Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences

Isolating microorganisms from marine and marine-associated samples

– A targeted search for novel natural antibiotics

Tua Lilja

Department of Microbiology
Independent project • 15 hec • First cycle, G2E
Biotechnology bachelor programme • Examensarbete/Sveriges lantbruksuniversitet,
Institutionen för mikrobiologi: 2013:9 • ISSN 1101-8151
Uppsala 2013

Isolating microorganisms from marine and marine-associated samples

Tua Lilja

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
Course code: EX0689
Programme/education: Biotechnology bachelor programme

Place of publication: Uppsala
Year of publication: 2013
Title of series: Examensarbete/Sveriges lantbruksuniversitet, Institutionen för mikrobiologi
No: 2013:9
ISSN: 1101-8151
Online publication: http://stud.epsilon.slu.se

Keywords: antibiotics, natural products, marine microorganisms, isolation methods, actinomycetes, rare actinomycetes, whole-cell screening, antimicrobials, antibiotic resistance

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

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

ABSTRACT

The search for antibiotic compounds from the natural environment has been going on for seven decades, ever since penicillin entered the market and antibiotic treatments became routine. The evolutionary pressure put on the pathogenic microorganisms induced a rapid spreading of naturally occurring resistance genes, leaving only the option of finding new antibiotics to treat the resistant pathogens. Microorganisms have been extensively mined for their biosynthetic abilities to produce biologically active compounds. To date, more than 23 000 microbial natural products have been discovered. The *Actinomycetales* are ubiquitous bacteria that have been used for antibiotic discovery for more than half a century, and over 10 000 natural products have been identified from the order. The genus of *Streptomyces* is acknowledged as the most prolific producer of natural products, but lately, non-*Streptomyces* species have been on the rise in novel drug research. Mainly terrestrial species have been studied, but the marine environment offer just as much, if not more, biodiversity to mine for new natural products. In this study, five marine and marine-associated samples were collected from south Shetland archipelago and southern Sweden coastal areas. The samples were subjected to seven selective treatments of varying chemical or physical nature. The samples were subsequently incubated on one general and six selective agar media of varying compositions of nutrients to enrich mainly for bacteria of more advanced metabolic capacity. Bacterial isolates (340 and counting) were recovered from the cultures, and the 16S rDNA of 96 strains was sequenced. A phylogenetic tree analysis of 91 sequences identified to the genus or species level was carried out. Biological assays were carried out on a few selected isolates, and their antibiotic potential was assessed. One strain analyzed by HPLC was found to produce the known antibiotic compound oxydifficidin.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Antibiotika är ett av de viktigaste medel vi har för att bekämpa infektioner. Tyvärr har våra möjligheter att använda olika antibiotika kraftigt begränsats på grund av att mikroorganismerna har utvecklat olika resistensmekanismer vilka också sprids mellan olika arter. Resistensspridningen är i dag ett globalt problem vilket lett till att man inom forskningen aktivt försöker hitta nya typer av antibiotika. Om nya mediciner och behandlingar mot infektioner inte upptäcks och utvecklas riskerar mänskligheten att klockan vrids tillbaka till den pre-antibiotiska eran och att enkla infektioner därmed kan bli livshotande.

Nya antibiotika kan upptäckas och vidareutvecklas med hjälp av flera metoder. Den vanligaste och äldsta metoden är att leta i naturen efter mikroorganismer som uppvisar en hämmande aktivitet mot andra mikroorganismer. En alternativ metod är att modifiera existerande antibiotika på kemisk väg, och på så sätt skapa olika versioner av en och samma substans. Ofta används en kombination av båda metoderna, där man först hittar en naturlig substans som man sedan kan modifiera.

Metoden att leta efter mikroorganismer i naturen har hittills lett till upptäckten av 23 000 aktiva molekyler, och då inte bara antibiotika, utan också antivirala läkemedel, anti-cancerläkemedel, insektsmedel, industriella enzymer, etc. Av dessa 23 000 substanser kommer cirka 10 000 från en speciell bakteriegrupp som kallas aktinomyceter.

Aktinomyceter är bakterier som lever naturligt i alla miljöer över hela världen. De är ekonomiskt betydelsefulla eftersom de är exceptionellt duktiga på att producera molekyler som kan användas inom medicin, industri, och jordbruk. Jämfört med andra vanligt förekommande bakterier är aktinomyceter mycket komplexa, med en avancerad ämnesomsättning som gör dem till troliga producenter av ovanliga ämnen som ofta kan komma till användning. Dessa bakterier beräknas kunna producera hundra tusen olika sorters substanser vilket är en av anledningarna till att man aktivt letar efter nya arter av aktinomyceter

Den mest lovande miljön att undersöka idag är haven; de är de största ekosystemen som finns på planeten, och de minst utforskade. Forskare letar efter nya bakteriearter i allt från uråldriga glaciärer till heta undervattenskällor i havsbotten, och nya mikroorganismer upptäcks därför ideligen.

I det här forskningsprojektet analyserades sammantaget fem olika prover från fyra platser i två världsdelar: Whaler’s Bay på Deception Island och Yankee Harbour på Greenwich Island (Antarktis) samt från Mollösund i Skagerak och Torekov i Skälderviken (Sverige). Proven behandlades enligt sju olika kemiska och fysiska metoder och bakterier från proven odlades sedan upp på agarplattor med varierande typer och mängd av näringsämnen. Intressanta bakteriekolonier isolerades, och genom att sekvensera valda delar av bakteriernas DNA kunde de sorteras in i grupper av redan kända bakterier på släkt- eller artnivå. Över 300 bakterieisolat har tagits till vara och nära 100 av dessa har identifierats med hjälp av DNA-sekvensering. Vissa bakterieisolat testades även för antibiotisk aktivitet mot mikroorganismer som är sjukdomsframkallande hos människor. Hos ett isolat hittades flera aktiva substanser, varav en kunde identifieras som oxydifficidin, vilket är en tidigare känd antimikrobiell substans.

TABLE OF CONTENTS

Introduction 7
80 years of antibiotics 7
Approaches for discovery of antibiotic compounds 7
Whole-cell screening of natural antibiotics 8
Actinomycetes 8
Marine sources of new biochemical diversity 9
On the subject of unculturables 9
Experimental techniques: Enrichment and selection 10
Isolating microorganisms from marine samples 10
Materials and methods 11
Biological material 11
Sample treatments and preparation 11
Culture media and Cultivation 12
Selection and preservation of isolates 13
DNA analysis 13
Isolate identification and Phylogeny 14
Biological assays 14
Results 15
Cultivation 15
16S rDNA amplification 16
Sequencing 17
Isolate identity 17
Identity of Gram-negative bacteria 17
Identity of Gram-positive bacteria 18
Phylogenetic Analysis 19
Biological Assays 21
Discussion 22
Sampling the marine environment 22
The search for new antibiotics 23
Future perspectives 24
Acknowledgements 25
References 25
Appendix 31
Appendix 1 31
Appendix 2. 36

INTRODUCTION

80 YEARS OF ANTIBIOTICS

Antibiotic compounds are the weapons of chemical warfare among microorganisms. It is in the process of evolution that these compounds have evolved, allowing the survival of the fittest, or survival of the most prolific producer of toxic compounds. The fight for a territory is a reality not only for animals, but for microorganisms as well. The means to do this vary among the microbes, from finding an own environmental niche (as in the example of extremophiles) to producing secondary metabolites that kill intruders (as in antibiotics). The antibiotic compounds discovered from microorganisms revolutionized modern medicine when used as a weapon against bacterial infections.

Fleming found the first natural antibiotic, penicillin, in 1928. The compound was identified as a bacteriostatic and bactericidal secondary metabolite of the fungi *Penicillium notatum* (Ligon, 2004). The drug entered the market approximately ten years later, and redefined the way that infectious diseases were viewed. Penicillin provided a cure for bacterial pneumonia and meningitis (*Streptococcus pneumoniae*), diphtheria (*Corynebacterium diphtheriae*) and gonorrhea (*Neisseria gonorrhoeae*), among others (Fleming, 1929; Sykes, 2001). Following the discovery of penicillin, the researchers studying natural antibiotics begun to uncover more functional compounds from diverse microorganisms. The following decades are called the "golden age" of antibiotics. During the 1940's and 1950's almost all groups of important antibiotics against bacteria were found. These are the macrolides, tetracyclines, cephalosporines, and aminoglycosides (Bérdy, 2005). Other compounds discovered were also found to be effective against viruses, parasites, or tumors, etc. (Talbot et al., 2006).

Microbial antibiotics have been in use in medicine since the 1940's. Antibiotics decreased our vulnerability to common bacterial infections that had earlier been death sentences. The following over-use of antibiotics can, therefore, be easily understood. Unfortunately, the evolution of pathogenic microbes did not abruptly end, as all microbes carrying biosynthetic genes for a certain antibiotic, carry also resistance genes for the same compound; otherwise the organisms had not survived the production of their own antibiotics (D'Costa, 2006).

Continuous exposure to antibiotics led quickly to an increasing spread of such resistance genes among especially bacterial pathogens, rendering many antibiotics largely ineffective shortly after entering the market (Bush, 2004; Fenical and Jensen, 2006). The horizontal transfer of resistance genes between microbes is thought to be mainly responsible in most cases and was hurried on by the increased evolutionary pressure (Maplestone et al., 1992; Stone and Williams, 1992).

The genomic changes that cause resistance in pathogens, and the way that these changes spread so quickly, are yet not completely understood and this is a serious cause for concern (Liu and Pop, 2009). Cassell and Mekalanos (2001) suggested that drug-resistant bacterial pathogens might transfer us back to the pre-antibiotic era; and that is quickly becoming a reality. For example, methicillin-resistant *Staphylococcus aureus* (MRSA) cause nowadays more deaths than HIV-infections (Bancroft EA, 2007; Liu and Pop, 2009). The measures taken to decrease the damage caused by drug-resistant pathogens in the later years have not been effective enough to stop the spread of resistance. The remaining alternative is to stay ahead of the pathogens by continuously unearthing new compounds.

APPROACHES FOR DISCOVERY OF ANTIBIOTIC COMPOUNDS

The search for antibiotics was carried out in roughly the same manner throughout the 20th century, until the new techniques developed in the 1990's began to take over. The old techniques followed the same pattern: an environmental niche was sampled for microorganisms, and the compounds obtained from the isolated microorganisms were tested against pathogens. The newly emerged techniques were compiled of more high-tech methods, utilizing combinatorial chemistry and high-throughput screening against molecular targets. However, even though high-throughput screening of mass-produced combinatorial libraries has been used extensively, the expected increase of marketable compounds never came (Baltz, 2008; Newman et al., 2003; Payne et al., 2006).

Combinatorial biosynthesis utilizes existing compounds and increases the effectiveness of it by developing novel derivatives, or by increasing production yields. Gene inactivation, gene combination, mutasynthesis etc., are effective ways to improve compounds, their yields and their activity. Unfortunately, they all require the biosynthetic pathways to be previously known and these are not always elucidated (Floss, 2006; Olano et al., 2008, 2009, 2011). These methods are commonly applied by large pharmaceutical companies, however, the more straightforward search for novel natural compounds from microorganisms is far from obsolete (Clardy et al., 2006) and is still preferred by many (Henkel et al., 1999).

WHOLE-CELL SCREENING OF NATURAL ANTIBIOTICS

In whole-cell screening the fermentation products of the isolate are tested against live target organisms, and any antimicrobial properties can be identified by the rate of growth (or lack of growth) of the target. This strategy allows identification of antimicrobials in their physiological context. Also, the strategy eliminates all potentially active compounds that cannot function in the live target or enter the target cells (Payne et al., 2006). In target-based screening it is instead a biochemical target that the fermentation products are tested against. The targets are enzymes or hormones collected from chemical libraries (Baltz, 2008). The target-based screening often allows for a higher throughput, but it is the whole-cell screening that directly displays antimicrobial potential by affecting a whole target organism.

Whole-cell screening was used in the early drug discovery projects: it is simple and effective, but the low throughput has made it too slow to be an economical approach for working with larger collections of compounds. High-throughput *in vitro*-screening projects has failed to be the good investments that it was earlier believed to be, as the time and money spent is not correlated with the amounts of novel finds (Baltz, 2008; Payne et al., 2006).

ACTINOMYCETES

Of the approximately 23 000 microbial antibiotics found today, an estimated number of 10 000 have been isolated from the order *Actinomycetales* (Manivasagan et al., 2013). The actinomycetes are the most important prokaryotes both economically and biotechnologically as their ability to produce bioactive compounds is undefeated by any other group of organisms. They are the producers of approximately 45% of all discovered active natural products, and the genus *Streptomyces* is the top producer with 80% of the compounds within the actinomycetes to its name (Bérdy, 2005).

The actinomycetes species vary in morphology, growing as coccoid, rod-coccoid, hyphal form, or different degrees of differentiated mycelia (Atlas, 1997). The order was previously assumed to be fungi since only filamentous species had then been identified that showed all the characteristics of fungi. Molecular analysis has later grouped the actinomycetes with bacteria. Common traits among the actinomycetes are a large genome of up to nine megabases, and high GC content However, new studies on freshwater actinomycetes indicates that some instead have a low GC content (Ghai et al., 2012).

The actinomycetes are well-documented soil bacteria that used to be considered indigenous only to terrestrial environments. The actinomycete species recovered from marine sources were suggested to have come from terrestrial spores washed out to sea (Weyland, 1969). It was first in 1984 that a marine actinomycetes species of *Rhodococcus marinonascene* was characterized (Helmke and Weyland, 1984). After a series of studies of actinomycetes from marine sediments, the idea that the marine environment is colonized by its own indigenous actinomycetes started to gain evidence. Some actinomycetes found in marine sediments showed metabolic activity (Moran et al., 1995), and others displayed specific marine adaptation (Jensen et al., 1991). The first obligate marine actinomycete genus to be discovered was *Salinispora* (Mincer et al., 2002, 2005), and the two first sea-water obligate species found were *Salinispora arenicola* and *Salinispora tropica* (Maldonado et al., 2005a).

The major genus among the actinomycetes is the *Streptomyces*; this genus does not only contain many species, but these species are also exceptionally talented in secondary metabolite production. Streptomycetes are some of the most complex bacteria, and they have been extensively studied in terrestrial environments. The terrestrial *Streptomyces* species grow filamentously and form reproductive aerial branches, where sturdy spores form (Chater and Chandra, 2006; Flärdh and Buttner, 2009). The very first antibacterial agent from streptomycetes, streptomycin, was discovered in 1943. Since then, about 7600 bioactive secondary metabolites have been identified from *Streptomyces.* Computerized estimations propose

that the genus is capable of producing about 100 000 antibiotic compounds all in all (Mahajan and Balachandran, 2012; Watve et al., 2001). This means that only a tiny fraction of the potential natural products from this genus have, yet, been identified, which leaves a huge window open for new findings.

The secondary metabolites of streptomycetes have found industrial applications as antibiotics as well as antifungal, antiparasitic, antitumor and immunosuppressive drugs, among others (Schrempf and Dyson, 2011). The molecular structures of these compounds are diverse and have usually low toxicity which renders them good candidates for drug development (Bérdy, 2005). A range of industrially important enzymes are also produced, as well as enzyme inhibitors and insecticides (Goodfellow and Williams, 1986).

MARINE SOURCES OF NEW BIOCHEMICAL DIVERSITY

The ocean covers more than 70% of the surface of the earth. It is the least explored environment on the planet, especially so when looking at microorganisms. The deep oceans, abysses with depths greater than 2000 m, were considered biological deserts until a land-mark study (Grassle and Maciolek, 1992) estimated microbiological diversity in such habitats to exceed 10 million species (Bull et al., 2000). That extrapolation of data, the basis of the estimation, has later been questioned, but the fact remains that the ocean is a smorgasbord of undiscovered microbial diversity (Bull et al., 2000; Deming, 1998; Glöckner and Joint, 2010; Joint et al., 2010; Maldonado et al., 2005b).

The terrestrial environments have, successfully, been sampled for microorganisms with the ability to produce natural products for a long time. However, when the terrestrial samples begun to yield mostly rediscoveries, many begun to look at the marine environments. The actinomycetes of the sea have been shown to be taxonomically diverse and well distributed throughout all to date studied locations (Colquhoun et al., 1998; Magarvey et al., 2004; Weyland, 1969). However, one must not make the mistake of thinking that the marine environment is a continuous ecosystem. On the contrary, it is a collection of wide ranges of extreme niches, all with their own microbial diversity.

The oceans cover many ranges of extreme environmental factors. Viable microorganisms have been recovered from environments with temperatures ranging from as low as -32°C (Breezee et al., 2004; Cassell and Mekalanos, 2001; Price, 2000; Price and Sowers, 2004) to the surrounding of deep-sea hydrothermal vents where temperatures approach 400°C (Eecke et al., 2012; Gerday et al., 2007; Thornburg et al., 2010). As an example of extreme niches, deep-sea hydrothermal vents seem to be barren at the first look. But that hostile environment is home to a wide diversity of microorganisms that have acclimatized to the high temperatures, high pressure, and acidity (Gerday et al., 2007; Merkel et al., 2012; Tarasov et al., 2005; Valverde et al., 2012; Vetriani et al., 2004). The specialization of the inhabitants has also yielded biosynthetic pathways that have just begun to be analyzed. The sampling of bacterial communities of extreme niches in marine environments carries great potential to be a major approach for unearthing new natural products (Deming, 1998; Maldonado et al., 2005b; Takai and Horikoshi, 1999; Thornburg et al., 2010).

