Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences

Antibiotics from the nature - culturing of genetically talented bacterial isolates

Elinor Bertholtz

Department of Microbiology
Independent project • 15 hec • First cycle, G2E
Biology with specialisation in Biotechnology - Bachelor's Programme •
Examensarbete/Sveriges lantbruksuniversitet,
Institutionen för mikrobiologi, 2014:8 • ISSN 1101-8151
Uppsala 2014

Antibiotics from the nature - culturing of genetically talented bacterial isolates

Elinor Bertholtz

Supervisor: Joakim Bjerketorp, Swedish University of Agricultural Sciences, Department of Microbiology

Assistant Supervisor: Jolanta Levenfors, Swedish University of Agricultural Sciences, Department of Microbiology

Examiner: Bengt Guss, Swedish University of Agricultural Sciences, Department of Microbiology

Credits: 15 hec
Level: First cycle, G2E
Course title: Independent project in Biology - bachelor project
Course code: EX0689
Programme/education: Biology with specialisation in Biotechnology - Bachelor's Programme

Place of publication: Uppsala
Year of publication: 2014
Title of series: Examensarbete/Sveriges lantbruksuniversitet, Institutionen för mikrobiologi
No: 2014:8
ISSN: 1101-8151
Online publication: http://stud.epsilon.slu.se

Keywords: antimicrobial, antibiotics, actinomycetes, secondary metabolites, NRPS, PKS-I, PKS-II, DTS

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences
Uppsala BioCenter
Department of Microbiology

ABSTRACT

Bacterial infections are a threat to the modern healthcare because of the increase of the antibiotic resistance that many bacteria have developed during the history of antibiotics that began with the discovery of penicillin 1928. In order to respond to the accumulating resistance, novel antibiotics have to be discovered constantly. Therefore, there is an increasing need to exploit the ability of microorganisms to produce secondary metabolites with an antimicrobial effect. The current report addresses methods to activate silent genes coupled to the production of secondary metabolites. These procedures involve usage of microbial culture media with various composition, different culturing conditions such as high or low oxygen level as wells as addition of previously reported supplements that may trigger the expression of silent metabolic pathways for novel antibiotics. The different approaches used affected the metabolite production both by activating or suppressing the observed antimicrobial effects and possibly also resulted in the production of previously not detected substances. Some of the obtained substances will be further investigated in order to determine their structure and putative antimicrobial effects in more detail.

TABLE OF CONTENTS

INTRODUCTION

Infectious diseases caused by bacteria and fungi resistant to antibiotics are an increasingly severe threat to the modern standards of healthcare. The morbidity and mortality continue to rise worldwide as well as the costs coupled to the treatment of these infections. Finding treatments for these diseases has been and still are of great importance. In the early mid 20´th century the development of antimicrobial medicines experienced a breakthrough after Alexander Fleming found out that the mold *Penicillium notatum* produced a substance currently known as penicillin that inhibited the growth of *Staphylococcus aureus* on plates (Fleming 1929). Along with the emerging use of antibiotics the bacteria became resistant to the treatments. As early as 1944, *S. aureus* was reported to make use of the enzyme β- lactamase to eliminate the effect of penicillin. To deal with this problem, scientists created semisynthetic variants of penicillin, for example methicillin, that *S. aureus* eventually also became resistant to (Neu 1992). Nowadays disease causing bacteria resistant to various antibiotics is a serious problem in many hospitals, not to mention infections caused by Methicillin Resistant *S. aureus* (MRSA). These bacteria are resistant to virtually all types of antibiotics (Enright et al. 2002). Hence there is a critical need to find new antibiotics, and one of the places to investigate is once again among the secondary metabolites produced by microorganisms. One approach that is used in the search of new antibiotics is testing the influence of different culturing conditions such as culture media with different composition, aeration and so forth to investigate the impact on the production of secondary metabolites. Research conducted by systematically varying the culture conditions has shown that one talented microbial species can produce surprisingly many different compounds. This methodology is called One Strain- Many Compounds (OSMAC) (Bode et al. 2002).

Another approach for the discovery of new antibiotics is to detect genes that are connected to the synthesis of bioactive compounds such as secondary metabolites, and then try to activate them in order to obtain more interesting substances (Ayuso-Sacido and Genilloud, 2005, Xie et al. 2014). There are still enormous amounts of bacterial species left to be explored and even more substances to discover from these species that may be used for various applications in both industry and healthcare.

Examples of antibiotic- producing organisms

Intense investigation in the last almost 80 years has resulted in the screening of many microorganisms. Particularly genera of filamentous bacteria and fungi have been attracting much interest in the search for active compounds. Also *Pseudomonas, Bacillus* and the cyanobacteria are included in the list of potential producers of valuable substances (Donadio et al., 2002). Frequently mentioned in antibiotic discovery related publications are species belonging to the phylum Actinobacteria. They are Gram- positive bacteria known to produce not only antibiotics but also metabolites active against cancer and suppressors of the immune system (Baltz 2008, Zotchev 2012). Of all antibiotics that were discovered in the years between 1940 and 1960, 70-80% was isolated from *Streptomyces* species (Bérdy 2005, Bush 2010). Streptomycin, produced by *Streptomyces griseus*, is one example of an antibiotic that is produced by *Actinobacteria* spp.

Other examples of antibiotics produced by actinobacteria are Tetracycline, Erythromycin and Daptomycin (Mahajan and Balachandran 2012). Both actinobacteria of marine origin (reviewed by Manivasagana et al. 2013, Zotchev 2012,) and terrestrial origin (reviewed by Kumar et al. 2013) have been revealed to produce antimicrobial substances against both Gram-positive and Gram-negative bacteria that are multi-drug resistant. Recent studies have been focused on rare actinobacteria also called non-streptomycetes in the search for novel secondary metabolites as reviewed by Tiwari and Gupta (2012).

Genetic approach in the discovery of novel antimicrobial agents

The enzyme families type I polyketide synthase (PKS-I), type II polyketide synthase (PKS-II), non-ribosomal peptide synthetase (NRPS) and diterpene synthases (DTS) has been linked to the synthesis of secondary metabolites from different microorganisms (Ayuso-Sacido and Genilloud 2005, Xie et al. 2014).These large enzymes are considered to be multifunctional, meaning that one single protein has the ability to generate various compounds. PKS-I manufacture reduced polyketides, PKS-II produce aromatic polyketides and NRPS create non-ribosomal peptides. The fourth mentioned enzyme DTS generate diterpenoides (Xie et al. 2014). A recent report proposes a method to achieve metabolites from the respective enzymatic class by using primers targeted to the corresponding gene as a criterion which isolate to cultivate (Xie et al. 2014). After screening 100 strains of actinobacteria, the authors choose 16 strains that had the potential to produce all the four classes of natural products. One of them, a strain of *Streptomyces griseus,* was showcased to produce several metabolites from all these different classes of natural products. The advent of sequencing has facilitated the search of genes responsible of secondary metabolite production. Bentley et al. (2002) reported the whole genome sequencing of *Streptomyces coelicolor* and found out that its genome possess a large number of potentially interesting genes for antibiotic production. Baltz (2008) compiled the total genome sizes and compared that to the size of PKS-I/-II and NRPS- genes of several bacterial species. In addition to actinobacteria, species from the genus *Pseudomonas* showed big potential for antibiotic production due to its relatively large proportions of PKS- and NRPS-genes in their genomes.

Activating silent genes

Sequencing of *Streptomyces* species reveals that they do contain many genes coding for the enzymatic pathways for the synthesis of bioactive compound, but only a few of these end products were actually detected when the organism was cultured at standard laboratory conditions (Bentley et al. 2002). The silent genes can hold great potential to encode for enzymes that make novel antibiotics and several methods to activate these pathways have been reported. For example, by introducing mutations in gene *rpoB* which encode for the β- subunit in RNA polymerase, the production of secondary metabolites by some microbes increased (Ochi and Hosaka 2013). Other approach for activating genes involved in secondary metabolite expression is adding selected supplements that affect the relevant gene expression in the cultures. Rare earth elements such as Scandium have been shown to trigger the production of both certain enzymes and also the production of secondary metabolites in microorganisms (Inaoka and Ochi 2011). Triclosan is a formerly

used antimicrobial ingredient in toothpaste (which is still used in parts of the world) that recently has also been examined for its effect as a trigger of antibiotic production (Craney et al. 2012).

