strains ΔtagTV and ΔtagUV. Active compound extraction and purification of these strains produced fractions that were differentially active. A semipure compound was isolated from C17P2 strain that exhibited differential activity against the mutant strains and mass spectrophotometry analysis on this semipure compound was conducted.

Time limitation prevented a MS analysis on the active chromatographic fractions obtained from strain 2161. HPLC analysis was conducted on these fractions, but these fractions were not clean enough to make any conclusion. The reasons for obtaining unclean fractions can be attributed to improper separation, which might have increased the chances of the other contaminants from the reaction mixture to elute with our compound of interest or due to the similar retention times of the components in the aqueous fraction, making them co elute at the same time. Moreover it has been observed throughout this study that this strain clearly

produced a red pigment which might have interfered with the purification. As the HPLC analysis yielded unclean fractions, they were not progressed further for MS analysis.

Active compound produced by C17P2 strain

Preliminary mass spectrophotometry analysis based on the semipure compound extracted from strain C17P2 showed a peak (Fig) indicating mass with hydrogen ion (M+H+). This lead to the identification of the accurate mass of 520.6026. A literature search using the dictionary of natural products obtained two close hits produced by actinomycetes (Table 8) namely alisamycin (accurate mass =520.60) isolated from *S.actuosus* (46) and albofungin (accurate mass = 520.49) isolated from *S. tumermacerans* (47) Activity of Alisamycin against *B.subtilis* has been previously reported (ref).

As the fraction used for MS analysis was only semipure further work needs to be conducted to confirm this and structure elucidation should be attempted using NMR and Xray crystallography.

Conclusion

To summarize, the actinomycetes strains exhibited differential inhibitory activity against B.subtilis mutant strains, when grown on optimized media(Table 6 and 7). Detailed phylogenetic tree was constructed for each of the strains. Actinomycetes strain 2161 was mostly active against ΔtagTV mutant strain and media optimization and upscale was successfully conducted howerver due to time constrains a successful identification of the active compound was not achieved. After media optimization, upscale and compound isolation, a semipure fraction from isolate C17P2 was obtained, a compound with a mass of 520.2406 was identified, which might be one of the two compounds alisamycin or albofungin.

Table 8: Summary Table:

Strain	Preliminary plug assay from GYM plate	Plug assay from optimized media	Liquid Culture assay from optimized media	Scale up in lab scale(15l wv) Bioreactors	Organic/ Amberlite extraction	Purification/MS
A263	NA	Inhibits best on INA5 media	Inhibits best on INA5	ND	ND	ND
B2121	Didn't grow in GYM	Inhibits best on INA5 media	Didn't grow on the liquid optimized media.	ND	ND	ND
C17P2	NA	Inhibits best on RA3 media	Inhibits best on RA3 media.	Successful	Crude extract differentially active on both mutants.	Semi pure fraction obtained. A compound with mass 520.60 identified.
T471	Differential activity observed	Inhibits more on RA3 media	ND	ND	ND	ND
S34	Slightly active	NA	NA	ND	ND	ND
N1095	NA	NA	NA	ND	ND	ND
2161	Unclear zones of inhibition	Inhibits mostly *ΔtagTV on* V6, M8, RA3 media	Inhibits best on V6 media.	Successful	Crude extract differentially active on both mutants.	Purification not successful, unclean fractions obtained post HPLC.

NA- No activity ND- Not done

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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