ON THE SUBJECT OF UNCULTURABLES

In microbiology there is a phenomenon called ”the great plate count anomaly“, a term coined by Staley and Konopka in 1985. The phrase describes the fact that, when culturing bacteria, only a fraction of the cells in the original sample are recovered in culture. Bacterial cells visible and countable using light microscopy are by several order of magnitudes more abundant than the cells counted using the plating method (Staley and Konopka, 1985). This discrepancy had been observed much earlier; it was first reported by Razumov in 1932. The bacteria and even other microorganisms that do not readily grow on plates are commonly called unculturable.

The idea that some bacteria are unculturable is today somewhat outdated. The unculturable bacteria are likely to be perfectly able to grow in culture if the right conditions are found and their metabolic requirements are met (Button et al., 1993; Connon and Giovannoni, 2002; Vartoukian et al., 2010; Watve et al., 2000). The problem lies instead in finding what the bacteria require for their growth. One successfully applied approach to grow difficult species is to simulate the natural environment, from which the species originate. This method allows the bacteria to grow in the complex environment that they require without having to identify and recreate the missing parameters in the lab (Gavrish et al., 2008; Kaeberlein et al., 2002; Polsinelli and Mazza, 1984).

EXPERIMENTAL TECHNIQUES: ENRICHMENT AND SELECTION

There are many ways to increase the yield of actinomycete and even other bacterial isolates from complex samples. The specific abilities of the actinomycete species can be used to aid their cultivation. For example, a range of different treatments and substrates can be used to weed out the unwanted species in a sample. By modifying the methods to specifically select for actinomycete growth, the number of unique isolates retrieved can increase considerably.

Many actinomycete species form sturdy spores when facing difficult environments, this trait can be utilized in sample enrichment. Heat-treating the samples or exposing the material to other stress factors can initiate such sporulation. A harsh enough treatment would kill the unwanted organisms in the sample, rendering mostly the viable spores to germinate and form colonies (Terahara et al., 2013; Wakisaka et al., 1982).

ISOLATING MICROORGANISMS FROM MARINE SAMPLES

In this study, three marine samples from Whaler's bay and Yankee Harbour from the South Shetland archipelago in Antarctica and two samples from Mollösund (Skagerak) and Torekov (Skälderviken), in Sweden were analyzed for the presence of actinomycete species. The samples varied in their biological nature: one sample consisted of sea water, one of beach sand, one of mostly penguin guano, one of mussels, and one of macroalgea. The study provided a chance to analyze a broad range of bacterial species, from sessile species to species associated with mollusks and plants.

The five collected samples were processed with a range of physical and chemical treatments in order to enrich for actinomycetes. The samples were, afterwards, cultivated on media with varying nutrient sources, e.g. complex carbon sources, trace minerals, as well as with and without selective antibiotics against Gram-negative bacteria and fungi. The samples were also cultivated in modified actinomycete traps, a kind of diffusion chamber constructed with Millipore membranes (Kaeberlein et al., 2002; (Gavrish et al., 2008). Some promising isolates were subsequently sequenced and their phylogeny was elucidated. The ability to produce antimicrobial metabolites was estimated for few selected isolates by means of the bioassay against selected bacterial and fungal pathogens, some carrying on the resistant genes.

MATERIALS AND METHODS

BIOLOGICAL MATERIAL

Five samples of various biological natures were collected from marine environments and marine-influenced terrestrial locations. Three samples (Y1, W1, and W2) were collected in the South Shetland archipelago (Antarctica) and two (E1 and T1) in southern coastal areas in Sweden. Additionally, samples Y1 and W1 were collected above water, while the samples W2, E1 and T1 were collected from sub-surface locations. Table 1 provides a detailed description of the samples as some of the samples were of a complex biological nature. The samples were frozen at -50° C directly upon their arrival to the laboratory. No prior treatment was applied at this point.

Table 1. Content, amount, and origin of the samples analyzed in this study.

Sample	Amount	Contents	Origin
Y1	15 g	Sand, shell fragments, penguin guano, seaweed, etc.	Yankee Harbour, Greenwich Island, Antarctica
E1	>50 g	*Mytilus edulis*, blue mussels	Mollösund, Skagerak, Sweden
T1	>100g	Macroalgea (species undetermined)	Torekov, Skälderviken, Sweden
W1	30 g	Volcanic black sand, etc.	Whalers' Bay, Deception Island, Antarctica
W2	25 g	Sea water	Whalers' Bay, Deception Island, Antarctica

SAMPLE TREATMENTS AND PREPARATION

Samples Y1, E1, T1, and W1 were subjected to seven diverse treatments of physical, physical and chemical as well as mechanical matter (B1 through B7, Table 2). Sample W2 was subjected to treatments B3 through B7 (Table 2). Each treatment was carried out using 0.5 g of sample material. Control treatment (Table 2) consisted of each respective sample (0.5g frozen material) proceeded without prior treatment and suspended in 4.5 ml SSW (Sea Salt Water; 16.5 g sea salt, 1 L deionized sterile H_2O, a salt solution of approximately half the oceanic salt concentration). .

Table 2. Treatments of the samples.

Manner of treatment	Treatment name	Treatment
	K	Control (no treatment)
Physical	B1	Drying in laminar flow bench (LAF) at room temperature (Jensen et al., 2005)
	B2	Drying in LAF at room temperature, heat treated at 120°C for 30 min (Bredholt et al., 2008)
	B3	Suspended in 4.5 ml SSW, heat treated at 60°C for 10 min (Jensen et al., 2005)
	B4	Suspended in 4.5 ml SSW, UV-radiation treated for 15 s (Bredholdt et al., 2007)
	B5	Suspended in 4.5 ml SSW, microwave treated at 80 W for 45 s (Bredholdt et al., 2007)
Physical and chemical	B6	Suspended in 4.5 ml 1.5% phenol, kept at 30°C for 30 min (Bredholt et al., 2008)
Mechanical	B7	Suspended in 4.5 ml SSW, rotated with 25 glass beads for 30 min at room temperature (Maldonado et al., 2008)

Following sample treatments were applied:

- B1 - the frozen sample was transferred to a petri dish. The sample was kept in a laminar flow bench (LAF) for drying overnight. The sample was subsequently gently crushed, and the dry material was used for stamping of plates (see under Cultivation). The remaining material was suspended in 4.5 ml SSW.
- B2 – the treatment was carried out as B1. When the sample was completely dry, the glass petri dish was transferred to a 120°C heat cabinet for 30 min. The material was subsequently gently crushed and used for stamping of plates. The remaining material was suspended in 4.5 ml SSW.
- B3 - the frozen sample was directly suspended in 4.5 ml SSW in a falcon tube. The sample was incubated in a water bath at 60°C for 10 min.
- B4 and B5 - the frozen samples were first suspended in 4.5 ml SSW, then transferred to petri dishes. In treatment B4, the petri dish was exposed to UV-radiation for 15 s. In treatment B5, the petri dish was irradiated with microwaves at 80 W for 45 s.
- B6 - the frozen sample was suspended in 4.5 ml 1.5% phenol in a falcon tube. The sample was subsequently incubated at 30°C for 30 min.
- B7, the sample was suspended in 4.5 ml SSW in a glass tube. Twenty-five glass beads were added to the tube, and the tube was kept on a rotator for 30 min.

After completed treatments, the samples were diluted in SSW by using a standard dilution protocol until a dilution of 10^{-5} was reached. The dilutions 10^{-3} to 10^{-5} (300 µl) were used to inoculate agar plates with diverse culture media. Suspension was spread onto each plate using a bent plastic inoculation loop. The plating was done in duplicates.

Additionally, the dry material from treatments B1 and B2 were used for inoculation of plates by stamping. The stamping was carried out using autoclaved foam plugs. The plugs were pressed into the dry material and the plates were stamped in a clockwise direction, creating a slight dilution effect.

Actinomycete traps were constructed by attaching Millipore membranes to plastic washers (Gavrish et al., 2008; Hirsch and Christensen, 1983). Membranes of pore sizes 0.05, 0.2, and 0.4 µm were used. The traps were inoculated with 100 µl of sample dilutions 10^{-1} and 10^{-2}. Subsequently, the traps were filled with 2.5 ml sterile water agar (1.5 % agar, deionized H_2O) placed on WA plates and incubated at 20°C.

CULTURE MEDIA AND CULTIVATION

Seven agar based culture media were used to cultivate samples out of which six media were selective and one was non-selective general culture medium. The detailed description of media composition is given in Table 3. The selective media were supplemented with antibiotics against Gram-negative bacteria and fungi. The antibiotics used were cycloheximidine (50 µg/ml), nystatin (50 µg/ml), and nalidixic acid (30 µg/ml). Vitamin supplements (ATCC Vitamin Supplements) added to the WA, IM6, IM7, and IM8 consisted of: folic acid (2 mg/L), pyridoxine hydrochloride (10 mg/L), riboflavin (5 mg/L), biotin (2 mg/L), thiamine (5 mg/L), nicotinic acid (5 mg/L), calcium pantothenate (5 mg/L), vitamin B12 (0.1 mg/L), p-aminobenzoic acid (5 mg/L), thioctic acid (5 mg/L), and monopotassium phosphate (900 mg/L).

Table 3. Culture media used for isolating microorganism from the samples.

Media	Antibiotics	Composition
VPB	No	Vegetable Peptone Broth 10g, Agar 15g, H_2O 1L
WA	Yes	Agar 15g, H_2O 1L, Vitamins 1ml
SCN	Yes	Starch 10g, Casein 0.3g, K_2HPO_4 2g, $KN0_3$ 2g, NaCl 2g, $MgSO_4$x7H2O, 0.05g, $CaCO_3$ 0.02g, $FeSO_4$ x7H_2O 0.01g, Agar 15g, H_2O 1L (Küster and Williams, 1964)
SCN-SW	Yes	Starch 10g, Casein 0.3g, K_2HPO_4 2g, $KN0_3$ 2g, NaCl 2g, $MgSO_4$x7H2O, 0.05g, $CaCO_3$ 0.02g, $FeSO_4$ x7H_2O 0.01g, Agar 15g, Sea salt 33g, H_2O 1L (Küster and Williams, 1964)
IM6	Yes	Glycerol 0.5g, Starch 0.5g, Sodium propionate 0.5g, $KN0_3$ 0.1g, asparagine 0.1g, casein 0.3g, K_2HPO_4 0.5g, $FeSO_4$ x7H_2O 1mg, Agar 18g, Sea salt 16g, H_2O 1L, Vitamins 1ml (adapted from Bredholt et al., 2008)
IM7	Yes	Chitin 2g, K_2HPO_4 0.5g, $FeSO_4$ x7H_2O 1mg, Agar 18g, Sea salt 23g, H_2O 1L, Vitamins 1ml (adapted from Bredholt et al., 2008)
IM8	Yes	Malt extract 1g, Glycerol 1g, Glucose 1g, Peptone 1g, Yeast extract 1g, Agar 18g, Sea salt 16g, H_2O 1L (adapted from Bredholt et al., 2008)

After plating, all plates were placed in a growth chamber at 20°C. Upcoming bacterial isolates were and are picked up continuously in order to obtain a collection of fast and slow growing organisms.

SELECTION AND PRESERVATION OF ISOLATES

In general, isolates for preservation were selected based on their morphology, e.g. color, shape of colony, and texture. Ability to grow on complex media as well as which specific treatment the colony survived was also taken into account. The selected colonies were transferred to NBC (1 g nutrient broth, 1 g casamino acids, 10 g agar, 1 L H_2O) and NBC-SW (NBC medium supplemented with 33 g sea salt), respectively. NBC-SW plates were, routinely, used to estimate an ability of a given strain to grow in the presence of salt. Two preservation methods were used to maintain purified isolates: i) aliquots of pure isolates were frozen in 50 % salt water for following DNA purification; ii) aliquots of isolates were frozen in 50 % glycerol for long-time storage at -70°C.

DNA ANALYSIS

The DNA was extracted using the Fast DNA SPIN Kit for Soil (MP Biomedicals) according to the manufacturer's description; approximately 200 µl of culture suspension in 50% salt water was used for DNA extraction. The 16S rDNA sequence was subsequently PCR-amplified using Fermentas DreamTaq DNA polymerase mix (Thermo Scientific). The primers used were 27F (5'- AGAGTTTGATCMTGGCTCAG - 3') and 1495R (5'- GGTTACCTTGTTACGACTT - 3') (Lane, 1991; Turner et al., 1999). Each PCR reaction contained 12.5 µl DreamTaq (2X), 0.4 µM of forward and reverse primer, respectively, 10 ng/µl DNA template, and 12.5 µl distilled H_2O giving a total reaction volume of 25 µl. The PCR program used was 95°C for 5 min, 30 cycles of 94°C for 40s, 55°C for 40s, 72°C for 80s, ending with 72°C for 7 min, and then kept at 4°C.

Some isolates that did not amplify using the original PCR program with the primer set given above were afterwards amplified using the primer sets 27F against 765R (5′ - CTGTTTGCTCCCCACGCTTTC - 3'), and 704F (5′ - GTAGCGGTGAAATGCGTAGA - 3') against 1492R, respectively (Coombs and Franco, 2003).

The PCR products were visualized using gel electrophoresis (1% agarose with EtBr) with GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific) and subsequently purified using QIAquick PCR Purification kit (Qiagen) according to the manufacturer's description.

The purified DNA was transferred into two 96-well plates as templates for sequencing (5 µl of appr. 50 ng/µl). The forward (27F) and reverse (1492R) primers (5 µl of 10 µM) were added to 48 wells on each plate. The following DNA sequencing was done by Macrogen.

ISOLATE IDENTIFICATION AND PHYLOGENY

The sequences from the 16S rRNA gene were from both forward (27F) and reverse (1492R) primers, and thus had an overlapping center. The chromatograms were firstly analyzed using Chromas software (McCarthy), where unreliable parts of sequences were trimmed off. The forward and reverse sequences were assembled using MEGA5.2 (Tamura et al., 2011). The full-length sequences were annotated using BLAST (nucleotide BLAST against highly similar sequences) (Altschul et al., 1990; Ye et al., 2006).

An alignment of the complete sequences was constructed using MEGA5.2. After trimming the alignment, it was used to construct a phylogenetic tree. The tree was calculated using Maximum Parsimony, with model Tamura-Nei (Tamura and Nei, 1993) and bootstrap testing of 5000. The branches with bootstrap support lower than 75% were collapsed.

BIOLOGICAL ASSAYS

Biological assays were carried out by using two methods: i) crosswise interaction plate assay and ii) 96-wells microtiter plate assay.

Crosswise interaction assay plates were constructed by inoculating VPB agar plates (15 cm) with rows of eight bacterial isolates (5 µl isolate suspended in 50% salt water). The isolates were positioned in rows, so that each isolate come in contact with the seven others (Seyedsayamdost et al., 2012). The plates were incubated for about 14 days and observed for visible antagonistic activity.

The ability to produce antimicrobial metabolites of one selected isolate (T74) was analyzed by microtiter plate assay (Pohanka et al., 2005). Prior to analysis, the isolate was cultured in 50% Vegetable Peptone Broth (VPB, (Oxoid Ltd), 15.5 g VPB, 1L deionized H_2O) and in modified Mineral Medium (Pohanka et al., 2005; Stanier et al., 1966), both in baffled and non-baffled Erlenmayer flasks, (96h, 20°C, 160 rpm). Six to twenty-four hours after inoculation, nylon bags with a sterile polymeric resin Amberlite XAD 16 (Sigma-Aldrich; approximately 5g per bag) were submerged to cultures in order to collect metabolites produced during culturing. Prior to the use, the polymeric resin was activated as advised by manufacturer.

At the end of cultivation Amberlite bags were removed from the cultures, washed with deionized H_2O (approximately 80 to 100 ml) and extracted with methanol (40 to 80 ml depending on the sample). The solvents were removed by overnight drying (30°C, under flow of N_2) and dissolved in an appropriate solvent prior to HPLC- analysis. The extracts were fractionated using preparative HPLC (C18 column with increasing gradient of acetonitrile), and the fractions were transferred to a set of 96-wells microtiter plates. The extracts were bioassayed against several target microbial pathogens according to previously described method (Pohanka et al., 2005). Fractions with activity against one or more target pathogens were analyzed with the help of LC-MS (Liquid Chromatography-Mass Spectrometry). Active compounds were identified by comparison of their molecular weight with molecular weights of known natural products available at a range of chemical databases. All chemical analysis was carried at the Department of Chemistry, SLU.

RESULTS

CULTIVATION

The cultivation yielded so far a broad range of diverse microbial isolates. The amount of colonies, and their morphology, varied several log units between samples, treatments, and media composition (data not shown). Figure 1 shows a comparison of colony yields from the sample Y1 after treatments B1 and B3, respectively. A wide range of colonies with divergent morphology appeared after cultivation of the sample Y1B1 on the non-selective VPB agar medium. The colonies varied in size and they were more or less fast growing. Colonies from this sample tended to be rich in pigments, often dark yellow, orange, or red (Fig. 1a). In contrast, sample Y1B3 that was also cultured on VPB agar displayed more pale beige colonies (Fig. 1b). This clearly demonstrates that the different treatments strongly affected the bacterial composition of the samples.