As a defense mechanism, bacteria can use signaling substances as pheromones in a way called quorum sensing, that allows them to communicate with each other. Sufficient concentration of these pheromones can either activate or suppress genes encoding for enzymes involved in e.g. secondary metabolite production (de Kievit and Iglewski 2000). One example of signaling molecules that particularly Gram-negative bacteria use is N-Acylhomoserine lactones (AHLs), which chemical structure have been described (Yajima 2014). Gram-positive bacteria in contrariwise, use γ-butyrolactone (GBL) as signal substance (Takano et al. 2000).

Culturing of talented bacterial isolates

When the technologies that are known as high-throughput screening (HTS) were introduced in the beginning of 1990´s it was believed to revolutionize the search for new drugs (Persidis 1998). Older methods like fermentation and testing against target pathogens had to step aside for the modern techniques. Unfortunately HTS did not reach the expected success (Livermore 2011). Very few new antibiotics have been discovered since the antibiotic golden age in the 1940´s, and nowadays the majority of the current antibiotics are derivatives of the ones that were originally discovered as secondary metabolites of actinobacteria. Currently, it is believed that lots of the antibiotics that can be produced by actinobacteria are still left to be discovered. Research at companies and universities must only put more efforts and re-introduced screening of enough numbers actinobacteria with improved methods (Baltz 2008).

Scope of this investigation

In the current report, a few Gram-positive isolates of actinobacteria that hopefully might be used as putative antimicrobial agents were chosen for their potential ability to produce secondary metabolites. The choice of microbes for antibiotic research, nowadays, can be based on their genetic potential to produce metabolites. Genes that are linked to the production of metabolites can be screened in order to investigate if they are present within the organism. Sometimes these genes can be weakly expressed or silent when culturing in standard conditions. It is therefore desirable to try to switch on these genes using various culturing conditions (Baltz 2008).

As a comparison, a representative of the Gram- negative bacterial species (*Pseudomonas)* belonging to the phylum Proteobacteria was included in the set of experiments. Previous research has shown that species from the genus *Pseudomonas* produce antibiotics against plant pathogens such as fungi (Raaijmakers et al. 1997).

The bacterial isolates were tested to see if the genes encoding for PKS-I, PKS-II, NRPS and DTS were present in their genome, followed by methods aimed to activate or enhance their potential for production of antimicrobial secondary metabolites. The basic hypothesis was that when talented microorganisms are cultured in different conditions and with various supplements a change in antimicrobial activity may be detected similar to what have been observed by previous research efforts.

MATERIALS AND METHODS

Growth media

The following culture media reported by Bisht et al. (2013) with some modifications were used in this study: Arginine glycerol salt broth (AGS), Glucose soybean meal (GS) and Fermentation Medium 4 (FM4) but without glycerol. A commercial Vegetable Peptone Broth (VPB) was used as a standard medium to allow various comparisons. Afterwards, an additional medium denoted Glucose soy peptone (GSP), was composed on the basis of some other media. The media composition is presented in Table 1**.** Glycerol (25 ml, 50% w/v) was added to AGS medium after autoclaving.

For the inhibition zone bioassay two different media were used: Vegetable Peptone Agar (VPA) and COR (Table 1). COR medium was composed as a mixture of three different media. At first, Mineral Medium (MM, Table 1) previously described by Stainer et al. (1966) with modifications by Pohanka et al. (2005) was made in 800 ml of deionized H_2O. This medium was, afterwards, complemented by adding 100 ml each of the two other media: Tryptone broth (O) and Casamino Acids- Dextrose Broth (R, Table 1). When needed 15 g agar was added to solidify the growth media. After autoclaving, 2 % (20 g/L) of glycerol was added to the mixture.

Table 1: Media composition

Medium	**Composition (gram/liter H_2O)**
AGS	1.0 L-arginine (SIGMA-ALDRICH), 1.0 K_2HPO_4(MERCK), 1.0 NaCl (SIGMA-ALDRICH), 0.5 $MgSO_4 \cdot 7H_2O$ (SIGMA-ALDRICH), 0.001 $CuSO_4 \cdot 5H_2O$ (SIGMA-ALDRICH), 0.01 $Fe_2(SO_4)_3 \cdot 6H_2O$ (SIGMA-ALDRICH) and 0.001 $ZnSO_4 \cdot H_2O$ (MERCK)
GS	10.0 glucose (VWR), 10.0 soybean meal (Grocery brand Risenta), 10.0 NaCl (SIGMA-ALDRICH) and 1.0 $CaCO_3$ (SIGMA-ALDRICH)
VPB	15.0 Vegetable Peptone Broth (OXOID)
FM 4	10.0 glucose (VWR), 10.0 soluble starch (SIGMA-ALDRICH), 2.5 corn steep liquor (SIGMA-ALDRICH), 5.0 peptone (BD), 2.0 yeast extract (BD), 1.0 NaCl (SIGMA-ALDRICH) and 3.0 $CaCO_3$ (SIGMA-ALDRICH)
GSP	20.0 glucose (VWR), 10.0 soy peptone (BD), 10.0 NaCl (SIGMA-ALDRICH) and 1.0 $CaCO_3$ (SIGMA-ALDRICH)
VPA	10.0 Vegetable Peptone Broth (OXOID), 15.0 agar (SIGMA-ALDRICH)
MM	40 ml $Na_2HP0_4 \cdot KH_2P0_4$ (SIGMA-ALDRICH), 25 ml mineral stock A, 25 ml mineral stock B, 1.0 $(NH_4)_2SO_4$ (SIGMA-ALDRICH), 20 glycerol (Stainer et al., 1966, Pohanka et al., 2005)
O	10.0 Tryptone (BD), 1 L-Tryptophan (SIGMA-ALDRICH), 5.0 NaCl (SIGMA-ALDRICH)
R	10.0 Casamino Acids (BD), 2.0 Dextrose (VWR), 1.25 K_2HPO_4 (SIGMA-ALDRICH), 1.0 yeast extract (BD)

Bacterial isolates

The different bacterial isolates were selected for their potential to produce secondary metabolites. The isolate identities and origin is shown in Table 2**.**

Table 2: The identity of bacterial isolates and their origin

Isolate denotation	**Isolate identity**	**Origin**
AV130	*Streptomyces sp.*	Coffee plant
GB 34:1	*Nocardia sp.*	*Geodia barretti* (sponge)
GB 43	*Pseudomonas sp.*	*Geodia barretti* (sponge)
T110	*Streptomyces sp.*	Seaweed
T339	*Brevibacterium sp.*	Seaweed
T306	*Salinibacterium sp.*	Seaweed
AV226:2	*Serratia sp.*	Grass
GB39	*Mycobacterium sp.*	*Geodia barretti* (sponge)
GB59	*Brevibacterium sp.*	*Geodia barretti* (sponge)
GB7	*Bacillus sp.*	*Geodia barretti* (sponge)
MF503	*Serratia sp.*	Plant material from field
CCUG11104T	*Streptomyces griseus*	Reference strain*
CCUG11110TT	*Streptomyces coelicolor*	Reference strain*

* Obtained from Culture Collection, University of Gothenburg, Sweden (CCUG)

Screening for the presence of genes involved in secondary metabolism

DNA was isolated from the bacterial isolates AV130, GB34:1, GB43, T110, T339, CCUG11104T and CCUG11110TT using FASTDNA SPIN kit for soil. The PCR primers targeting the four genes PKS-I, PKS-II, NRPS and DTS are presented in Table 3 (Xie et al. 2014, Ayuso-Sacido and Genilloud, 2005). For the PCR reaction DreamTaq master mix (Thermofisher Scentific) was used together with forward and reverse primer with a final concentration of 0.4 µM each. The PCR program was as follows: initial melting at 95 ºC for 5 min, followed by 30 cycles with 94 ºC for 30 sec, 52 ºC for 30 sec and 72 ºC for 1 min, and finally an elongation step at 72 ºC for 7 min.

Table 3: Primers for PCR

Gene	Direction	Primers
PKS-1	Forward	5´TSAAGTCSAACATCGGBCA3´
PKS-1	Reverse	5´CGCAGGTTSCSGTACCAGTA3´
PKS-2	Forward	5´GGCAGCGGITTCGGCGGITTCCAG3′
PKS-2	Reverse	5´CGITGTTIACIGCGTAGAACCAGGCG3′
DTS	Forward	5´CGCTCAGTGCGGTSGAG3′
DTS	Reverse	5´GGIGAGGCGTGCCACTTGTC3′
NRPS	Forward	5´GCSTACSYSATSTACACSTCSGG3´
NRPS	Reverse	5´SASGTCVCCSGTSCGGTAS3´

S=G/C, B=C/G/T, Y=C/T, I=Inosine

Culturing conditions and media composition

In the first experiment (experiment 1) the selected bacteria; AV130, GB34:1, GB43, T110, T339, CCUG11104T and CCUG11110T were all pre-cultured in a mixture of 80 % VPB (Table 1) and 20 % Malt Extract (15 g Malt Extract, (Difco Ltd) in 1 L deionized H_2O) in order to obtain well growing inoculum. The cultures were incubated at 20 ºC on a rotary shaker (135 rpm) for 24 h. Fifteen ml of the pre-cultured bacteria were inoculated in 500 ml E-flasks, each containing 150 ml FM 4 (Table 1) and incubated at 20 ºC on a rotary shaker (210 rpm) for 6 days. For each isolate one baffled (high oxygen access) and one regular E-flask (low oxygen access) was used. After incubation overnight, one nylon tea bag containing approximately 5 grams of polymer resin Amberlite XAD16N (SIGMA-ALDRICH) was placed in each flask to allow the absorption of any produced secondary metabolites.
The Amberlite bags were harvested at the end of cultivation and washed by deionized water to remove any bacterial cells attached to the bag. The accumulated metabolites were then extracted stepwise in tubes with 20 ml methanol followed by 20 ml acetonitrile, and the extract was dried under nitrogen gas. The dried extracts containing secondary metabolites were later tested for their antimicrobial properties in a bioassay against non-pathogenic bacteria.

For the second experiment (experiment 2), the bacterial isolates GB43, CCUG1110T, GB34:1 and GB59 were inoculated in two 500 ml baffled flasks, each containing 100 ml of respectively the growth media AGS and GS (Table 1). The cultures were incubated, treated and extracted as described above with exception that they were incubated at 20 ºC on a rotary shaker (190 rpm).

In the third experiment (experiment 3) the bacterial isolates GB43, GB34:1, T110 and CCUG11110T were grown in 150 ml of GSP medium (Table 1) with addition of the three various supplements: γ-butyrolactone

GBL and $ScCl_3$ (SIGMA-ALDRICH), and Triclosan (MERCK). The isolates GB34:1, T110 and CCUG11110T were inoculated in baffled flasks and isolate GB43 was inoculated in regular E-flasks. GBL (0.5 µM), Triclosan (1 µM) and $ScCl_3$ (0.2 mM) were added each to one set of cultures. Controls were cultures without any additives. Cultures were in total incubated for a period of 9 days (20 ºC, 190 rpm). Three days after inoculation supplements were added and 24 h after the Amberlite bags. Extraction procedure was as described above.

Zone inhibition assay

The two test organisms *Staphylococcus warneri* and *Pseudomonas resinovorans* were diluted in VPB to a concentration of $5.0 \cdot 10^5$ cfu/ml and $2.6 \cdot 10^5$ cfu/ml respectively. The bacterial solutions were each spread onto two agar plates (Ø 14 cm), one containing the media VPA and the other one COR. Suspensions (10 µl) of the isolates AV130, GB34:1, GB43, T110, T339, CCUG11104T, CCUG11110T, T306, AV226:2, GB39, GB7 and MF503 were then spotted in a square pattern on the total four different plate/test organism combination and then incubated at 25 ºC for 3 days.

Microtiter plate bioassay against non-pathogenic microorganisms

The ability of extracts obtained in all culturing experiments to inhibit the growth of two tested organisms *S. warneri* and *P. resinovorans* was estimated in microtiter plate assay. Extracts from the experiment 1 (varying oxygen levels) were dissolved in 5 ml methanol plus 5 ml acetonitrile, whereas extracts from experiments 2 (two culture media) and 3 (specific supplements) were dissolved in 2.5 ml methanol. Aliquots of extracts (100, 80, 60, 40, 20 and 10 µl) were distributed to the wells (96-wells microtiter plates) and dried overnight. Suspensions (100 µl) of *S. warneri* 3.0×10^4 cfu/ml (experiment 1) and 3.3×10^5 cfu/ml (experiment 2 and 3) and of *P. resinovorans* 4.3×10^4 cfu/ml (experiment 1) and $2{,}9 \times 10^5$ cfu/ml (experiment 2 and 3) were added to the wells. Plates were cover with adhesive plastic covers (NUNC) and incubated at 37 °C overnight. Inhibition of bacterial growth was estimated by eye using following scale: 0 – bacterial growth not inhibited at all; 1 – little inhibition of bacterial growth; 2 – intermediate inhibition of bacterial growth; 3 – full inhibition and no bacterial growth at all.

High- Performance Liquid Chromatography (HPLC)

In order to detect the possible metabolites with antimicrobial activity, the extracts collected from the supplement test (experiment 3) were separated by HPLC with gradient 10-95 % acetonitrile during 10 min with a flow of 10 ml min^{-1} (injection volume 1 ml, column HypersilGold, C-18, 5µm, 100 x 21.2 mm, and guard column 10 x 10 mm). Aliquots of the fractions (100µl) were transferred to seven identical replica 96-well microtiter plates, and chromatograms showing the fractionated compounds absorbance at 210 nm wavelength were recorded. Solvents were, afterwards, evaporated in the fume cupboard.

Microtiter plate assay against pathogenic microorganisms

Suspensions (100µl, approximately 10^4 cfu/ml) of six different bacterial pathogens and one fungal pathogen were distributed into wells. The plates were incubated at 37 ºC overnight. Inhibition of bacterial growth was estimated as described above.

LC-MS analysis of active fractions

The fractions that showed the interesting activity profile against the pathogens were analyzed by using Liquid Chromatography- Mass Spectrometry (LC-MS). The column used was Accucore RP-MS (50 x 3 mm, particle size 2.6 µm). The eluent consisted two parts: A (H_2O, 0.2 % FA) and B (MeCN, 0.2 % FA). One microliter of the active fractions were injected and analyzed with the gradient of 10-95 % eluent during 4 min, followed by 95 % eluent for 3 min, and ended with 10 % eluent for 3 min. The flow was set at 0.8 ml/min. The substances were detected in the mass spectrometer Bruker maXis Impact, Q- TOF (electrospray ionization with positive ions), and compared against known microbial metabolites from the database Antibase.

RESULTS

PCR screening of genes for secondary metabolites

Seven of the bacterial isolates in this report (AV130, GB34:1, GB43, T110, T339, CCUG11104T and CCUG11110T) were tested if they harbor genes that encode for secondary metabolites associated enzymes PKS-1, PKS-2, NRPS and DTS. A gel of the resulting PCR-products is shown in Figure 1. These results are converted into a gene grading scale that corresponds to the number of PCR products obtained from each bacterial isolates (Figure 2). For the bacterial isolate GB43 the results strongly indicate that is possesses all 4 genes, and the bacterial isolate GB34:1 seems to have all genes with doubts concerning PKS-II. The gene encoding for DTS is present both in isolate CCUG11104T and T110, and probably also in the reference strain CCUG11110TT but not in isolate AV130. In the strain CCUG11104T the presence of PKS-II could be detected but not in the isolate T339. The remaining gene NRPS could be weakly detected in the isolates T339, T110 and AV130.