The nutrient composition and availability of the cultivation media strongly affected the outcome of isolation. It could be observed that the colonies grown on different media varied in their morphology, even though they were cultivated from the same sample and treatment. This effectively demonstrates the selective power of various nutrient sources. Particularly the isolates cultivated on non-selective VPB plates differed greatly in morphology when compared to isolates obtained from all selective media.

In Figure 2, a large, watery colony appeared to be utilizing the starch and/or casein in the medium, surrounding itself with a clear zone area. The small surrounding colonies appear to utilize the carbon degraded by the large colony, which seems to excrete some kind of digestion enzymes.

A total number of 340 isolates were recovered and purified up to date. A complete table of all preserved isolates is enclosed as Appendix 1. Some points considered when choosing isolates were morphology and size of the colony, nutrition requirements, and sample treatment, etc. Upon transfer to NBC media with and without sea salt, the sodium requirements of the isolates were noted as well, and taken into account when isolates were chosen for further analysis by 16S rDNA sequencing and fermentation studies, i.e. production or antibiotic compounds.

The isolation of microorganisms will continue over a period of few more months, in order to select the ones that grow slowly and especially these from the selective media. The data set collected is of a great value for the future work on microbial diversity of samples originating from marine environments. The preserved microbial collection will also serve as a valuable source in other projects.

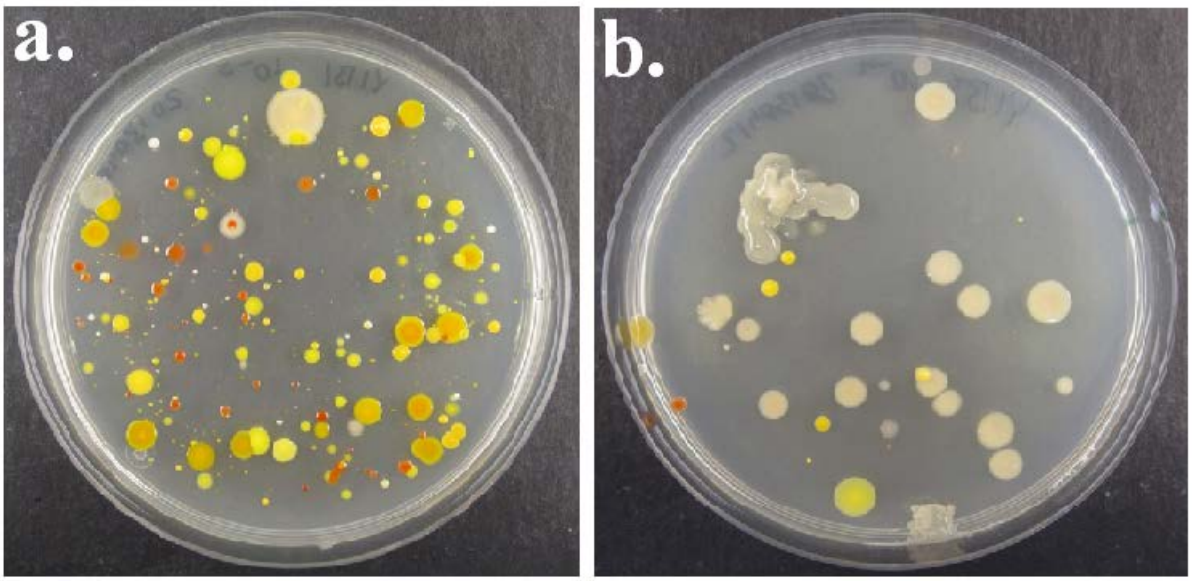

Figure 1. Comparison of the colony morphology of a) sample Y1B1, to b) sample Y1B3, both cultured on the non-selective VPB agar plates.

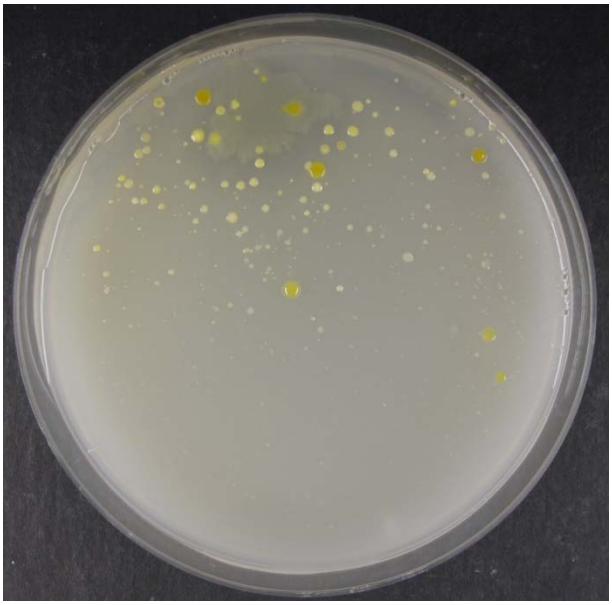

Figure 2. Colony morphology of isolates obtained from the sample Y1K cultured on SCN agar plates. A large, watery and irregular colony in the left upper corner of the plate has an ability to utilize the starch and/or casein. This is visualized by the clear zone area surrounding the colony.

16S rDNA AMPLIFICATION

Most isolates were successfully amplified using the primer set 27F and 1492R (Fig. 3a). The modified program (using 27F against 765R, and 704F against 1492R) was successfully applied on some isolates as well (Fig. 3b). By using both primer pairs in the second program, the faulty primer binding site could in a few cases be identified. Such an example could be seen in Fig. 3b, where isolate 130 was successfully amplified using primer pair 704F/1492R, but not using 27F/765R. A few isolates did not amplify with the use of either of the two methods, these isolates were at the time omitted from further analysis.

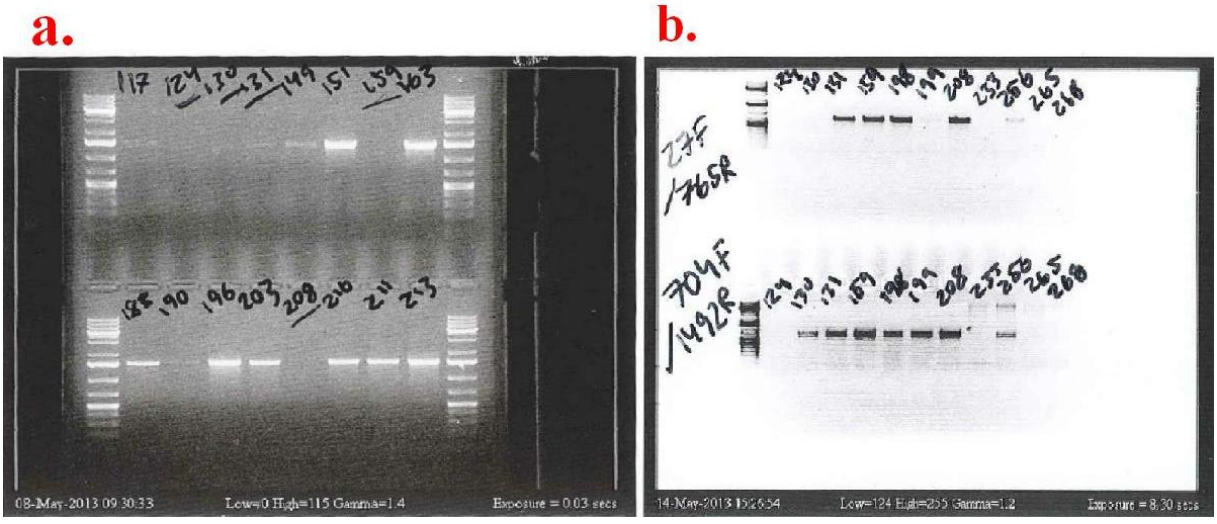

Figure 3. The gel images of 16S rDNA PCR products that did or did not amplify with **a)** the primer set of 27F/1492R. and **b)** the primer pairs 27F/765R (top) and 704F/1492R (bottom). The numbers are the isolate identification numbers.

SEQUENCING

Out of 96 isolates that were sequenced, 91 yielded good-quality chromatograms. These sequences were used to identify isolates to genus and/or species level, if possible. Identification was done with the help of BLAST database (Ye et al., 2006). The identity of recovered sequences with sequences available in the NCBI database ranged from 98% to 100%. However, the best hits in the database were often non-cultivated environmental isolates and the species-determined sequences could only be found further down the list. A table with identity of selected isolates is enclosed as Appendix 2.

ISOLATE IDENTITY

The DNA sequencing revealed that of the 91 sequences, 34 were of the order *Actinomycetales.* The most commonly occurring species among these were *Rhodococcus* sp. and *Micrococcus* sp., but also *Arthrobacter* sp. and a few others were found. Notably, the found actinomycetes were all so called rare-actinomycetes, i.e. non-*Streptomyces* species.

In total, 39 out of 91sequences recovered belonged to various species of Gram-negative bacteria was (Fig. 4), while 52 belonged to the Gram-positive (Fig. 5).

IDENTITY OF GRAM-NEGATIVE BACTERIA

Thirty-nine of Gram-negative bacterial isolates belonged mainly to the following genera/species:

- *Chryseobacterium*
- *Erythrobacter*
- *Hymenobacter*
- *Pseudomonas*
- *Methylobacterium*
- *Granulosicoccus*
- *Psychrobacter*

All isolates were selected from the diverged samples/treatments. Figure 4 shows distribution of Gram- negative isolates among genera/species in percent of total number of Gram-negative isolates sequenced.

For example *Chryseobacterium* and *Hymenobacter* isolates were obtained from different treatments of the sample Y1. Two *Erythrobacter* species were found in the sample T1, and treatments B4 and B7. Unexpectedly they grew on IM7 and SCN media that both contain antibiotics against Gram-negatives bacteria which indicate that these can be resistant to the antibiotics used. Two *Methylobacterium adhaesivum* strains were isolated from Y1B1 on WA.

Four *Pseudomonas* species were isolated from the samples Y1 and E1, mostly on non-selective VPB medium, but one grew on SCN medium containing antibiotics against Gram-negative bacteria.

Seventeen *Granulosicoccus* strains were isolated from sample T1; several of them appeared to be identical clones. All isolates grew best on NBC-SW, containing 3.3% sea salt.

Five *Psychrobacter* strains were isolated from samples E1 and T1. The *Psychrobacter* strain denoted T74 showed signs of antibacterial activity and was subsequently tested for production of antimicrobial metabolites.

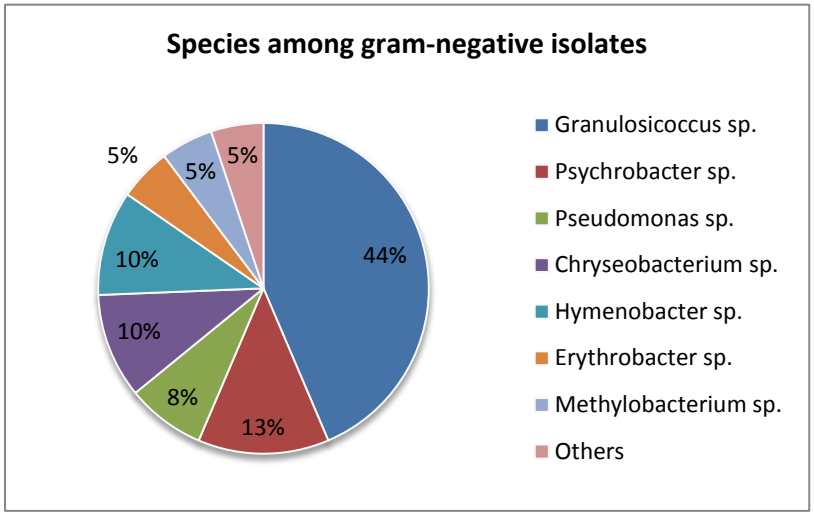

Figure 4. Distribution of 39 Gram-negative isolates among identified genera/species (%).

IDENTITY OF GRAM-POSITIVE BACTERIA

Fifty-two of Gram-positive bacterial isolates belonged mainly to the following orders/genera/species:

- Bacillales with predominantly various species of *Bacillus* and *Paenibacillus*
- *Staphylococcus*
- *Actinomycetales* with various species of *Micrococcus*, *Rhodococcus*, *Agreia*, *Pseudoclavibacter*, *Brevibacterium*, *Salinibacterium* and *Mycobacterium*

All isolates were selected from the diverged samples/treatments. Figure 5 shows distribution of Gram- positive isolates among genera/species in percent of total number of Gram-positive isolates sequenced.

Out of 52 sequenced isolates, 17 different isolates from varying samples were found to belong to the order of Bacillales; 8 isolates could be identified as *Bacillus* species.

One *Deinococcus* strain was isolated from the control Y1 sample cultured on VPB. The closest identity of this specific strain in comparison to a described species was 96%, and the identity to uncultured clones was 99%.

Five *Paenibacillus* strains were isolated from samples Y1, E1, and T1 cultured on VPB plates. The samples had undergone varying treatments; the isolates expressed a typical colony morphology that was pale and flat.

A total number of 34 sequences belonged to various Actinomycetales. Eleven strains of *Micrococcus* species were isolated from all five samples, and from varying treatments. All but one was isolated from VPB agar plates; the remaining one was isolated from IM8. Among the 7 isolated *Arthrobacter* strains, 6 were isolated from the sample Y1 while the remaining one was isolated from the sample E1. One of the strains had grown on VPB, two on IM8, and the remaining had grown on IM6 media. All 10 *Rhodococcus* strains were isolated from samples Y1 and W1. Two were isolated from IM6, and remaining once from VPB medium.

The remaining actinomycete species found were two *Agreia* species, one *Pseudoclavibacter* species, one *Brevibacterium* species, one *Salinibacterium* species, and one *Mycobacterium neglectum*. They were isolated from samples Y1, T1, and E1, and grown on IM7, IM8, and VPB media.

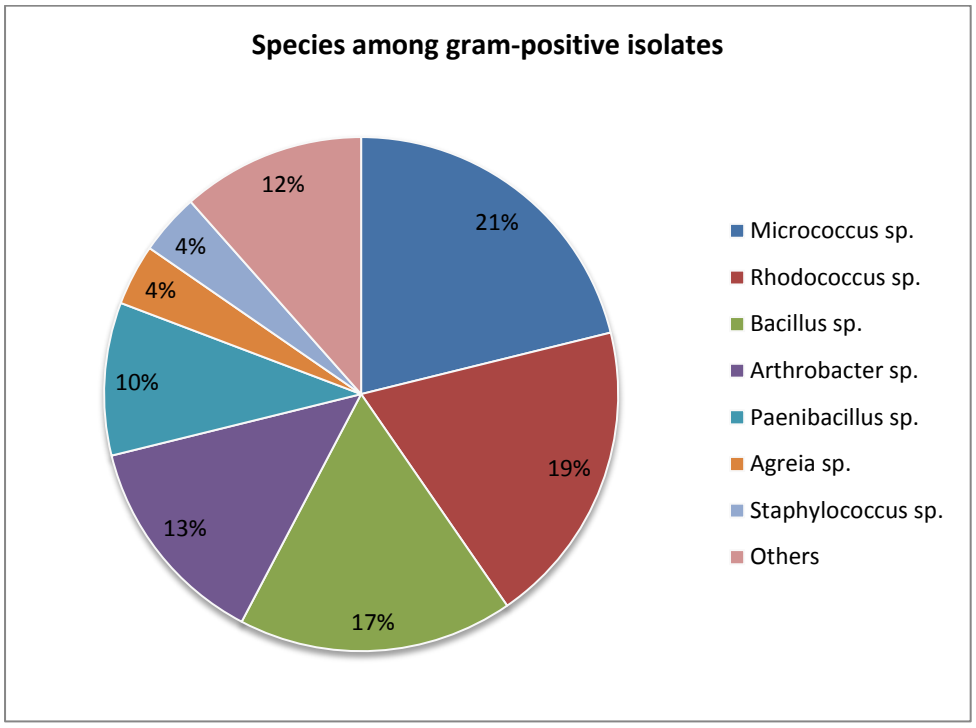

Figure 5. Distribution of 52 Gram-positive isolates among identified genera/species (%).

PHYLOGENETIC ANALYSIS

A broad range of isolates belonging to diverse bacterial species were isolated and identified in this study. A complete phylogenetic tree constructed with the use of 88 full-length 16S sequences of isolates recovered during this study is shown in Figure 6. Figure 7 shows a separate tree constructed for the branch of all isolates of Actinomycetes.