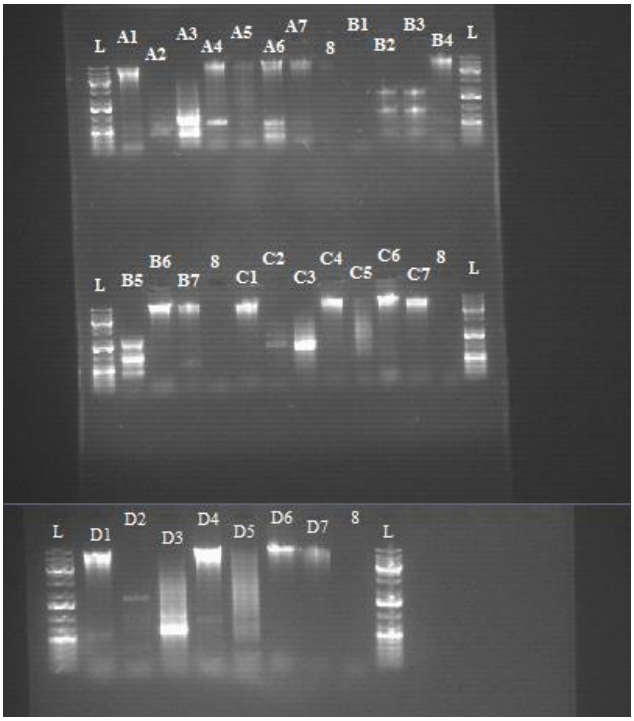

Figure 1: Gel image of PCR products. L (ladder) A (PKS-II), B (DTS), C (PKS-I) and D (NRPS). 1(AV130), 2 (GB34:1), 3 (GB43), 4 (T110), 5 (T339) 6 (CCUG11104T), 7 (CCUGG11110T), 8 (Negative control).

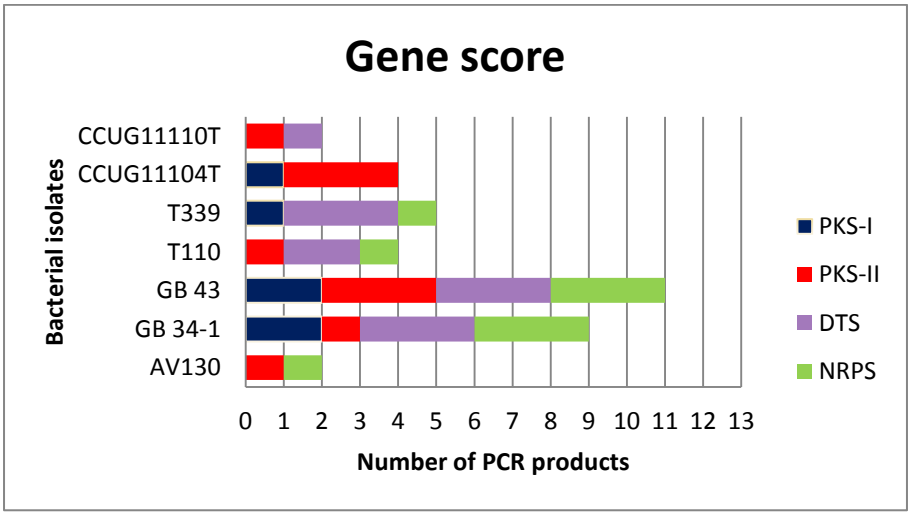

Figure 2: Genetic potential of the tested isolates estimated after converting the bands for PCR- products on the gel image in Figure 1. The following scale was used for conversion: 0- no PCR-product, 1- only one PCR-product, 2- two PCR-products, 3- three or more PCR-products.

Zone inhibition assay

The bacterial isolates AV130, GB34:1, GB43, T110, T339, CCUG11104T and CCUG11110T were cultured in baffled flasks at a relatively high speed on a rotary shaker for increased oxygen exposure. The results of the zone inhibition assay that was performed on plates with different substrates are shown in Figure 3 and 4**.** The bacterial isolate GB43 and two other isolates included in the test AV226:6 and MF503 showed inhibition zones against *S. warneri* on both COR and VPA plates. The bacterial isolate CCUG11110T also

created an inhibition zone against *S. warneri* but only on the plate with COR. The isolate AV226:2 only showed a narrow inhibition zone against *P. resinovorans* (result not shown).

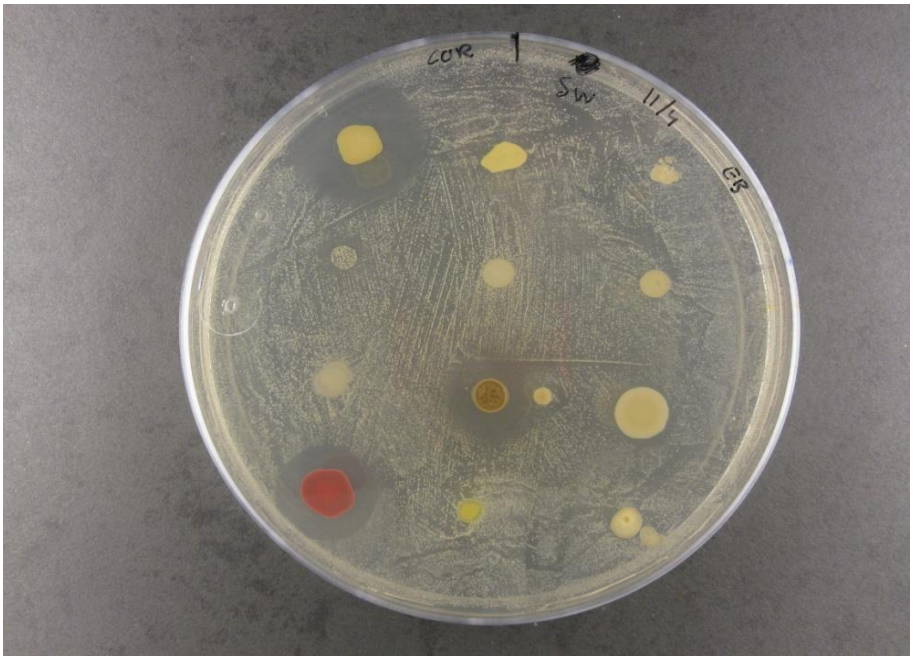

Figure 3: Inhibition zone bioassay. Suspensions of the bacterial isolates (starting from the top left) AV226:2, T339, AV130, GB39, 11104 T, GB34:1, GB7, CCUG11110T, GB43, MF503, T306 and T110 were spotted (10 µl) on a COR plate with *S. warneri* as test organism. Plates were inspected after 72 h at 25 ºC.

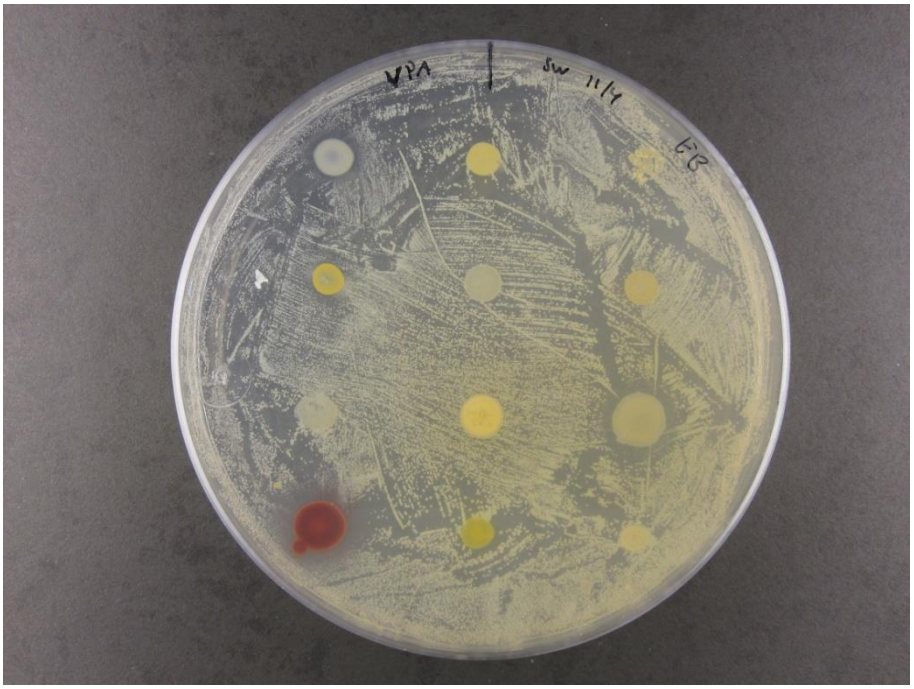

Figure 4**:** Inhibition zone bioassay. Suspensions of the bacterial isolates (starting from the top left) AV226:2, T339, AV130, GB39, 11104 T, GB34:1, GB7, CCUG11110T, GB43, MF503, T306 and T110 were spotted (10 µl) on a VPA plate with *S. warneri* as test organism. Plates were inspected after 72 h at 25 ºC.

Influence of culturing conditions on production of secondary metabolites

Experiment 1- Oxygen exposure

The results of the 96-well microtiter plate bioassay with extracts from the cultures exposed to different levels of oxygen are shown in Figure 5. Bacterial isolate GB43 produced secondary metabolites that inhibit the growth of both test organisms *S. warneri* and *P. resinovorans* when it was cultivated in non-baffled flasks. Higher concentration of extracts of the isolate GB34:1 obtained after cultivation in baffled flasks also inhibits the growth of both test organisms. Extracts from bacterial isolate CCUG11110T cultivated in baffled flasks displayed activity against *S. warneri* at all tested concentrations.

Test organism *S. warneri*

Regular E-flasks	µl											
	100	100	80	80	60	60	40	40	20	20	10	10
AV130	0	0	0	0	0	0	0	0	0	0	0	0
GB 34:1	3	3	2	2	1	1	0	0	0	0	0	0
GB 43	3	3	3	3	3	3	3	3	3	3	3	3
T110	2	2	1	1	1	1	1	1	0	0	0	0
T339	0	0	0	0	0	0	0	0	0	0	0	0
CCUG11104 T	0	0	0	0	0	0	0	0	0	0	0	0
CCUG11110T	1	1	0	0	0	0	0	0	0	0	0	0

Test organism *P. resinovorans*

Regular E-flasks	µl											
	100	100	80	80	60	60	40	40	20	20	10	10
AV130	0	0	0	0	0	0	0	0	0	0	0	0
GB 34:1	3	3	1	2	0	0	0	0	0	0	0	0
GB 43	3	3	3	3	3	3	3	3	3	3	3	3
T110	0	0	0	0	0	0	0	0	0	0	0	0
T339	0	0	0	0	0	0	0	0	0	0	0	0
CCUG11104 T	0	0	0	0	0	0	0	0	0	0	0	0
CCUG11110T	0	0	0	0	0	0	0	0	0	0	0	0

Test organism *S. warneri*

Baffled flasks	µl											
	100	100	80	80	60	60	40	40	20	20	10	10
AV130	3	3	2	2	1	1	0	0	0	0	0	0
GB 34:1	3	3	1	1	0	0	0	0	0	0	0	0
GB 43	1	1	0	0	0	0	0	0	0	0	0	0
T110	3	3	3	3	2	2	2	2	0	0	0	0
T339	0	0	0	0	0	0	0	0	0	0	0	0
CCUG11104 T	0	0	0	0	0	0	0	0	0	0	0	0
CCUG11110T	3	3	3	3	3	3	3	3	2	2	1	1

Test organism *P. resinovorans*

Baffled flasks	µl											
	100	100	80	80	60	60	40	40	20	20	10	10
AV130	0	0	0	0	0	0	0	0	0	0	0	0
GB 34:1	3	3	3	3	0	0	0	0	0	0	0	0
GB 43	0	0	0	0	0	0	0	0	0	0	0	0
T110	2	2	2	2	2	2	2	2	1	1	0	0
T339	0	0	0	0	0	0	0	0	0	0	0	0
11104 T	1	1	1	1	0	0	0	0	0	0	0	0
CCUG11110T	0	0	0	0	0	0	0	0	0	0	0	0

Figure 5: Inhibition of the *S. warneri* and *P. resinovorans* growth by extracts of isolates cultured with varying oxygen access. The activity was graded with following scale: 0 – bacterial growth not inhibited at all; 1 – little inhibition of bacterial growth; 2 – intermediate inhibition of bacterial growth; 3 – full inhibition, no bacterial growth at all.

Experiment 2- Different growth media composition

In general, only the extracts of isolates CCUG11110T and GB43 inhibited the growth of both test organisms. The bacterial isolate CCUG11110T produced metabolites that inhibited the growth of both test organisms when cultivated in the medium AGS whereas metabolites from GB43 cultured in AGS and GS correspondingly inhibited the growth of test organisms *S. warneri* and *P. resinovorans* (Figure 6).

Test organism *S. warneri*		µl											
		100	100	80	80	60	60	40	40	20	20	10	10
AGS	GB43	3	3	3	3	3	3	2	2	2	2	1	1
	CCUG11110T	3	3	3	3	3	3	3	2	1	1	1	1
	GB34:1	1	1	1	1	1	1	1	0	0	0	0	0
	GB59	0	0	0	0	0	0	0	0	0	0	0	0
GS	GB43	3	3	3	3	3	3	3	3	0	0	0	0
	CCUG11110T	1	1	0	0	0	0	0	0	0	0	0	0
	GB34:1	0	0	0	0	0	0	0	0	0	0	0	0
	GB59	0	0	0	0	0	0	0	0	0	0	0	0

Test organism *P. resinovorans*		µl											
		100	100	80	80	60	60	40	40	20	20	10	10
AGS	GB43	3	3	3	3	3	3	3	3	3	3	0	0
	CCUG11110T	2	2	1	1	1	1	1	1	1	1	1	1
	GB34:1	0	0	0	0	0	0	0	0	0	0	0	0
	GB59	0	0	0	0	0	0	0	0	0	0	0	0
GS	GB43	3	3	3	3	3	3	2	3	0	0	0	0
	CCUG11110T	0	0	0	0	0	0	0	0	0	0	0	0
	GB34:1	0	0	0	0	0	0	0	0	0	0	0	0
	GB59	0	0	0	0	0	0	0	0	0	0	0	0

Figure 6: Inhibition of the *S. warneri* and *P. resinovorans* growth by extracts of isolates cultured in different culture media. The activity was graded with following scale: 0 – bacterial growth not inhibited at all; 1 – little inhibition of bacterial growth; 2 – intermediate inhibition of bacterial growth; 3 – full inhibition, no bacterial growth at all.

Experiment 3- Culturing in the presence of various supplements

The bacterial isolate T110 produced metabolite(s) active against *S. warneri* after being exposed to the supplements compared to control (Figure 7). The addition of $ScCl_3$ down-regulated the activity of the bacterial isolates GB34:1 against *S. warneri* (Figure 7), and the same observation was noticed for bacterial isolate T110 against the other test organism *P. resinovorans* (Figure 7). Also GBL deactivated the metabolites from T110 whereas Triclosan triggered the activity against *P. resinovorans* (Figure 7).