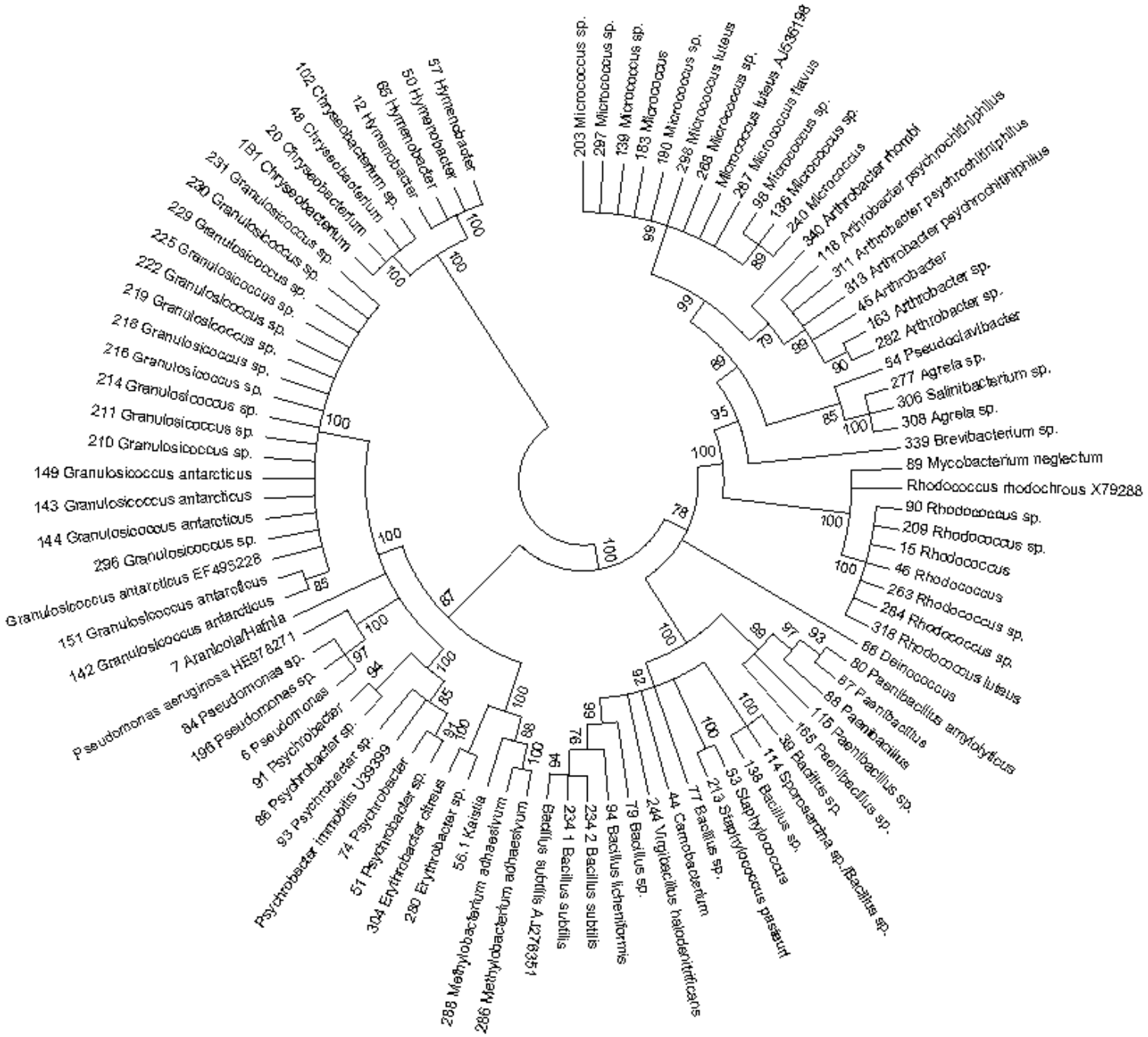

Figure 6. A phylogenetic tree of based on 88 sequences of isolates recovered in this study and constructed using the Maximum Likelihood analysis. Substitution model Tamura-Nei was used with 5000 bootstrap replicates. The branches with bootstrap support below 75% were collapsed. Six type strains included in analysis are marked with their accession numbers. The branch labels describe isolate number and identity at the genus or species level.

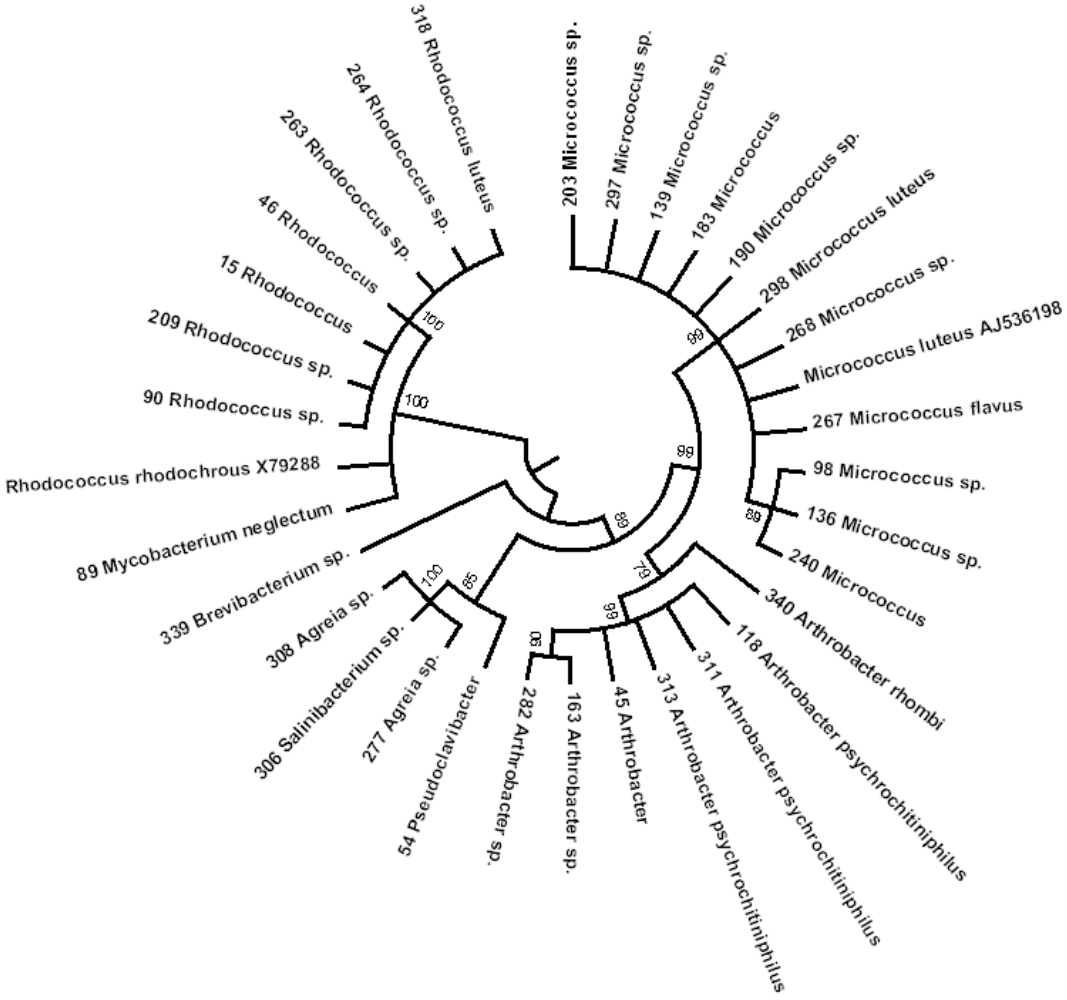

Figure 7. A phylogenetic tree of 34 Actinomycetes recovered in this study based on comparison of their 16 S-rDNA sequences. Two type strains of *Micrococcus* and *Rhodococcus* are marked with their accession numbers. All branches with bootstrap support lower than 75% are omitted.

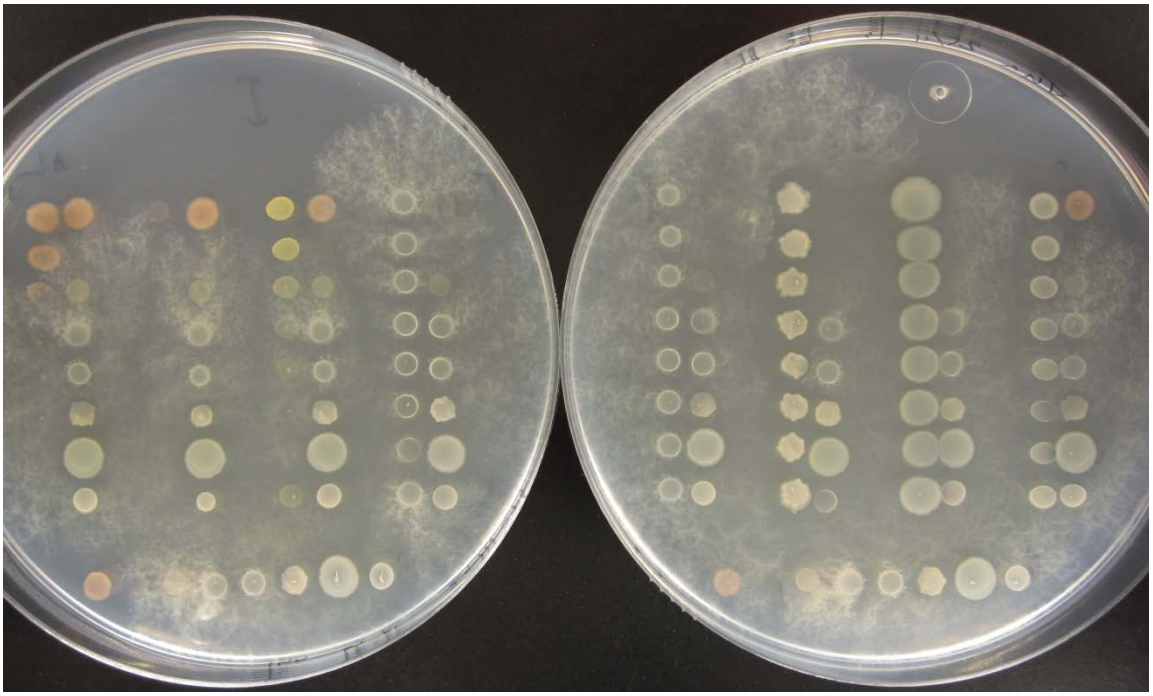

Figure 8. The crosswise interaction assay plates, with 8 isolates tested against each other. The extensive growth of one *Bacillus* isolate is controlled by some tested isolates, what is visualized by appearance of clearing zone areas around bacterial colonies.

BIOLOGICAL ASSAYS

Both the crosswise interaction assay and microtiter plate assay showed that some of recovered isolates have a potential to produce antimicrobial metabolites.

The crosswise interaction assay plates (Fig. 8) were quickly over-grown by an aggressive isolate of the *Bacillus* species. However, some other isolates managed to fend off the *Bacillus* and created clearing zones surrounding their colonies. The isolates might have a potential to produce metabolites active against Gram-positive bacteria.

The antimicrobial activity of the isolate 74, of a *Psychrobacter* species, against several pathogenic microorganisms indicated that the isolate produced compounds with antibiotic properties. The isolate inhibited growth of several pathogens, but its activity depended on the tested pathogen. The HPLC chromatogram (Fig. 10) shows several different areas of activity suggesting that the isolate produces several antimicrobial molecules. The active compound corresponding to at least one peak could be identified by its molecular weight. The compound was traced to be oxydifficidin, a known substance with antimicrobial activity (Zimmerman et al., 1987). The position of oxydifficidin is arrow-marked in Fig. 10. Corresponding antimicrobial activity is shown below the chromatogram.

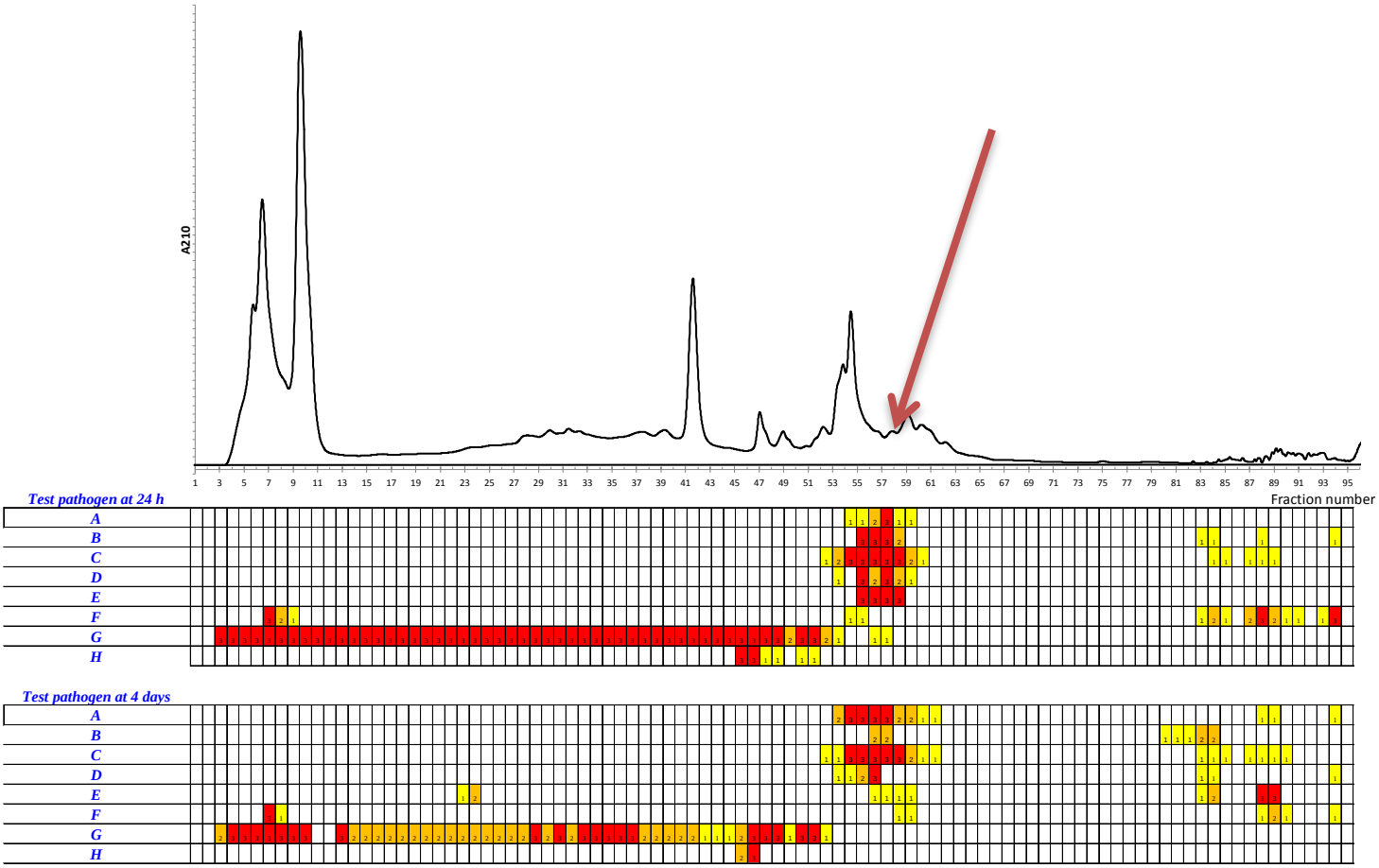

Figure 10. The HPLC chromatogram over a range of metabolites produced by the isolate T74 is shown coupled to the activity against eight pathogens. The antimicrobial activity detected after 24 hours and 4 days of incubation is shown below the HPLC chromatogram. The red boxes indicate complete inhibition of the target organism, whereas dark yellow indicate intermediate inhibition, and light yellow some inhibition. The red arrow points to approximately fraction 58 where the antibiotic compound oxydifficidin was detected.

DISCUSSION

SAMPLING THE MARINE ENVIRONMENT

The aim of this study was to isolate strains of actinomycetes from marine samples. The samples were taken from Whaler's bay and Yankee Harbor in Antarctica, as well as from southwestern coastal areas of Sweden. The samples were of diverse biological natures, consisting of seawater, beach sand, penguin guano, blue mussels, and macroalgea. The diversity of the samples allowed a series of niches to be studied simultaneously; species ranging from planktonic to sessile to mollusk- and macroalgea-associated life styles were targeted. The treatments of the samples allowed different genera to flourish, as the treatments divided the sensitive species from the insensitive. This could easily be observed as the diversity of the colonies decreased as the harshness of the treatments increased.

All the different treatments of the samples yielded viable colonies with sizes visible to the naked eye, although, in very varying amounts. It seems rather obvious that the harsher treatments (e.g. high temperatures, mechanical disruption) would yield fewer viable survivors than milder treatments (e.g. quick UV-radiation, low temperatures), and that was indeed confirmed in this study. The sturdy cells of the well-acclimatized species are very interesting from the biochemical point of view as their survival talents may indicate additional capabilities. The extremophiles of the marine environments are thought to hold great potential of new natural products (Hamedi et al., 2013; Wilson and Brimble, 2009).

In the attempts to amplify the 16S rRNA gene from the isolates using PCR, it could be noted that some isolates were not amplified using the general 16S rDNA primers. As the primers were substituted with two alternative primer pairs, fragmenting the 16S rRNA gene into two separate sequences, PCR products from some isolates could be recovered. In some cases, only one of the two fragments could be amplified. This indicates a mutation in the primer binding site, creating a mismatch that weakens primer binding. As this problem could be noted in such a small-scale study, while working with only about 100 isolates, one might stop to consider what the accumulated effect would be when screening marine communities. However, the main bias when culturing microbial samples for analysis is the plate-count anomaly, the fact that the main part of the microorganisms cannot be easily cultivated with standard procedures (Vartoukian et al., 2010). The general cultivability of soil microorganism has been estimated to be 0.30% (Amann et al., 1995; Torsvik et al., 1990); the marine microorganisms from sediments or sea water have an estimated cultivability as low as 0.25% (Amann et al., 1995; Jones, 1977) and 0.001-0.1% (Amann et al., 1995; Ferguson et al., 1984; Kogure et al., 1979, 1980), respectively. The culture-independent methods were developed to avoid this problem, and a much greater diversity can indeed be observed by analyzing the microorganisms without the limiting culturing steps (Hugenholtz et al., 1998; Wagner et al., 1993; Weisburg et al., 1991). However, if the PCR bias is as relatively common as detected in this study, then the diversity of microbial communities might be underestimated. Another explanation of the problems accounted during PCR amplification is a large variability of 16S rDNA sequences and copy numbers among various bacterial taxa. Analysis based on sequenced bacterial genomes show that only a few identical 16S rDNA copies can be detected and that sequence diversity is higher with increasing copy number (Větrovský and Baldrian, 2013). This suggests that universal primers might not necessarily amplify the 16S rDNA gene in all microorganisms. Highly dissimilar 16S rDNA sequences were previously reported (Yap et al., 1999) .