Test organism *S. warneri*		µl											
		100	100	80	80	60	60	40	40	20	20	10	10
CCUG11110T	Control	3	3	3	3	3	3	3	3	2	2	1	1
	Triclosan	3	3	3	3	3	3	3	3	3	3	3	3
	ScCl3	3	3	3	3	3	3	2	2	1	1	1	0
	GBL	3	3	3	3	3	3	3	3	1	1	1	1
GB34:1	Control	3	3	3	3	2	2	2	2	0	0	0	0
	Triclosan	3	3	3	3	3	3	3	3	3	3	3	3
	$ScCl_3$	0	0	0	0	0	0	0	0	0	0	0	0
	GBL	3	3	3	3	3	3	3	2	0	0	0	0
T110	Control	2	2	1	1	1	0	0	0	0	0	0	0
	Triclosan	3	3	3	3	3	3	3	3	3	3	2	2
	ScCl3	3	3	3	3	3	3	3	3	3	3	3	3
	GBL	3	3	3	3	3	3	3	3	3	3	3	2
GB43	Control	3	3	3	3	3	3	3	3	1	1	0	0
	Triclosan	3	3	3	3	3	3	3	3	3	3	3	3
	ScCl3	3	3	3	3	3	3	3	3	0	0	0	0
	GBL	3	3	3	3	3	3	3	3	0	0	0	0

Test organism *P. resinovorans*		µl											
		100	100		80	60	60	40	40	20	20	10	10
CCUG11110T	Control	3	3	3	3	3	3	3	3	3	3	2	2
	Triclosan	3	3	3	3	3	3	3	3	3	3	3	3
	$ScCl_3$	3	3	3	3	3	3	2	1	0	0	0	0
	GBL	3	3	3	3	3	3	3	3	3	3	1	1
GB34:1	Control	3	3	3	3	3	3	3	3	1	1	0	0
	Triclosan	3	3	2	2	2	2	2	1	0	0	0	0
	$ScCl_3$	1	1	0	0	0	0	0	0	0	0	0	0
	GBL	3	3	3	3	3	3	3	3	3	3	0	0
T110	Control	1	1	0	0	0	0	0	0	0	0	0	0
	Triclosan	3	3	3	3	3	3	3	3	0	0	0	0
	$ScCl_3$	0	0	0	0	0	0	0	0	0	0	0	0
	GBL	0	0	0	0	0	0	0	0	0	0	0	0
GB43	Control	3	3	3	3	3	3	3	3	3	3	0	0
	Triclosan	3	3	3	3	3	3	3	3	3	3	0	0
	$ScCl_3$	3	3	3	3	3	3	3	3	3	3	0	0
	GBL	3	3	3	3	3	3	3	3	3	3	0	0

Figure 7: Inhibition of the *S. warneri* and *P. resinovorans* growth by extracts of isolates cultured on GPS medium with different supplements. The activity was graded with following scale: 0 – bacterial growth not inhibited at all; 1 – little inhibition of bacterial growth; 2 – intermediate inhibition of bacterial growth; 3 – full inhibition, no bacterial growth at all.

Microtiter plate assay against pathogenic organisms

The HPLC separated fractions of extracts from the supplement experiment were tested against several pathogenic microorganisms. The additions of Triclosan to the culture with bacterial isolate CCUG11110T resulted in two new substances active against pathogen F (red encircled activity area Figure 8). The active substance (blue encircled activity area Figure 8) was established to be Triclosan.

Separation of extracts produced by the bacterial isolate T110 and T110 supplemented with $ScCl_3$ both resulted in detecting antimicrobial affect. However, the retention time for active fractions was different for these two samples suggesting production of two different active metabolites (orange and purple encircled activity area Figure 9).

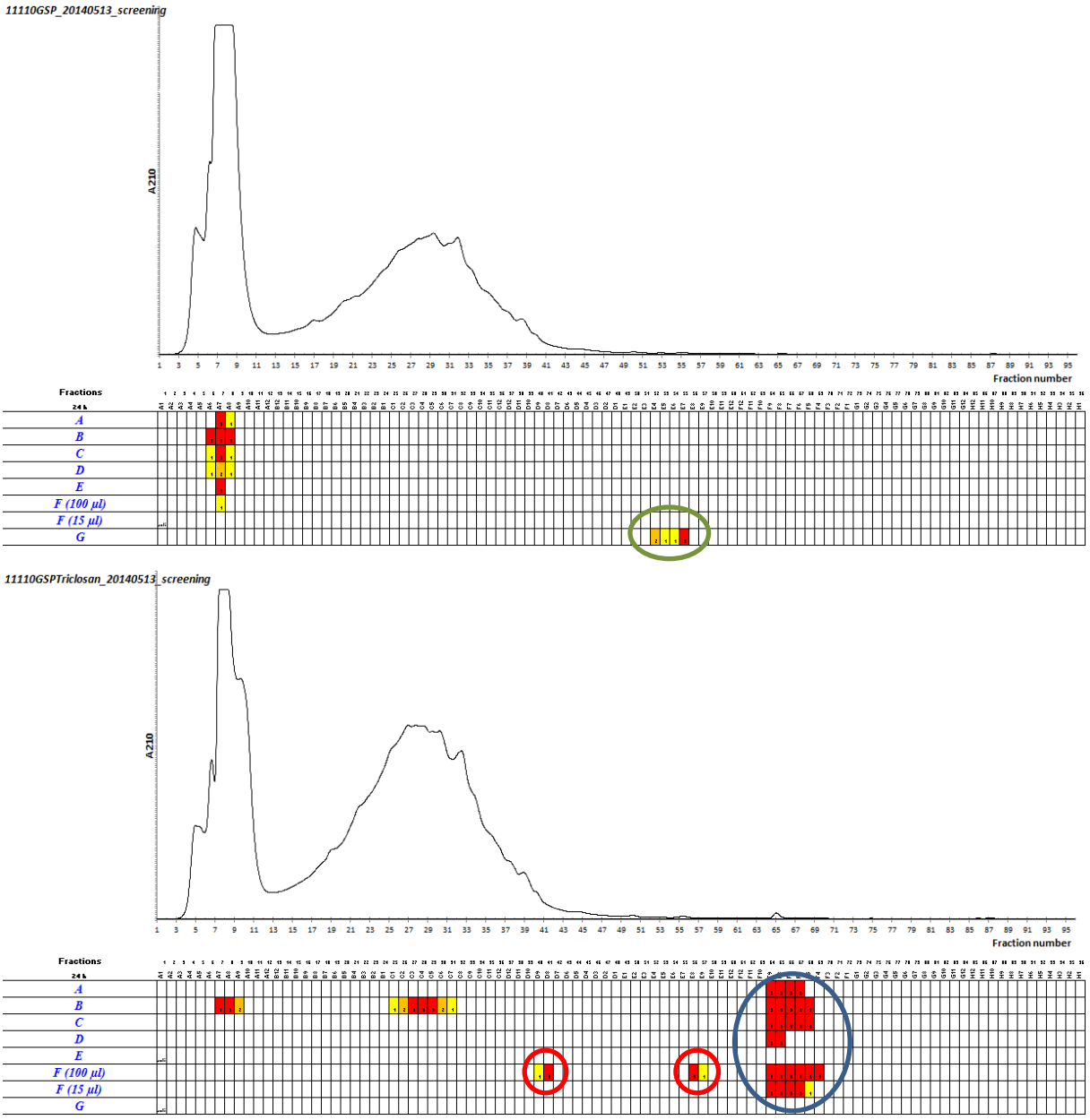

Figure 8: HPLC chromatograms of the isolate CCUG11110T extracts cultured without supplements (top) and with addition of Triclosan (bottom) and corresponding activity against a panel of pathogenic microorganisms. The green encircled fractions in the top chromatogram show activity against pathogen G. The blue encircled fractions in bottom chromatogram were active against almost all of the test pathogens and the two red encircled fractions were only active against pathogen F. The remaining detected activity was most likely due to cyclic dipeptides derived from media components.

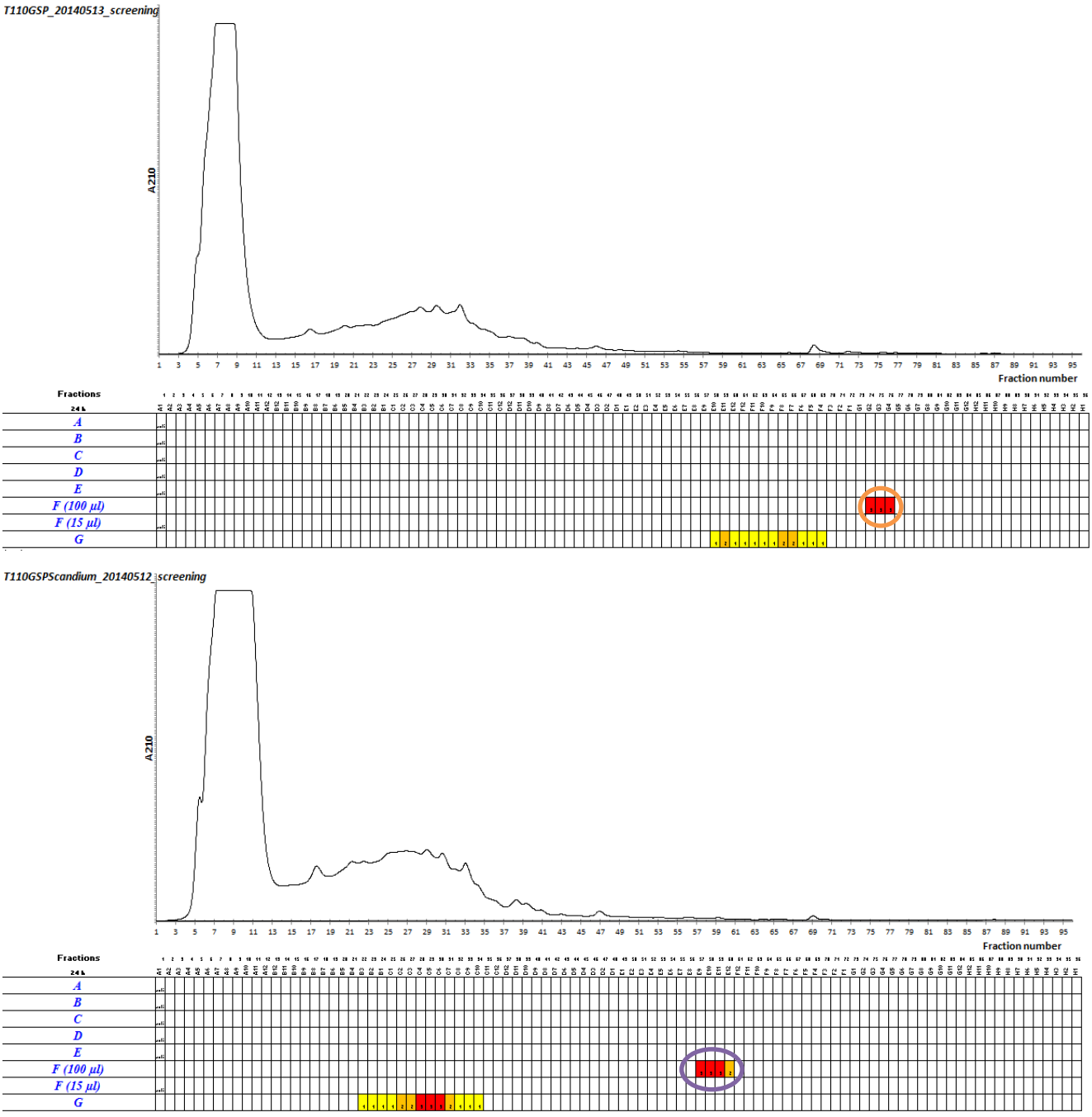

Figure 9: HPLC chromatograms of the isolate T110 extracts cultured without supplements (top) and with addition of $ScCl_3$ (bottom) and corresponding activity against a panel of pathogenic microorganisms. The orange and purple circles show the activity against the test organism F at different retention time for top and bottom chromatogram. The remaining detected activity was most likely due to cyclic dipeptides derived from media components.

DISCUSSION

The aim of this study was to trigger several bacterial isolate to produce different secondary metabolites, hopefully also including novel compounds that may be used as future antibiotics as a countermeasure to the increasing antibiotic resistance among an alarmingly large proportion of microbial pathogens. Three experiments were performed, in which the influence of different oxygen levels, different media composition and adding different supplements to the cultures on the production of active metabolites was tested. The hypothesis was that a change in activity against the test organisms should be distinguished if it is as agreed with similar research from the literature (Bode et al. 2002, Craney et al. 2012, de Kievit and Iglewski 2000, Inaoka and Ochi 2011). Overall it seemed that the experiment with varying supplements added to the cultures generated the most powerful antimicrobial effect for the highest number of tested isolates. Especially, addition of Triclosan yielded a number of active substances in the extracts of all bacterial isolates. One of the detected substances was established as Triclosan (Figure 8) after LC- MS analysis and molecular weight comparison with substances available for comparison in the database Antibase. Also the determination of substances by LC-MS revealed the higher diversity of produced secondary metabolites. It can confirm the approach referred to as OSMAC (Bode et al. 2002): that one microorganism is capable of to generate several active metabolites under varying culture conditions.

The reference isolate CCUG11110T, denotation for *Streptomyces coelicolor*, showed its potential to produce antibiotics, particularly in the experiments using supplements (Figure 7). Similar experiments in reviews have shown the same outcome in successful activity against several test organisms (Craney et al. 2012, Kawai et al. 2007 and Takano et al. 2000). Concerning non-Streptomyces and their optimistically capacity to produce novel antibiotics (Tiwari and Gupta 2012), bacterial isolate GB34:1 (representing *Nocardia* sp) displayed promising antibiotic production in the current research both in terms of the detected genes coupled to the secondary metabolism (Figure 2) and activity against the test organisms (Figure 5 and 7). It must be further investigated what kind of active metabolites were produced by this organism.

The initial test consisted of performing PCRs of four genes (PKS-1, PKS-2, NRPS and DTS) encoding for enzymes that have been shown to be involved in the production of secondary metabolites. Isolate GB43, belonging to the genus *Pseudomonas*, was tested positive for all four genes. Its potential was also reflected in the bioassays. Metabolites produced by the isolate GB43 showed activity against both *S. warneri* and *P. resinovorans* in all bioassays except in the inhibition zone test against *P. resinovorans* and also in the microtiter plate inhibition assay cultured in baffled flasks (Figure 5). Since none of the isolates apart from one (AV226:2, figure not shown) were inhibiting the growth of *P. resinovorans* in the inhibition zone test, it can be concluded that the growth of the test organism may have been too strong or simply that no effective antibiotics against *P. resinovorans* could be found. Based on the results from the bioassay of the extracts produced by this isolate that originated from cultures grown at different oxygen level, one can conclude that

Pseudomonas sp. apparently grows well in the presence of high oxygen level but are not able to produce antimicrobial metabolites.

GBL used in this report as a supplement has a similar core structure as the family of signal molecule AHL. Mostly gram-negative bacteria use AHL in the quorum-sensing signaling (Yajima, A. 2014) whereas many Gram-positive bacteria use GBLs as signaling molecules for regulation of antibiotic production (Takano et al. 2000). It is consistent that isolate GB43, which is a Gram-negative bacterium, were activated in the production of metabolites in the supplement experiment after DHM- furanone was added to the culture. DHM- furanone activated the secondary metabolite production for all bacterial isolates except T110 against *P. resinovorans.* Both that absence of activity for bacterial isolate T110 and the poor activity of all isolates against *P. resinovorans* in general could possibly be explained by that *P. resinovorans* may have used a quorum-sensing mechanism that protected its growth in the presence of active metabolites. *Pseudomonas* species, like other Gram-negative bacteria, are also known to use efflux pumps to effectively reject the added antimicrobial agents, which had led to the enormous problems with nosocomial infections caused by antibiotic resistant bacteria (Köhler et al. 1997, Nikaido 1996).

CONCLUSION AND FUTURE PERSPECTIVES

Different culturing conditions and added supplements positively affected the cultivated bacterial isolates in a way that the production of secondary metabolites with antimicrobial properties was in general enhanced. However, the experiments have to be repeated in order to obtain more reliable result. Also, more various culture media and culture conditions should be included to clearer detect differences. The substances that were separated after the supplement experiments will be further investigated by Nuclear Magnetic Resonance (NMR) to closer elucidate their chemical structures. If they prove to be novel they will undergo additional tests such as for example Minimum Inhibitory Concentration (MIC) that allows to established the minimal concentration of the compound, at which it totally inhibit the growth of tested pathogen.