Of the 96 isolates that were sequenced, 90 yielded good-quality sequences that could be identified against the BLAST database. The number of gram-positive isolates was somewhat larger than the number of gram-negatives. Due to the fact that many isolates were taken from the non-selective VPB plates, i.e. the control plates, the amount of gram-negatives is larger than expected from the selective plates. The growth-rate of the colonies on media containing limited organic carbon, low nutrition, and antibiotics was slower; with a longer incubation time, the number of isolates from selective plates is likely to increase. Among the slow-growing colonies on selective plates, the majority of isolated bacteria are expected to be gram-positive actinobacteria.

All of the identified actinomycete isolates to date were so called rare actinomycetes, i.e. non-Streptomyces species. These isolated actinomycete strains hold potential of novelty as producers of natural products (Lazzarini et al., 2000; Tiwari and

Gupta, 2011, 2012). The role of rare actinomycetes as natural product producers has become more and more important in the later years. In 1970 only 11 species of rare actinomycetes had been documented as producers of a sum of 50 bioactive compounds, whereas in 2005 as many as 50 species had been found to produce a total of 2500 compounds (Bérdy, 2005). The rare actinomycetes are recognized as good sources of novel natural products, but to increase the discovery rate of these products, optimized methods of isolation (Maldonado et al., 2005b), fermentation (Genilloud et al., 2011), and screening (Bérdy, 2005) must be developed. By trying out several different fermentation conditions, previously undetected metabolites can be extracted from both old and new actinomycete species (Bode et al., 2002; Genilloud et al., 2011) .

The methods of isolation and cultivation that were used in this study were chosen in an attempt to select for rarer organisms. Fewer, but rarer, isolates are more suitable to be screened by the version of small-scale whole-cell screening mainly used by smaller research groups. By using samples from less-studied ecological niches, the chance of encountering novel organisms can be increased, and even more so by pre-treating the samples by various methods. Cultivating the samples on selective media containing different sources of organic carbon and varying concentrations of nutrients may further increase the chances of isolating organisms that are not usually found using standard laboratory culturing (Button et al., 1993; Connon and Giovannoni, 2002) .

THE SEARCH FOR NEW ANTIBIOTICS

The antibiotic resistance genes in microorganisms are part of an ancient evolutionary process; they are not something that has emerged recently by the use of antibiotics in medicine. However, the use of antibiotics in modern medicine and agriculture as well as increased travelling has increased the evolutionary pressure to develop antibiotic resistance and the speed of dispersal of these genes. Today, 70 years after the first introduction of antibiotics on the market, infectious diseases still are the major cause of death world-wide and account for 13.3 million deaths per year (Selvameenal et al., 2009). Resistant strains of previously treatable pathogens emerge constantly. The pursuit of new antibiotic compounds will be continuing in all foreseeable future unless completely new methods to efficiently control the presence and/or growth of microorganisms are invented.

Most probably, the outlook for environmental niches to sample for novel microbial metabolites and the use of combinatorial biosynthesis will be used side by side in future research. Both techniques have their pros and cons to be considered. Combinatorial biosynthesis requires good background libraries, and advanced know-how of both biological and chemical nature. When those criteria are met, the work that has to be done is basically an attempt to outsmart millions of years of evolution. Seen in that light, it is a miracle that this has actually been done, creating novel molecules able to combat pathogens. The synthetic and semisynthetic antibiotics also carry an extra positive: the resistance against synthetic drugs is not already common in the environment, and this may lead to a somewhat slower dispersal of resistance among targeted pathogens (D'Costa, 2006). The rediscovery rate of natural products antibiotics when isolating microorganisms has been steadily increasing; this in turn decreases the effectiveness of the research and renders such studies uneconomical (Fenical et al., 1999). But the microbiome of the planet is vast, and only a fraction of it has been explored to date (Cragg and Newman, 2002; Harvey, 2000). By targeting new ecosystems, including extreme environments, the chance of isolating novel organisms increases and novel microbes are potential gold mines of secondary metabolites and biosynthetic gene diversity. It might not be too bold to think that the quite substantial amount of natural products that has been found to date could easily be multiplied by a factor of ten, when considering that we have not by far studied all accessible ecosystems. Also, many of the cultivable organisms have the ability to produce many more secondary metabolites than previously shown when they are grown under special conditions. By varying nutrition and stress factors, as well as growth temperature, some organisms can shift their metabolisms and reveal more of their biosynthesis potential (Bode et al., 2002; Higgs et al., 2001).

FUTURE PERSPECTIVES

Further studies of marine environments are essential. The abyssal depths of the oceans are mostly unexplored and are likely to harbor a great diversity of microorganisms yet unseen. Methods for culturing and isolating microorganisms need to be further developed if we should have any chance to investigate the full diversity of microorganisms and their biosynthetic abilities (Huber et al., 1995, 1998). Also, microorganisms from the extreme environments might require the development of completely new methods of cultivation. For example, cultivation procedures at high pressure and high temperatures may need to be developed and made available for standard laboratory settings (Houghton et al., 2007). On the other hand, previous studies suggest that many extreme microbes do not always require extreme culturing settings (Pettit, 2011). Genomic studies of uncultivated microbes may in parallel not only identify biosynthetic pathways, but also help to uncover metabolic pathways than can reveal what factors are missing for successful isolation. The amount of time and energy put into the exploration of the terrestrial part of the planet throughout the last centuries has been substantial. Similarly, huge resources will also be needed to explore the oceans, but the rewards can be hoped to be even greater.

ACKNOWLEDGEMENTS

I would like to extend my sincerest thanks to my supervisor Joakim Bjerketorp, assistant supervisor Jolanta Levenfors, and examiner Bengt Guss, all at the Department of Microbiology at the Swedish University of Agricultural Sciences. I would also like to thank Pierre Andersson, Elsa Coucheney, and Anders Broberg at the Department of Chemistry at the Swedish University of Agricultural Sciences.

This research project was carried out as collaboration between researchers at SLU and the pharmaceutical company Medivir AB, Stockholm, Sweden.

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. *59*, 143–169.

Atlas, R.M. (1997). Principles of microbiology (Dubuque, IA: WCB, Brown).

Baltz, R.H. (2008). Renaissance in antibacterial discovery from actinomycetes. Anti-Infect. Technol. *8*, 557–563.

Bancroft, E.A. (2007). Antimicrobial resistance: It's not just for hospitals. JAMA *298*, 1803–1804.

Bérdy, J. (2005). Bioactive microbial metabolites. J. Antibiot. (Tokyo) *58*, 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 Eur. J. Chem. Biol. *3*, 619–627.

Bredholdt, H., Galatenko, O.A., Engelhardt, K., Fjaervik, E., Terekhova, L.P., and Zotchev, S.B. (2007). Rare actinomycete bacteria from the shallow water sediments of the Trondheim fjord, Norway: isolation, diversity and biological activity. Environ. Microbiol. *9*, 2756–2764.

Bredholt, H., Fjaervik, E., Johnsen, G., and Zotchev, S.B. (2008). Actinomycetes from Sediments in the Trondheim Fjord, Norway: Diversity and Biological Activity. Mar. Drugs *6*, 12–24.

Breezee, J., Cady, N., and Staley, J.T. (2004). Subfreezing growth of the sea ice bacterium "Psychromonas ingrahamii."Microb. Ecol. *47*, 300–304.

Bull, A.T., Ward, A.C., and Goodfellow, M. (2000). Search and Discovery Strategies for Biotechnology: the Paradigm Shift. Microbiol. Mol. Biol. Rev. *64*, 573–606.

Bush, K. (2004). Antibacterial drug discovery in the 21st century. Clin. Microbiol. Infect. *10*, 10–17.

Button, D.K., Schut, F., Quang, P., Martin, R., and Robertson, B.R. (1993). Viability and Isolation of Marine Bacteria by Dilution Culture: Theory, Procedures, and Initial Results. Appl. Environ. Microbiol. *59*, 881–891.

Cassell, G.H., and Mekalanos, J. (2001). Development of antimicrobial agents in the era of new and reemerging infectious diseases and increasing antibiotic resistance. Jama J. Am. Med. Assoc. *285*, 601–605.

Chater, K.F., and Chandra, G. (2006). The evolution of development in Streptomyces analysed by genome comparisons. Fems Microbiol. Rev. *30*, 651–672.

Clardy, J., Fischbach, M.A., and Walsh, C.T. (2006). New antibiotics from bacterial natural products. Nat. Biotechnol. *24*, 1541–1550.

Colquhoun, J.A., Mexson, J., Goodfellow, M., Ward, A.C., Horikoshi, K., and Bull, A.T. (1998). Novel rhodococci and other mycolate actinomycetes from the deep sea. Antonie Van Leeuwenhoek *74*, 27–40.

Connon, S.A., and Giovannoni, S.J. (2002). High-Throughput Methods for Culturing Microorganisms in Very-Low-Nutrient Media Yield Diverse New Marine Isolates. Appl. Environ. Microbiol. *68*, 3878–3885.

Coombs, J.T., and Franco, C.M.M. (2003). Isolation and Identification of Actinobacteria from Surface-Sterilized Wheat Roots. Appl. Environ. Microbiol. *69*, 5603–5608.

Cragg, G.M., and Newman, D.J. (2002). Chemical diversity: a function of biodiversity. Trends Pharmacol. Sci. *23*, 404–405; author reply 405.

D'Costa, V.M. (2006). Sampling the Antibiotic Resistome. Science *311*, 374–377.

Deming, J.W. (1998). Deep ocean environmental biotechnology. Curr. Opin. Biotechnol. *9*, 283–287.

Eecke, H.C.V., Butterfield, D.A., Huber, J.A., Lilley, M.D., Olson, E.J., Roe, K.K., Evans, L.J., Merkel, A.Y., Cantin, H.V., and Holden, J.F. (2012). Hydrogen-limited growth of hyperthermophilic methanogens at deep-sea hydrothermal vents. Proc. Natl. Acad. Sci. *109*, 13674–13679.

Fenical, W., and Jensen, P.R. (2006). Developing a new resource for drug discovery: marine actinomycete bacteria. Nat. Chem. Biol. *2*, 666–673.

Fenical, W., Baden, D., Burg, M., Goyet, C.V., Grimes, J.D., Katz, M., Marcus, N.H., Pomponi, S., Rhines, P., Tester, P., et al. (1999). From monsoons to microbes: Understanding the ocean's role in human health. Mar. Deriv. Pharm. Relat. Bioact. Compd. 71–86.

Ferguson, R.L., Buckley, E.N., and Palumbo, A.V. (1984). Response of marine bacterioplankton to differential filtration and confinement. Appl. Environ. Microbiol. *47*, 49–55.

Fleming, A. (1929). On the antibacterial action of cultures of a penicillium with special reference to their use in the isolation of B. influenzae. Br. J. Exp. Pathol. *1929*, 226–236.

Floss, H.G. (2006). Combinatorial biosynthesis—Potential and problems. Bioperspectives—from Basic Res. Ind. Prod. Bioperspectives Congr. *124*, 242–257.

Flärdh, K., and Buttner, M.J. (2009). Streptomyces morphogenetics: dissecting differentiation in a filamentous bacterium. Nat. Rev. Microbiol. *7*, 36–49.

Gavrish, E., Bollmann, A., Epstein, S., and Lewis, K. (2008). A trap for in situ cultivation of filamentous actinobacteria. J. Microbiol. Methods *72*, 257–262.

Genilloud, O., González, I., Salazar, O., Martín, J., Tormo, J., and Vicente, F. (2011). Current approaches to exploit actinomycetes as a source of novel natural products. J. Ind. Microbiol. Biotechnol. *38*, 375–389.

Gerday, C., Glansdorff, N., and American Society for Microbiology (2007). Physiology and biochemistry of extremophiles (Washington, D.C: ASM Press).

Ghai, R., McMahon, K.D., and Rodriguez-Valera, F. (2012). Breaking a paradigm: cosmopolitan and abundant freshwater actinobacteria are low GC. Environ. Microbiol. Reports *4*, 29–35.

Glöckner, F.O., and Joint, I. (2010). Marine microbial genomics in Europe: current status and perspectives. Microb. Biotechnol. *3*, 523–530.

Goodfellow, M., and Williams, E. (1986). New Strategies for the Selective Isolation of Industrially Important Bacteria. Biotechnol. Genet. Eng. Rev. *4*, 213–262.

Grassle, J.F., and Maciolek, N.J. (1992). Deep-Sea Species Richness: Regional and Local Diversity Estimates from Quantitative Bottom Samples. Am. Nat. *139*, 313–341.

Hamedi, J., Mohammadipanah, F., and Ventosa, A. (2013). Systematic and biotechnological aspects of halophilic and halotolerant actinomycetes. Extrem. Life Extreme Cond. *17*, 1–13.

Harvey, A. (2000). Strategies for discovering drugs from previously unexplored natural products. Drug Discov. Today *5*, 294–300.

Helmke, E., and Weyland, H. (1984). Rhodococcus marinonascens sp. nov., an Actinomycete from the Sea. Int. J. Syst. Bacteriol. *34*, 127–138.

Henkel, T., Brunne, R.M., Müller, H., and Reichel, F. (1999). Statistical Investigation into the Structural Complementarity of Natural Products and Synthetic Compounds. Angew. Chem. Int. Ed. *38*, 643–647.

Higgs, R.E., Zahn, J.A., Gygi, J.D., and Hilton, M.D. (2001). Rapid Method To Estimate the Presence of Secondary Metabolites in Microbial Extracts. Appl. Environ. Microbiol. *67*, 371–376.

Hirsch, C.F., and Christensen, D.L. (1983). Novel method for selective isolation of actinomycetes. Appl. Environ. Microbiol. *46*, 925–929.

Houghton, J.L., Seyfried, W.E., Jr, Banta, A.B., and Reysenbach, A.-L. (2007). Continuous enrichment culturing of thermophiles under sulfate and nitrate-reducing conditions and at deep-sea hydrostatic pressures. Extrem. Life Extreme Cond. *11*, 371–382.

Huber, R., Burggraf, S., Mayer, T., Barns, S.M., Rossnagel, P., and Stetter, K.O. (1995). Isolation of a hyperthermophilic archaeum predicted by in situ RNA analysis. Nature *376*, 57–58.

Huber, R., Dyba, D., Huber, H., Burggraf, S., and Rachel, R. (1998). Sulfur-inhibited Thermosphaera aggregans sp. nov., a new genus of hyperthermophilic archaea isolated after its prediction from environmentally derived 16S rRNA sequences. Int. J. Syst. Bacteriol. *48 Pt 1*, 31–38.

Hugenholtz, P., Goebel, B.M., and Pace, N.R. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. *180*, 4765–4774.

Jensen, P.R., Dwight, R., and Fenical, W. (1991). Distribution of actinomycetes in near-shore tropical marine sediments. Appl. Environ. Microbiol. *57*, 1102–1108.

Jensen, P.R., Gontang, E., Mafnas, C., Mincer, T.J., and Fenical, W. (2005). Culturable marine actinomycete diversity from tropical Pacific Ocean sediments. Environ. Microbiol. *7*, 1039–1048.

Joint, I., Mühling, M., and Querellou, J. (2010). Culturing marine bacteria - an essential prerequisite for biodiscovery. Microb. Biotechnol. *3*, 564–575.

Jones, J.G. (1977). The effect of environmental factors on estimated viable and total populations of planktonic bacteria in lakes and experimental enclosures. Freshw. Biol. *7*, 67–91.

Kaeberlein, T., Lewis, K., and Epstein, S.S. (2002). Isolating “Uncultivable” Microorganisms in Pure Culture in a Simulated Natural Environment. Science *296*, 1127–1129.

Kogure, K., Simidu, U., and Taga, N. (1979). A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. *25*, 415–420.

Kogure, K., Simidu, U., and Taga, N. (1980). Distribution of viable marine bacteria in neritic seawater around Japan. Can. J. Microbiol. *26*, 318–323.

Küster, E., and Williams, S.T. (1964). Selection of Media for Isolation of Streptomycetes. Nature *202*, 928–929.

Lane, D.J. (1991). 16S/23S rRNA sequencing. In Nucleic Acids Techniques in Bacterial Systematics, (Chichester.: John Wiley & Sons), pp. 115–147.

Lazzarini, A., Cavaletti, L., Toppo, G., and Marinelli, F. (2000). Rare genera of actinomycetes as potential producers of new antibiotics. Antonie Van Leeuwenhoek *78*, 399–405.

Ligon, B.L. (2004). Penicillin: its discovery and early development. Probl. Solutions Antimicrob. Resist. Pediatr. Respir. Tract Nosocomiall Pathog. *15*, 52–57.

Liu, B., and Pop, M. (2009). ARDB—Antibiotic Resistance Genes Database. Nucleic Acids Res. *37*, D443–D447.