ACKNOWLEDGMENT

I would like to thank my supervisor Joakim Bjerketorp and assistant supervisor Jolanta Levenfors at the Department of Microbiology at Swedish University of Agriculture Sciences (SLU) for their most appreciated help during this project. Also Pierre Andersson and Anders Broberg at the Department of Chemistry at SLU will be thanked for their help to interpret the results from the chemical analysis. Lastly I will also thank my examiner Bengt Guss and all of the other employees at the Department of Microbiology at SLU.

REFERENCES

Ayuso-Sacido, A. and Genilloud, O. (2005) New PCR Primers for the Screening of NRPS and PKS-I Systems in Actinomycetes: Detection and Distribution of These Biosynthetic Gene Sequences in Major Taxonomic Groups. *Microbial Ecology.* 49(1):10-24

Baltz, R. H. (2008) Renaissance in antibacterial discovery from actinomycetes. *Current opinion in Pharmacology.* 8(5): 557-563

Bentley, SD., Chater, KF., Cerdeño-Tárraga, AM., Challis, GL., Thomson, NR., James, KD., Harris, DE., Quail, MA., Kieser, H., Harper, D., Bateman, A., Brown, S., Chandra, G., Chen, CW., Collins, M., Cronin, A., Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang CH, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream MA, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, Hopwood DA. (2002) Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2). *Nature.* 417(6885):141-147.

Bérdy, J. (2005) Bioactive Microbial Metabolites. *Journal of Antibiotics*. 58(1): 1–26

Bode, H. B., Bethe, B., Höfs, R. and Zeeck, A. (2002) Big Effects from Small Changes: Possible Ways to Explore Nature's Chemical Diversity. *Chembiochem*. 3(7): 619-27.

Bush, K. (2010) The coming of age of antibiotics: discovery and therapeutic value. *Annals of the New York academy of sciences.* 1213(1): 1-4

Craney, A., Ozimok, C., Pimentel-Elardo, SM., Capretta, A. and Nodwell, JR. (2012) Chemical perturbation of secondary metabolism demonstrates important links to primary metabolism. *Chemistry and Biology.* 19(8): 1020-1027

Donadioa, S., Monciardini, P., Alduina, R., Mazzaa, P., Chiocchini, C., Cavaletti, L., Sosioa, M. and Puglia, A. (2001) Microbial technologies for the discovery of novel bioactive metabolites. *Journal of Biotechnology* 99 (3): 187-198

de Kievit T. R. and Iglewski B. H. (2000) Bacterial Quorum Sensing in Pathogenic Relationships. *Infection and Immunity*. 68(9): 4839-4849

Enright, M. C., Robinson, A. D., Randle, G., Feil, E. J., Grundmann, H. and Spratt, B. G. (2002) The Evolutionary History of Methicillin-Resistant *Staphylococcus aureus* (MRSA). *National Academy of Sciences.* 99(11): 7687-7692

Fleming, A. (1929) On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to their Use in the Isolation of B. influenzæ. *The British Journal of Experimental Pathology*. 10(3): 226–236.

Gajraj, S. B., Alpana, B., Vijay, K. & Omprakash, G. (2013) Isolation, purification and partial characterization of an antifungal agent produced by salt-tolerant alkaliphilic *Streptomyces violascens. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences.* 83(1): 109-117

Genilloud, O., González, I., Salazar, O., Martín, J., Tormo, J. R. and Vicente, F. (2011) Current approaches to exploit actinomycetes as a source of novel natural products. *Journal of Industrial Microbiology & Biotechnology*. 38(3): 375-389

Inaoka, T. and Ochi, K. (2011) Scandium Stimulates the Production of Amylase and Bacilysin in *Bacillus subtilis. Applied and Environmental Microbiology.* 77(22): 8181-8183

Kawai, K., Wang, G., Okamoto, S. and Ochi K. (2007) The rare earth, scandium, causes antibiotic overproduction in *Streptomyces* spp. *FEMS Microbiology Letters.* 274(2): 311–315

Koehn, F. E. and Carter, G. T. (2005) The evolving role of natural products in drug discovery. *Nature Reviews Drug Discovery*. 4(3): 206-220

Kumar, V., Bisht, G. S. and Gusain, O. (2013) Terrestrial actinomycetes from diverse locations of Uttarakhnad, India: Isolation and screening for their antibacterial activity. *Iran Journal of Microbiology. 5(3): 299–308.*

Köhler, T., Michéa-Hamzehpour, M., Henze, U., Gotoh, N., Kocjancic Curty, L. and Pechère, J-C. (1997) Characterization of MexE–MexF–OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. *Molecular Microbiology.* 23(2): 345-354

Livermore, D. M. (2011) Discovery research: the scientific challenge of finding new antibiotics. *Journal of Antimicrobial Chemotherapy.* 66 (9): 1941-1944.

Mahajan, GB. and Balachandran, L. (2012) Antibacterial agents from actinomycetes - a review. *Frontier in Bioscience (Elite Ed).* 1(4): 240-53.

Manivasagana, P., Venkatesanb, J., Sivakumarc, K., Kima S-K. (2013) Marine Actinobacterial metabolites: Current status and future perspectives. *Microbiological Research.* 168 (6): 311-32

Nikaido, H. (1996) Multidrug Efflux Pumps of Gram-Negative Bacteria. *Journal of Bacteriology*, 178(20):5853–5859

Neu, H. C. (1992) The Crisis in Antibiotic Resistance. *Science.* 257(5073): 1064-1073

Ochi, K., Hosaka, T. (2013) New strategies for drug discovery: activation of silent or weakly expressed microbial gene clusters. *Applied Microbiological and Biotechnology.* 97(1): 87-98

Persidis, A. (1998) High-throughput screening. *Nature biotechnology.* 16(2): 488-489

Pohanka, A., Broberg, A., Johansson, M., Kenne, L., and Levenfors, J. (2005) Pseudotrienic Acids A and B, Two Bioactive Metabolites from Pseudomonas sp. MF381-IODS. *Journal of Natural Products*. 68(9): 1380-1385

Raaijmakers J. M., Weller D. M. and Thomashow L. S. (1997) Frequency of Antibiotic-Producing Pseudomonas spp. in Natural Environments. *Applied and Environmental Microbiology*. 63(3): 881-887

Subramani, R., Aalbersberg, W. (2012) Marine actinomycetes: An ongoing source of novel bioactive metabolites. *Microbiological Research.* 167(10): 571–580

Stainer, R. Y., Palleroni, N. J., Doudoroff, M. (1966) The aerobic pseudomonas: A taxonomic study. *Journal of General Microbiology.* 43(2): 159-271

Takano, E., Nihira, T., Hara, Y., Jones, J. J., Gershater, C. J. L., Yamada, Y. and Bibb, M. (2000) Purification and Structural Determination of SCB1, a γ-Butyrolactone That Elicits Antibiotic Production in *Streptomyces coelicolor* A3(2). *The Journal of Biological Chemistry.* 275(15): 11010–11016

Tiwari, K. and Gupta, R. K. (2012) Rare actinomycetes: a potential storehouse for novel antibiotics. *Critical Reviews in Biotechnology.* 32(2): 108–132

Xie, P., Ma, M., Rateb, M. E., Shaaban, K. A., Yu, Z, Huang, S., Zhao, L., Zhu, X., Yan, Y., Peterson, R. M., Lohman, J. R., Yang, D.,Yin, M., Rudolf, J. D., Jiang, Y., Duan, Y., and Shen, B. (2014) Biosynthetic Potential-Based Strain Prioritization for Natural Product Discovery: A Showcase for Diterpenoid-Producing Actinomycetes. *Journal of Natural Products.* 77(2): 377-87

Yajima, A. (2014) Recent progress in the chemistry and chemical biology of microbial signaling molecules: quorum-sensing pheromones and microbial hormones. *Tetrahedron Letters*. 55(17): 2773–2780

Zotchev, S. B. (2012) Marine actinomycetes as an emerging resource for the drug development pipelines. *Journal of Biotechnology*. 158 (4): 168– 175