Magarvey, N.A., Keller, J.M., Bernan, V., Dworkin, M., and Sherman, D.H. (2004). Isolation and Characterization of Novel Marine-Derived Actinomycete Taxa Rich in Bioactive Metabolites. Appl. Environ. Microbiol. *70*, 7520–7529.

Mahajan, G.B., and Balachandran, L. (2012). Antibacterial agents from actinomycetes - a review. Front. Biosci. Elite Ed. *4*, 240–253.

Maldonado, L.A., Fenical, W., Jensen, P.R., Kauffman, C.A., Mincer, T.J., Ward, A.C., Bull, A.T., and Goodfellow, M. (2005a). Salinispora arenicola gen. nov., sp. nov. and Salinispora tropica sp. nov., obligate marine actinomycetes belonging to the family Micromonosporaceae. Int. J. Syst. Evol. Microbiol. *55*, 1759–1766.

Maldonado, L.A., Stach, J.E.M., Pathom-aree, W., Ward, A.C., Bull, A.T., and Goodfellow, M. (2005b). Diversity of cultivable actinobacteria in geographically widespread marine sediments. Antonie Van Leeuwenhoek *87*, 11–18.

Maldonado, L.A., Fragoso-Yáñez, D., Pérez-García, A., Rosellón-Druker, J., and Quintana, E.T. (2008). Actinobacterial diversity from marine sediments collected in Mexico. Antonie Van Leeuwenhoek *95*, 111–120.

Manivasagan, P., Venkatesan, J., Sivakumar, K., and Kim, S.-K. (2013). Marine actinobacterial metabolites: Current status and future perspectives. Microbiol. Res.

Maplestone, R.A., Stone, M.J., and Williams, D.H. (1992). The evolutionary role of secondary metabolites — a review. Gene *115*, 151–157.

McCarthy, C. Chromas (Technelysium Pty Ltd).

Merkel, A.Y., Huber, J.A., Chernyh, N.A., Bonch-Osmolovskaya, E.A., and Lebedinsky, A.V. (2012). Detection of Putatively Thermophilic Anaerobic Methanotrophs in Diffuse Hydrothermal Vent Fluids. Appl. Environ. Microbiol. *79*, 915–923.

Mincer, T.J., Jensen, P.R., Kauffman, C.A., and Fenical, W. (2002). Widespread and Persistent Populations of a Major New Marine Actinomycete Taxon in Ocean Sediments. Appl. Environ. Microbiol. *68*, 5005–5011.

Mincer, T.J., Fenical, W., and Jensen, P.R. (2005). Culture-dependent and culture-independent diversity within the obligate marine actinomycete genus Salinispora. Appl. Environ. Microbiol. *71*, 7019–7028.

Moran, M.A., Rutherford, L.T., and Hodson, R.E. (1995). Evidence for indigenous Streptomyces populations in a marine environment determined with a 16S rRNA probe. Appl. Environ. Microbiol. *61*, 3695–3700.

Newman, D.J., Cragg, G.M., and Snader, K.M. (2003). Natural products as sources of new drugs over the period 1981-2002. J. Nat. Prod. *66*, 1022–1037.

Olano, C., Lombó, F., Méndez, C., and Salas, J.A. (2008). Improving production of bioactive secondary metabolites in actinomycetes by metabolic engineering. Metab. Eng. *10*, 281–292.

Olano, C., Mendez, C., and Salas, J.A. (2009). Antitumor compounds from actinomycetes: from gene clusters to new derivatives by combinatorial biosynthesis. Nat. Prod. Rep. *26*, 628–660.

Olano, C., Méndez, C., and Salas, J. (2011). Molecular insights on the biosynthesis of antitumour compounds by actinomycetes. Microb. Biotechnol. *4*, 144–164.

Payne, D.J., Gwynn, M.N., Holmes, D.J., and Pompliano, D.L. (2006). Drugs for bad bugs: confronting the challenges of antibacterial discovery. Nat. Rev. Drug Discov. *6*, 29–40.

Pettit, R.K. (2011). Culturability and secondary metabolite diversity of extreme microbes: expanding contribution of deep sea and deep-sea vent microbes to natural product discovery. Mar. Biotechnol. New York N *13*, 1–11.

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. J. Nat. Prod. *68*, 1380–1385.

Polsinelli, M., and Mazza, P. g. (1984). Use of membrane filters for selective isolation of actinomycetes from soil. Fems Microbiol. Lett. *22*, 79–83.

Price, P.B. (2000). A habitat for psychrophiles in deep Antarctic ice. Proc. Natl. Acad. Sci. *97*, 1247–1251.

Price, P.B., and Sowers, T. (2004). Temperature dependence of metabolic rates for microbial growth, maintenance, and survival. Proc. Natl. Acad. Sci. U. S. A. *101*, 4631–4636.

Schrempf, H., and Dyson, P. (2011). Editorial – preview. Microb. Biotechnol. *4*, 138–140.

Selvameenal, L., Radhakrishnan, M., and Balagurunathan, R. (2009). Antibiotic pigment from desert soil actinomycetes; biological activity, purification and chemical screening. Indian J. Pharm. Sci. *71*, 499–504.

Seyedsayamdost, M.R., Traxler, M.F., Clardy, J., and Kolter, R. (2012). Old meets new: using interspecies interactions to detect secondary metabolite production in actinomycetes. Methods Enzymol. *517*, 89–109.

Staley, J.T., and Konopka, A. (1985). Measurement of in Situ Activities of Nonphotosynthetic Microorganisms in Aquatic and Terrestrial Habitats. Annu. Rev. Microbiol. *39*, 321–346.

Stanier, R.Y., Palleroni, N.J., and Doudoroff, M. (1966). The Aerobic Pseudomonads a Taxonomic Study. J. Gen. Microbiol. *43*, 159–271.

Stone, M.J., and Williams, D.H. (1992). On the evolution of functional secondary metabolites (natural products). Mol. Microbiol. *6*, 29–34.

Sykes, R. (2001). Penicillin: from discovery to product. Bull. World Health Organ. *79*, 778–779.

Takai, K., and Horikoshi, K. (1999). Genetic diversity of archaea in deep-sea hydrothermal vent environments. Genetics *152*, 1285–1297.

Talbot, G.H., Bradley, J., Edwards, J.E., Jr, Gilbert, D., Scheld, M., and Bartlett, J.G. (2006). Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. *42*, 657–668.

Tamura, K., and Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. *10*, 512–526.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. *28*, 2731–2739.

Tarasov, V.G., Gebruk, A.V., Mironov, A.N., and Moskalev, L.I. (2005). Deep-sea and shallow-water hydrothermal vent communities: Two different phenomena? Shallow-Water Hydrothermal Venting *224*, 5–39.

Terahara, T., Kobayashi, T., and Imada, C. (2013). An effective method based on wet-heat treatment for the selective isolation of Micromonospora from estuarine sediments. World J. Microbiol. Biotechnol.

Thornburg, C.C., Zabriskie, T.M., and McPhail, K.L. (2010). Deep-sea hydrothermal vents: potential hot spots for natural products discovery? J. Nat. Prod. *73*, 489–499.

Tiwari, K., and Gupta, R.K. (2011). Rare actinomycetes: a potential storehouse for novel antibiotics. Crit. Rev. Biotechnol. *32*, 108–132.

Tiwari, K., and Gupta, R.K. (2012). Diversity and isolation of rare actinomycetes: an overview. Crit. Rev. Microbiol.

Torsvik, V., Goksoyr, J., and Daae, F.L. (1990). High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. *56*, 782–787.

Turner, S., Pryer, K.M., Miao, V.P.W., and Palmer, J.D. (1999). Investigating Deep Phylogenetic Relationships among Cyanobacteria and Plastids by Small Subunit rRNA Sequence Analysis1. J. Eukaryot. Microbiol. *46*, 327–338.

Wagner, M., Amann, R., Lemmer, H., and Schleifer, K.H. (1993). Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. *59*, 1520–1525.

Wakisaka, Y., Kawamura, Y., Yasuda, Y., Koizumi, K., and Nishimoto, Y. (1982). A selective isolation procedure for Micromonospora. J. Antibiot. (Tokyo) *35*, 822–836.

Valverde, A., Tuffin, M., and Cowan, D.A. (2012). Biogeography of bacterial communities in hot springs: a focus on the actinobacteria. Extrem. Life Extreme Cond. *16*, 669–679.

Vartoukian, S.R., Palmer, R.M., and Wade, W.G. (2010). Strategies for culture of "unculturable" bacteria. Fems Microbiol. Lett. *309*, 1–7.

Watve, M., Shejval, V., Sonawane, C., Rahalkar, M., Matapurkar, A., Shouche, Y., Patole, M., Phadnis, N., Champhenkar, A., and Damle, K. (2000). The'K'selected oligophilic bacteria: a key to uncultured diversity? Curr. Sci. *78*, 1535–1542.

Watve, M.G., Tickoo, R., Jog, M.M., and Bhole, B.D. (2001). How many antibiotics are produced by the genus Streptomyces? Arch. Microbiol. *176*, 386–390.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. *173*, 697–703.

Vetriani, C., Speck, M.D., Ellor, S.V., Lutz, R.A., and Starovoytov, V. (2004). Thermovibrio ammonificans sp. nov., a thermophilic, chemolithotrophic, nitrate-ammonifying bacterium from deep-sea hydrothermal vents. Int. J. Syst. Evol. Microbiol. *54*, 175–181.

Větrovský, T., and Baldrian, P. (2013). The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. Plos One *8*, e57923.

Weyland, H. (1969). Actinomycetes in North Sea and Atlantic Ocean sediments. Nature *223*, 858.

Wilson, Z.E., and Brimble, M.A. (2009). Molecules derived from the extremes of life. Nat. Prod. Rep. *26*, 44–71.

Yap, W.H., Zhang, Z., and Wang, Y. (1999). Distinct types of rRNA operons exist in the genome of the actinomycete Thermomonospora chromogena and evidence for horizontal transfer of an entire rRNA operon. J. Bacteriol. *181*, 5201–5209.

Ye, J., McGinnis, S., and Madden, T.L. (2006). BLAST: improvements for better sequence analysis. Nucleic Acids Res. *34*, W6–W9.

Zimmerman, S.B., Schwartz, C.D., Monaghan, R.L., Pelak, B.A., Weissberger, B., Gilfillan, E.C., Mochales, S., Hernandez, S., Currie, S.A., and Tejera, E. (1987). Difficidin and oxydifficidin: novel broad spectrum antibacterial antibiotics produced by Bacillus subtilis. I. Production, taxonomy and antibacterial activity. J. Antibiot. (Tokyo) *40*, 1677–1681.

APPENDIX

APPENDIX 1

The table lists all isolates taken to date with their respective descriptions of their morphology. Table includes what sample, treatment, and medium the isolate was taken from, as well as the approximate colony age in days. Isolates were transferred to fresh plates of either VPB or NBC (with and without sea salt). All incubations were at 20°C.

From plate:	Isolate nr.	Medium 1	Temp	Time	Medium 2	Temp	Notes:
Y1B1	#1	VPB/VPA	20°C	4 d	VPB/VPA	20°C	Yellow/red, buttery, small, round
	#9	VPB/VPA	20°C	4 d	VPB/VPA	20°C	Pinkish, medium size, colony is radiating from sample fragment
	#10	VPB/VPA	20°C	4 d	VPB/VPA	20°C	Orange, small, colony is radiating from sample fragment
	#11	VPB/VPA	20°C	4 d	VPB/VPA	20°C	Bright pink, tiny colony
	#15	VPB/VPA	20°C	4 d	VPB/VPA	20°C	Fungal colony, white in color, large
Y1K1	#1	VPB/VPA	20°C	5d	VPB/VPA	20°C	Displays some inhibition behavior towards another, larger, colony. Colony is small and whiteish.
	#2	VPB/VPA	20°C	5d	VPB/VPA	20°C	Displays inhibition zone, small and yeasty in color
	#3	VPB/VPA	20°C	5d	VPB/VPA	20°C	Displays some inhibition, lemon yellow colony
Y1K2	#4	VPB/VPA	20°C	5d	VPB/VPA	20°C	Displays some inhibition, shiny small colony with white/yellow color
	#5	VPB/VPA	20°C	5d	VPB/VPA	20°C	Shiny, white, inhibits other colony. Yeasty in colour.
Y1K3	#6	VPB/VPA	20°C	5d	VPB/VPA	20°C	Displays inhibition, yeasty in color
	#7	VPB/VPA	20°C	5d	VPB/VPA	20°C	Displays inhibition, yeasty in colour, slightly yellow
	#8	VPB/VPA	20°C	5d	VPB/VPA	20°C	Displays inhibition, yeasty in colour, whitish
	#12	VPB/VPA	20°C	5d	VPB/VPA	20°C	Bright pink, tiny colony
	#13	VPB/VPA	20°C	5d	VPB/VPA	20°C	White/pink, medium sized colony, round, opaque
	#14	VPB/VPA	20°C	5d	VPB/VPA	20°C	Bright orange, round colony
From plate:	Isolate nr.	Medium 1	Temp	Time	Medium 2	Temp	Notes:
E1Skrap	#17	VPB	20°C	2d	VPB	20°C	Fungi? Weird mycelia/roots. White, matte, large (MYXOBACTERIA)
E1Skrap	#18	VPB	20°C	2d	VPB	20°C	Bacterial colony, white, shiny
Y1B1 1	#19	VPB	20°C	5d	VPB	20°C	Red/pink colony. Might have mycelial growth. Small, shiny.
Y1B1 2	#20	VPB	20°C	5d	VPB	20°C	Orange/yellow. Might be inhibiting another colony, streak is probably of both. Small.
Y1B1 2	#21	VPB	20°C	5d	VPB	20°C	Pale pink, large. Shiny. Somewhat see-thru.
From plate:	Isolate nr.	Medium 1	Temp	Time	Medium 2	Temp	Notes:
Y1B1	30	SCN	20°C	11 d	VPB	20°C	Clearing zone area around colony. Colony is large, watery and yellow.
Y1B1	31	SCN	20°C	11 d	VPB	20°C	Small yellow colony
Y1B1	32	SCN	20°C	11 d	VPB	20°C	Large yellow colony, very watery, and flat.
Y1B1	33	SCN	20°C	11 d	VPB	20°C	Isolate #31 uppslammat with small surrounding colony. Probably failed, is an experimental technique.
Y1B5	34	VPB	20°C	5 d	VPB	20°C	Filamentous growth pattern, white, large.
Y1B5	35	VPB	20°C	5d	VPB	20°C	Filamentous growth pattern, white, small
Y1B5	36	VPB	20°C	5d	VPB	20°C	Filamentous growth pattern, white, small
Y1B2	37	VPB	20°C	11d	VPB	20°C	Lemon yellow, might be inhibiting other colony.
Y1B1	38	VPB	20°C	10 d	VPB	20°C	Orange, might be growing filamentously.
Y1B1	39	VPB	20°C	10 d	VPB	20°C	Pale pink, waxy. Colony grows unevely, might be filamentously growing.
Y1B1	40	VPB	20°C	10 d	VPB	20°C	Orange, uneven colony. Might be growing filamentously.
Y1B1	41	VPB	20°C	10 d	VPB	20°C	Orange, waxy, small colony. Is inhibiting other colony.
Y1B1 #2	42	VPB	20°C	10 d	VPB	20°C	Large, filamentous.
Y1B1 #2	43	VPB	20°C	10 d	VPB	20°C	Large, filamentous.
Y1B1 #2	44	VPB	20°C	10 d	VPB	20°C	Yellow, uneven colony. Might be inhibiting.
Y1B1 #2	45	VPB	20°C	10 d	VPB	20°C	Lemon yellow, might be inhibiting.
Y1B1 #2	46	VPB	20°C	10 d	VPB	20°C	Tiny, orange colony. Inhibiting other colony.
Y1B1 #2	47	VPB	20°C	10 d	VPB	20°C	Yellow/orange colony. Might be growing filamentously. Waxy. Uneven growth.
Y1B7	48	VPB	20°C	4 d	VPB	20°C	Large, white. Filamentous.
Y1B7	49	VPB	20°C	4d	VPB	20°C	Pink, uneven colony. "Strange" look.
Y1B7	50	VPB	20°C	4d	VPB	20°C	Pale, pink, see-through, uneven colony.
E1B1	52	VPB	20°C	6d	VPB	20°C	Waxy
E1B1	53	VPB	20°C	6d	VPB	20°C	Orange
E1B1	54	VPB	20°C	6d	VPB	20°C	Yellow
Y1B7	55	VPB	20°C	4 d	VPB	20°C	Pale pink, opaque, waxy, uneven
Y1B7	56.1	VPB	20°C	4 d	VPB	20°C	White, filamentous
Y1B7	56.2	VPB	20°C	4 d	VPB	20°C	White, filamentous
Y1B7	57	VPB	20°C	4 d	VPB	20°C	Pink, filamentous.
Y1K #3	58	VPB	20°C	12 d	VPB	20°C	Pink, uneven, filamentous?
Y1K #3	59	VPB	20°C	12 d	VPB	20°C	Orange, might be inhibiting
Y1K #3	60	VPB	20°C	12 d	VPB	20°C	Orange/yellow
Y1K #3	61	VPB	20°C	12 d	VPB	20°C	Yellow, might be inhibiting
Y1K	62	IM8	20°C	12 d	VPB	20°C	Pale yellow, migh be inhibiting
Y1K	63	IM8	20°C	12 d	VPB	20°C	Yellow, inhibiting
Y1B4	64	VPB	20°C	4 d	VPB	20°C	Pale pink, uneven growth, might be filamentous.
Y1B4	65	VPB	20°C	4 d	VPB	20°C	Paler pink, uneven growth, might be filamentous.
Y1K	66	VPB	20°C	12 d	VPB	20°C	Dark purple, tiny colony.
Y1K	67	VPB	20°C	12 d	VPB	20°C	Pale pink/white. Opaque.
Y1K	68	VPB	20°C	12 d	VPB	20°C	Pale orange, waxy.
Y1K	69	VPB	20°C	12 d	VPB	20°C	Pale orange/red, inhibiting other colony.
E1 skal	70	VPB	20°C	5d	VPB	20°C	#17.2 Myxobacter

E1 skal	71	VPB		20°C	5d	VPB	20°C	#18.2 white
E1K	72	VPB		20°C	7 d	VPB	20°C	Pink, see-through. Inhibiting.
E1K	73	VPB		20°C	7d	VPB	20°C	White, filamentous.
E1K	74	VPB		20°C	7d	VPB	20°C	Pale pink, opaque, inhibiting.
Y1K	75	SCN	4	20°C	12 d	VPB	20°C	Yellow, watery, large.
T1B1	76	VPB		20°C	4 d	VPB	20°C	Pale pink, opaque, uneven colony. Colony radiating from fragment.
T1B1	77	VPB		20°C	4 d	VPB	20°C	Myxobacteria? Looks like #70, #17.2
From plate:	Isolate nr.	Medium 1	Conc.	Temp	Time	Medium 2	Temp	Notes:
E1B1 1	78	VPB		20°C	7 d	MEA	20°C	Fungi w/ pink center, green ring around center, white radiating mycelia
E1B1 1	79	VPB		20°C	7d	VPB	20°C	Uneven growth, white/pink, starshape in middle of colony
E1B1 1	80	VPB		20°C	7d	VPB	20°C	Flat, beige colony, w/ dark spot in middle, uneven growth
E1B2	81	VPB		20°C	7d	VPB	20°C	Uneven, pink center (raised), waxy
E1B2	82	VPB		20°C	7d	VPB	20°C	Dense raised center, circular surrounding center, radiating uneven hyphae
E1B2	83	VPB		20°C	7d	VPB	20°C	Look alike to #82, but more even growth
E1B2	84	VPB		20°C	7d	VPB	20°C	Pale orange raised center, see-through radiating even colony
E1B2	85	VPB		20°C	7d	VPB	20°C	Pink center (raised), radiating hyphae-looking colony, uneven
E1B2	86	VPB		20°C	7d	VPB	20°C	Pink dense colony, uneven edges (might be filamentous)
E1B5	87	VPB	4		14	NBC +- SW	20°C	Small, uneven, opalescent, flat
T1B3	88	VPB	4		14	NBC +- SW	20°C	Small, uneven, see-through
Y1B1	89	VPB	6		19	NBC +- SW	20°C	Bright orange, waxy, slightly uneven
Y1B1	90	VPB	6		19	NBC +- SW	20°C	Light orange, waxy, even
E1B4	91	VPB	4		9	NBC +- SW	20°C	Papery, folded, dry, white
Y1B2	92	VPB	6		19	NBC +- SW	20°C	White/yellow, very small, has friend colony
T1B1	93	VPB	4		12	NBC +- SW	20°C	Pink, uneven colony with raised darker center
Y1B3	94	VPB	5		12	NBC +- SW	20°C	Bubbly bacteria, white stiff (like #35)
T1B1	95	VPB	6		12	NBC +- SW	20°C	Actinomycete-look, murkla, white, soft, raised
T1B1	96	VPB	6		12	NBC +- SW	20°C	Actinomycete-look, murkla, white, soft, raised
T1B1	97	VPB	4		12	NBC +- SW	20°C	Large, beige, excretes liquid
Y1B3	98	VPB	5		12	NBC +- SW	20°C	Light yellow, butter
Y1K	99	SCN	5		21	NBC +- SW	20°C	Yellow, very watery, has clearing zone (metabolism), helps other small colonies grow
Y1B4	100	VPB	5		13	NBC +- SW	20°C	Brown (probably dirty streak)
Y1B4	101	VPB	6		13	NBC +- SW	20°C	Small, pink, uneven
Y1B4	102	VPB	6		13	NBC +- SW	20°C	Orange, medium size, round, with darker orange center
Y1B6	103	VPB	4		13	NBC +- SW	20°C	Bubbly, looks like a flower
From plate:	Isolate nr.	Medium 1	Conc.	Temp	Time	Medium 2	Temp	Notes:
T1B2	103	IM6		20°C	14	NBC +- SW	20°C	Pastel pink/salmon, radiating from fragment
T1B2	104	SCN		20°C	14	NBC +- SW	20°C	White/yellow, radiating from fragment
T1B2	105	SCN		20°C	14	NBC +- SW	20°C	Salmon pink, radiating from sample
T1B2	106	SCN		20°C	14	NBC +- SW	20°C	Mold?
T1B2	107	IM8		20°C	14	NBC +- SW	20°C	Yellow, uneven, radiating from fragment
T1B2	108	IM8		20°C	14	NBC +- SW	20°C	Salmon pink
T1B2	109	VPB		20°C	14	NBC +- SW	20°C	White colony, circle of exudate
T1B1	110	IM7		20°C	14	NBC +- SW	20°C	Mold, small, radiating from fragment (transfer may be unsuccessful)
T1B1	111	SCN		20°C	14	NBC +- SW	20°C	tiny colony, pale, (transfer may be unsuccessful)
T1B1	112	SCN		20°C	14	NBC +- SW	20°C	tiny colony, pale (transfer may be unsuccessful)
Y1B5	113	VPB	5	20°C	15	NBC +- SW	20°C	pink, uneven, opaque, smooth
Y1B5	114	VPB	4	20°C	15	NBC +- SW	20°C	small, beige, inhibiting
Y1B5	115	VPB	4	20°C	15	NBC +- SW	20°C	flat, see-through, even
Y1B1	116	IM6	5	20°C	20	NBC +- SW	20°C	Bright yellow, shiny (major colonies represented on plate)
Y1B1	117	IM6	5	20°C	20	NBC +- SW	20°C	Orange, shiny (major colonies represented on plate)
Y1B1	118	IM6	5	20°C	20	NBC +- SW	20°C	pale yellow (major colonies represented on plate)
Y1B4	119	IM8	4	20°C	14	NBC +- SW	20°C	White/beige (the only white on a yellow-colony plate)
Y1B6	120	VPB	4	20°C	14	NBC +- SW	20°C	bubbly white, single colony, clean
Y1B6	121	VPB	4	20°C	14	NBC +- SW	20°C	dark beige
Y1B6	122	VPB	4	20°C	14	NBC +- SW	20°C	pale, waxy, flat, beige
T1B2	123	VPB	4	20°C	13	NBC +- SW	20°C	Orange, slightly uneven
Y1B2	124	IM6		20°C	11	NBC +- SW	20°C	Orange, smooth, shiny
Y1B2	125	IM6		20°C	11	NBC +- SW	20°C	Yellow, smooth shiny
Y1B7	126	VPB	6	20°C	14	NBC +- SW	20°C	Yellow, uneven, waxy
Y1B7	127	VPB	5	20°C	14	NBC +- SW	20°C	Waxy, orange
Y1B7	128	VPB	5	20°C	14	NBC +- SW	20°C	Pale pink/red, flat, has exudate ring
Y1B7	129	VPB	6	20°C	14	NBC +- SW	20°C	Tiny, pink, uneven
Y1B7	130	VPB	6	20°C	14	NBC +- SW	20°C	Pale orange, waxy, with raised center
Y1B7	131	VPB	6	20°C	14	NBC +- SW	20°C	Tiny orange, matte, uneven
Y1B7	132	VPB	6	20°C	14	NBC +- SW	20°C	Ruby red, domed, shiny
Y1B7	133	VPB	5	20°C	14	NBC +- SW	20°C	Red, flat, semi-shiny
Y1B7	134	VPB	5	20°C	14	NBC +- SW	20°C	White, mold-like, uneven
Y1B1	135	VPB	6	20°C	20	NBC +- SW	20°C	Orange, might be #89
From plate:	Isolate nr.	Medium 1	Conc.	Temp	Time	Medium 2	Temp	Notes:
W1B4	136	VPB	6	20°C	5	NBC +- SW	20°C	Yellow colony, smooth, shiny, white center
W1B4	137	VPB	5	20°C	5	NBC +- SW	20°C	Partially yellow, part white. Uneven. Dirty streak?
W1B4	138	VPB	4	20°C	5	NBC +- SW	20°C	Pale, see-through, round, shiny, flat
E1B6	139	VPB	6	20°C	17	NBC +- SW	20°C	Waxy, sharp middle peak, white, large
E1B6	140	VPB	4	20°C	17	NBC +- SW	20°C	large uneven colony -> yellow part
E1B6	141	VPB	4	20°C	17	NBC +- SW	20°C	large uneven colony -> white part
T1B4	142	VPB	5	20°C	13	NBC +- SW	20°C	Tiny, white, uneven (looks actinomycete-like)
T1B4	143	VPB	5	20°C	13	NBC +- SW	20°C	Tiny, white, uneven (looks actinomycete-like)
T1B4	144	VPB	5	20°C	13	NBC +- SW	20°C	Tiny, white, uneven (looks actinomycete-like) + creamy

T1B4	145	VPB	4	20°C	13	NBC +- SW	20°C	Salmon/orange/pink, shiny, even
T1B4	146	VPB	4	20°C	13	NBC +- SW	20°C	Yellow, uneven
T1B4	147	VPB	4	20°C	13	NBC +- SW	20°C	Tiny white, uneven
T1B4	148	VPB	4	20°C	13	NBC +- SW	20°C	Light yellow, opaque
T1B4	149	VPB	4	20°C	13	NBC +- SW	20°C	Tiny white, grows like a pillar, uneven
T1B4	150	VPB	4	20°C	13	NBC +- SW	20°C	Tiny white, grows like a pillar, uneven
T1B4	151	VPB	4	20°C	13	NBC +- SW	20°C	Tiny white, grows like a pillar, uneven
T1B4	152	VPB	4	20°C	13	NBC +- SW	20°C	Tiny white, grows like a pillar, uneven
T1B4	153	VPB	4	20°C	13	NBC +- SW	20°C	Tiny white, grows like a pillar, uneven
T1B4	154	VPB	5	20°C	13	NBC +- SW	20°C	Yellow, slightly uneven, slimy
T1B4	155	VPB	4	20°C	13	NBC +- SW	20°C	Mold, white aerial hyphae
Y1B1	156	IM8	5	20°C	24	NBC +- SW	20°C	Yellow, tiny, uneven
Y1B1	157	IM8	4	20°C	24	NBC +- SW	20°C	Yellow, tiny, uneven
Y1B1	158	IM8	4	20°C	24	NBC +- SW	20°C	Very tiny, yellow
Y1B1	159	IM8	4	20°C	24	NBC +- SW	20°C	Tiny, sandwiched between colonies, might be inhibitory
Y1B1	160	IM8	4	20°C	24	NBC +- SW	20°C	Light yellow
Y1B1	161	IM8	4	20°C	24	NBC +- SW	20°C	Large yellow, a slice of which is darker yellow
Y1B1	162	IM8	4	20°C	24	NBC +- SW	20°C	Pale yellow
Y1B1	163	IM8	4	20°C	24	NBC +- SW	20°C	Small, yellow, might be inhibitory
E1B4	164	VPB	5	20°C	14	NBC +- SW	20°C	Pink, shiny, dome, slightly uneven, slimy
E1B4	165	VPB	4	20°C	14	NBC +- SW	20°C	White with alternating dark rings within, looks inhibiting
T1K	166	VPB	4	20°C	14	NBC +- SW	20°C	Pink, pillar, uneven (mixed colony)
T1K	167	VPB	4	20°C	14	NBC +- SW	20°C	Pink, uneven
T1K	168	VPB	4	20°C	14	NBC +- SW	20°C	Pink, uneven
Y1B2	169	VPB	4	20°C	24	NBC +- SW	20°C	Pink, pillar, uneven, tiny
Y1B2	170	VPB	4	20°C	24	NBC +- SW	20°C	Pink, pillar, uneven, tiny
W1B6	171	VPB	4	20°C	5	NBC +- SW	20°C	Pale, flat, beige
W1B6	172	VPB	4	20°C	5	NBC +- SW	20°C	Pale, flat, beige, waxy
W1B7	173	VPB	4	20°C	5	NBC +- SW	20°C	Orange, tiny
W1B7	174	VPB	4	20°C	5	NBC +- SW	20°C	Pale yellow
Y1B7	175	IM6	4	20°C	18	NBC +- SW	20°C	Yellow, shiny
Y1B7	176	IM6	5	20°C	18	NBC +- SW	20°C	Pale pink, waxy
E1B3	177	VPB	4	20°C	20	NBC +- SW	20°C	Orange, waxy
E1B7	178	VPB	4	20°C	14	NBC +- SW	20°C	Yellow, shiny, uneven
Y1B7	179	SCN	5	20°C	22	NBC +- SW	20°C	Yellow, shiny, round
Y1B4	180	IM8	5	20°C	22	NBC +- SW	20°C	Pale yellow, semi-shiny
Y1B4	181	SCN	5	20°C	21	NBC +- SW	20°C	Yellow, see-through center, seems to digest substrate (has clearing zone)
T1B1	182	IM8	4	20°C	21	NBC +- SW	20°C	Tiny, yellow
Y1B5	183	IM8	5	20°C	22	NBC +- SW	20°C	Yellow, butter-y, murkla, actinomycete-look
E1K	184	IM8	4	20°C	24	NBC +- SW	20°C	Beige, uneven, might be a mixed colony
T1B1	185	SCN	4	20°C	21	NBC +- SW	20°C	Tiny, white/yellow
E1B1	186	SCN	5	20°C	23	NBC +- SW	20°C	Tiny, yellow
T1B1	187	SCN-SW	4	20°C	21	NBC +- SW	20°C	Tiny, bright yellow
Y1B5	188	VPB	4	20°C	22	NBC +- SW	20°C	Beige, tiny, might be inhibitory

From plate:	Isolate nr.	Medium 1	Conc.	20°C	Time	Medium 2	Temp	Notes:
T1B6	189	VPB	5	20°C	17	NBC +- SW	20°C	Yellow, large, circular but uneven, ribbed, topped
T1B6	190	VPB	5	20°C	17	NBC +- SW	20°C	Yellow, large, circular but uneven, ribbed, topped
T1B6	191	VPB	5	20°C	17	NBC +- SW	20°C	Yellow, large, circular but uneven, ribbed, topped
E1K	192	IM6	4	20°C	25	NBC +- SW	20°C	Yellow, looks filamentous, fades from center
E1K	193	IM6	4	20°C	25	NBC +- SW	20°C	Pale, uneven
E1K	194	SCN	4	20°C	25	NBC +- SW	20°C	Pale, whitish, uneven
E1K	195	SCN	4	20°C	25	NBC +- SW	20°C	Pale, whitish, uneven
E1K	196	SCN	4	20°C	25	NBC +- SW	20°C	Pale, whitish, uneven
E1K	197	SCN	4	20°C	25	NBC +- SW	20°C	Pale, whitish, uneven
T1B7	198	SCN	4	20°C	17	NBC +- SW	20°C	Yellow, uneven in colour, digests substrate
T1B7	199	SCN	4	20°C	17	NBC +- SW	20°C	White/yellow, round, digests substrate
T1B7	200	SCN	4	20°C	17	NBC +- SW	20°C	Pale orange, waxy, round
T1B3	201	IM6	4	20°C	21	NBC +- SW	20°C	Yellow, shiny
W1B2	202	VPB	4	20°C	14	NBC +- SW	20°C	Waxy, white, smooth
W1B2	203	VPB	5	20°C	14	NBC +- SW	20°C	Yellow, semi-waxy, round
T1B1	204	SCN		20°C	22	NBC +- SW	20°C	Yellow, uneven, grows into plate
Y1B1	205	VPB	6	20°C	28	NBC +- SW	20°C	Large, yellow, uneven, waxy
Y1B1	206	VPB	6	20°C	28	NBC +- SW	20°C	Orange, with darker raised center
Y1B1	207	VPB	6	20°C	28	NBC +- SW	20°C	Orange, with spots, raised center
Y1B4	208	VPB	6	20°C	22	NBC +- SW	20°C	Orange, murkla, uneven, raised
Y1B4	209	VPB	6	20°C	22	NBC +- SW	20°C	Orange, murkla, uneven, raised
T1B7	210	VPB	4	20°C	23	NBC +- SW	20°C	Tiny white murkla, raised
T1B7	211	VPB	4	20°C	23	NBC +- SW	20°C	Tiny white murkla, raised
T1B7	212	VPB	4	20°C	23	NBC +- SW	20°C	Tiny white murkla, raised
T1B7	213	VPB	4	20°C	23	NBC +- SW	20°C	Tiny white murkla, raised
T1B7	214	VPB	4	20°C	23	NBC +- SW	20°C	Tiny white murkla, raised
T1B7	215	VPB	4	20°C	23	NBC +- SW	20°C	Tiny white murkla, raised
T1B7	216	VPB	4	20°C	23	NBC +- SW	20°C	Tiny white murkla, raised
T1B7	217	VPB	4	20°C	23	NBC +- SW	20°C	Tiny white murkla, raised
T1B7	218	VPB	4	20°C	23	NBC +- SW	20°C	Tiny white murkla, raised
T1B7	219	VPB	4	20°C	23	NBC +- SW	20°C	Tiny white murkla, raised
T1B7	220	VPB	4	20°C	23	NBC +- SW	20°C	Tiny white murkla, raised
T1B7	221	VPB	4	20°C	23	NBC +- SW	20°C	Tiny white murkla, raised

T1B7	222	VPB	5	20°C	23	NBC +- SW	20°C	White, small, uneven
T1B7	223	VPB	5	20°C	23	NBC +- SW	20°C	White, small, uneven
T1B7	224	VPB	5	20°C	23	NBC +- SW	20°C	White, small, uneven
T1B7	225	VPB	5	20°C	23	NBC +- SW	20°C	White, small, uneven
T1B7	226	VPB	5	20°C	23	NBC +- SW	20°C	White, small, uneven
T1B7	227	VPB	5	20°C	23	NBC +- SW	20°C	White/yellow, uneven
TIB5	228	VPB	5	20°C	23	NBC +- SW	20°C	Weird, two-plane colony, previous contamination?
TIB5	229	VPB	4	20°C	23	NBC +- SW	20°C	Tydlig vit/beige murkla
TIB5	230	VPB	4	20°C	23	NBC +- SW	20°C	Tydlig vit/beige murkla
TIB5	231	VPB	4	20°C	23	NBC +- SW	20°C	Tydlig vit/beige murkla
TIB5	232	VPB	4	20°C	23	NBC +- SW	20°C	Tydlig vit/beige murkla
From plate:	Isolate nr.	Medium 1	Conc.	Temp	Time	Medium 2	Temp	Notes:
W2B3	233	VPB	5	20°C	14	NBC +- SW	20°C	Yellow, waxy, topped, round
W2B4	234	VPB	4	20°C	14	NBC +- SW	20°C	Beige, very uneven, excretes (mixed streak)
W2B4	234.1			20°C	14	NBC +- SW	20°C	Beige/white
W2B4	234.2			20°C	14	NBC +- SW	20°C	Beige/white
W2B4	235	VPB	4	20°C	14	NBC +- SW	20°C	Contamination? Colony grows under agar.
T1B3	236	VPB	4	20°C	25	NBC +- SW	20°C	Pale, uneven, "bubbly"
T1B3	237	VPB	4	20°C	25	NBC +- SW	20°C	Pale, uneven, "bubbly"
T1B3	238	VPB	4	20°C	25	NBC +- SW	20°C	Pale, uneven, "bubbly"
T1B3	239	VPB	4	20°C	25	NBC +- SW	20°C	Pale, uneven, "bubbly"
T1B3	240	VPB	4	20°C	25	NBC +- SW	20°C	Large, yellow, slightly raised, with raised spots
T1B3	241	VPB	4	20°C	25	NBC +- SW	20°C	Large, uneven, flat, beige, exudate (digests substrate)
T1B3	242	VPB	4	20°C	25	NBC +- SW	20°C	Like #241, but small and less uneven
T1B3	243	VPB	4	20°C	25	NBC +- SW	20°C	Like #236
T1B3	244	VPB	4	20°C	25	NBC +- SW	20°C	Like #236
W1B3	245	VPB	4	20°C	12	NBC +- SW	20°C	Pale, uneven, grows inside other colony
W1B3	246	VPB	4	20°C	12	NBC +- SW	20°C	Pale, beige, might be inhibitory, round
W1B5	247	VPB	4	20°C	13	NBC +- SW	20°C	Beige, see-through edges, rough center
W1B5	248	VPB	4	20°C	13	NBC +- SW	20°C	Beige, opalescent in circles
E1B4	249	VPB	4	20°C	22	NBC +- SW	20°C	Small, uneven, pale/whitish
T1K	250	VPB	4	20°C	22	NBC +- SW	20°C	Small, uneven, yellow
T1K	251	VPB	4	20°C	22	NBC +- SW	20°C	Small, uneven, white
Y1B3	252	VPB	4	20°C	26	NBC +- SW	20°C	Beige, small, might be inhibitory, darker center
Y1B3	253	VPB	4	20°C	26	NBC +- SW	20°C	White, uneven, leathery
Y1B3	254	VPB	4	20°C	26	NBC +- SW	20°C	Ruby red, semi-shiny, domed
Y1B3	255	VPB	5	20°C	26	NBC +- SW	20°C	Orange, round, shiny
E1B5	256	IM6	4	20°C	27	NBC +- SW	20°C	Tiny, yellow
Y1B6	257	VPB	4	20°C	27	NBC +- SW	20°C	Pale, leathery, uneven
E1B7	258	VPB	4	20°C	24	NBC +- SW	20°C	Pale, uneven, rough center
E1B7	259	VPB	4	20°C	24	NBC +- SW	20°C	Pale, beige, shiny, domed
E1B7	260	VPB	5	20°C	24	NBC +- SW	20°C	Beige, waxy, large, topped
E1B7	261	VPB	5	20°C	24	NBC +- SW	20°C	-||- beige part
E1B7	262	VPB	5	20°C	24	NBC +- SW	20°C	-||- white part
Y1B2	263	VPB	4	20°C	33	NBC +- SW	20°C	Orange, rough, uneven
Y1B2	264	VPB	4	20°C	33	NBC +- SW	20°C	Orange, watery, grainy
W2B6	265	VPB	4	20°C	15	NBC +- SW	20°C	Sunflower! Light yellow, round, ribbed
W2B6	266	VPB	4	20°C	15	NBC +- SW	20°C	Pale yellow/yellow, smooth, round, topped
W2B6	267	VPB	4	20°C	15	NBC +- SW	20°C	Pale yellow/yellow, shiny, round, domed
W2B6	268	VPB	4	20°C	15	NBC +- SW	20°C	White, rough surface, round, creamy
Y1B7	269	IM7	4	20°C	27	NBC +- SW	20°C	Tiny, yellow
Y1B7	270	IM7	4	20°C	27	NBC +- SW	20°C	Tiny, yellow
Y1B7	271	IM8	4	20°C	27	NBC +- SW	20°C	Tiny, yellow
T1B1	272	VPB	4	20°C	22	NBC +- SW	20°C	Small, white, uneven
T1B1	273	VPB	4	20°C	22	NBC +- SW	20°C	Small, white, uneven
T1B1	274	VPB	4	20°C	22	NBC +- SW	20°C	Small, white, uneven
T1B1	275	VPB	4	20°C	22	NBC +- SW	20°C	Small, white, uneven, looks inhibiting
From plate:	Isolate nr.	Medium 1	Conc.	Temp	Time	Medium 2	Temp	Notes:
T1B7	276	IM6	4	20°C	24	NBC +- SW	20°C	Light yellow, round, waxy
T1B7	277	IM6	5	20°C	24	NBC +- SW	20°C	Bright yellow, shiny
T1B7	278	IM8	4	20°C	24	NBC +- SW	20°C	yellow, small, uneven
T1B7	279	SCN	5	20°C	24	NBC +- SW	20°C	Tiny, orange/red, shiny
T1B7	280	IM7	4	20°C	24	NBC +- SW	20°C	Tiny, red, shiny
T1B7	281	IM7	4	20°C	24	NBC +- SW	20°C	Pale yellow, watery
Y1B7	282	IM8	5	20°C	29	NBC +- SW	20°C	Yellow, waxy, uneven surface, round
Y1B7	283	IM8	5	20°C	29	NBC +- SW	20°C	White, tiny, uneven
T1B7	284	IM8	5	20°C	24	NBC +- SW	20°C	Tiny, yellow
Y1B1	285	IM6	6	20°C	35	NBC +- SW	20°C	Tiny, red, matte
Y1B1	286	WA	4	20°C	35	NBC +- SW	20°C	Tiny, red, shiny
Y1B1	287	WA	4	20°C	35	NBC +- SW	20°C	Pale
Y1B1	288	WA	4	20°C	35	NBC +- SW	20°C	-
Y1B1	290	IM8	4	20°C	35	NBC +- SW	20°C	Yellow, uneven
Y1B1	291	IM8	4	20°C	35	NBC +- SW	20°C	Yellow, rough surface, uneven, shiny
Y1B1	292	IM8	4	20°C	35	NBC +- SW	20°C	Yellow/orange, uneven
W1B1	293	VPB	4	20°C	21	NBC +- SW	20°C	Orange, waxy, topped
W2B3	294	VPB	4	20°C	17	NBC +- SW	20°C	Microcolony, pale
T1B1	295	VPB	4	20°C	28	NBC +- SW	20°C	Pale yellow, round, waxy
T1B1	296	VPB	4	20°C	28	NBC +- SW	20°C	White, small (beige/pale), with raised white "hat"

T1B1	297	VPB	5	20°C	28	NBC +- SW	20°C	Yellow, smooth, waxy, round, topped
T1B1	298	VPB	5	20°C	28	NBC +- SW	20°C	White, smooth, waxy, round, topped
T1B4	299	SCN	4	20°C	24	NBC +- SW	20°C	Yellow, see-through, grows into plate like flower petals
T1B4	300	SCN	4	20°C	24	NBC +- SW	20°C	Yellow, see-through, grows into plate like flower petals
T1B4	301	SCN	4	20°C	24	NBC +- SW	20°C	tiny orange/salmon
T1B4	302	SCN	4	20°C	24	NBC +- SW	20°C	Pale, flat
T1B4	303	SCN	4	20°C	24	NBC +- SW	20°C	Pale, flat
T1B4	304	SCN	4	20°C	24	NBC +- SW	20°C	Bright yellow
T1B4	305	IM8	4	20°C	24	NBC +- SW	20°C	Salmon, watery
T1B4	306	IM8	4	20°C	24	NBC +- SW	20°C	Bright yellow, small
T1B4	307	IM8	4	20°C	24	NBC +- SW	20°C	Bright yellow, small
T1B4	308	IM8	6	20°C	24	NBC +- SW	20°C	Bright yellow, small
Y1B7	309	IM6	5	20°C	29	NBC +- SW	20°C	Bright yellow, shiny, round
Y1B7	310	IM6	5	20°C	29	NBC +- SW	20°C	Bright yellow, shiny, round, flat
Y1B7	311	IM6	5	20°C	29	NBC +- SW	20°C	Yellow, shiny, uneven
Y1B7	312	IM6	6	20°C	29	NBC +- SW	20°C	Orange, shiny
Y1B7	313	IM6	6	20°C	29	NBC +- SW	20°C	Yellow, slightly uneven
Y1B7	314	IM6	4	20°C	29	NBC +- SW	20°C	Pale yellow, opaque, shiny, round
Y1B7	315	IM6	4	20°C	29	NBC +- SW	20°C	Salmon, small, shiny, round, opaque
Y1B3	316	VPB	4	20°C	28	NBC +- SW	20°C	Orange, waxy
Y1B3	317	VPB	4	20°C	28	NBC +- SW	20°C	Orange, shiny
Y1B3	318	VPB	4	20°C	28	NBC +- SW	20°C	Orange, waxy
Y1B3	319	VPB	4	20°C	28	NBC +- SW	20°C	Orange, waxy/shiny
Y1B3	320	VPB	4	20°C	28	NBC +- SW	20°C	Red, shiny
Y1B3	321	VPB	4	20°C	28	NBC +- SW	20°C	Red, shiny, see-through
Y1B3	322	VPB	4	20°C	28	NBC +- SW	20°C	Red, shiny, see-through
Y1B3	323	VPB	4	20°C	28	NBC +- SW	20°C	Light yellow, opaque, slightly waxy
Y1B3	324	VPB	4	20°C	28	NBC +- SW	20°C	Waxy, white, round
Y1B3	325	VPB	5	20°C	28	NBC +- SW	20°C	Orange, semi-shiny, opaque
E1	326	SCN		20°C	32	NBC + SW	20°C	8 streaks from stamping plate, various colonies
E1	327	IM8		20°C	32	NBC + SW	20°C	8 streaks from stamping plate, various colonies
E1	328	IM7		20°C	32	NBC + SW	20°C	8 streaks from stamping plate, various colonies
E1	329	IM6		20°C	32	NBC + SW	20°C	8 streaks from stamping plate, various colonies
E1	330	WA		20°C	32	NBC + SW	20°C	8 streaks from stamping plate, various colonies
E1	331	SCN-SW		20°C	32	NBC + SW	20°C	8 streaks from stamping plate, various colonies
T1B1	332	IM6	4	20°C	29	NBC +- SW	20°C	Pale yellow, flat
T1B1	333	IM6	4	20°C	29	NBC +- SW	20°C	Pale, opaque, tiny
T1B4	334	VPB	6	20°C	24	NBC +- SW	20°C	Filaments. Bacillus?
W2B6	335	VPB	6	20°C	17	NBC +- SW	20°C	Pale, tiny, (several streaks)
E1	336	WA/NBC+SW		20°C	3	NBC +- SW	20°C	From 330, salmon, pale, opaque
E1	337	SCN/NBC+SW		20°C	3	NBC +- SW	20°C	From 326, yellow, opaque
E1	338	SCN-SW/NBC+SW		20°C	3	NBC +- SW	20°C	from 331, orange
E1	339	IM7/NBC+SW		20°C	3	NBC +- SW	20°C	from 328, pale salmon, opaque
E1	340	IM6/NBC+SW		20°C	3	NBC +- SW	20°C	from 329, yellow

APPENDIX 2.

The table lists all sequenced isolates, and their closest match in the BLAST database.

Isolate number	From sample	Media	Identity	Max score	Total score	Query cover	E-value	Max identi	Accession
1B1	Y1B1	VPB	Chryseobacterium sp. ARS145-11 16S ribosomal RNA gene, partial sequence	2399	2399	100%	0.0	98%	JX827616.1
6	Y1K	VPB	Pseudomonas sp. B7B 16S ribosomal RNA gene, partial sequence	2579	2579	99%	0.0	99%	KC433652.1
7	Y1K	VPB	Aranicola sp. NP34 16S ribosomal RNA gene, partial sequence	2462	2462	100%	0.0	99%	EU196321.1
12	Y1K	VPB	Hymenobacter sp. R-36616 partial 16S rRNA gene, strain R-36616	2460	2460	99%	0.0	99%	FR682737.1
15	Y1B1	VPB	Rhodococcus sp. ZS351 16S ribosomal RNA gene, partial sequence	2571	2571	100%	0.0	99%	JX428883.1
20	Y1B1	VPB	Sejongia jeonii strain AT1047 16S ribosomal RNA, partial sequence	2374	2374	97%	0.0	99%	NR_025810.1
39	Y1B1	VPB	Bacillus sp. SS14.36 16S ribosomal RNA gene, partial sequence	2632	2632	99%	0.0	99%	KC160805.1
44	Y1B1	VPB	Carnobacterium sp. NJ-46 16S rRNA gene, strain NJ-46	2571	2571	99%	0.0	99%	AM396913.1
45	Y1B1	VPB	Arthrobacter sp. R-36550 partial 16S rRNA gene, strain R-36550	2567	2567	99%	0.0	100%	FR682672.1
46	Y1B1	VPB	Rhodococcus sp. ZS351 16S ribosomal RNA gene, partial sequence	2316	2316	100%	0.0	99%	JX428883.1
48	Y1B7	VPB	Chryseobacterium sp. ARS145-11 16S ribosomal RNA gene, partial sequence	2398	2398	97%	0.0	99%	JX827616.1
50	Y1B7	VPB	Hymenobacter sp. R-36616 partial 16S rRNA gene, strain R-36616	2390	2390	99%	0.0	98%	FR682737.1
51	E1B1	VPB	Psychrobacter sp. enrichment culture clone B1-3 16S ribosomal RNA gene, partial sequence	2564	2564	100%	0.0	99%	GU570642.1
53	E1B1	VPB	Staphylococcus pasteuri strain M-S-MRS_7 16S ribosomal RNA gene, partial sequence	2615	2615	99%	0.0	99%	JQ795861.1
54	E1B1	VPB	Pseudoclavibacter helvolus strain CJ-G-TSA2 16S ribosomal RNA gene, partial sequence	2512	2512	100%	0.0	99%	HM584267.1
56.1	Y1B7	VPB	Kaistia sp. 5YN7-3 16S ribosomal RNA gene, partial sequence	2464	2464	100%	0.0	99%	EU723082.1
57	Y1B7	VPB	Hymenobacter sp. R-36616 partial 16S rRNA gene, strain R-36616	2440	2440	99%	0.0	99%	FR682737.1
65	Y1B4	VPB	Hymenobacter sp. R-36616 partial 16S rRNA gene, strain R-36616	2459	2459	99%	0.0	99%	FR682737.1
66	Y1K	VPB	Deinococcus sp. R-38476 partial 16S rRNA gene, strain R-38476	2525	2525	99%	0.0	99%	FR682757.1
68	Y1K	VPB	Rhodococcus yunnanensis partial 16S rRNA gene, strain R-36475	2486	2486	100%	0.0	99%	FR682691.1
74	E1K	VPB	Psychrobacter sp. KJF9-2 16S ribosomal RNA gene, partial sequence	2597	2790	89%	0.0	100%	JQ800140.1
77	T1B1	VPB	Bacillus sp. SG19 16S ribosomal RNA gene, partial sequence	2614	2846	90%	0.0	100%	JX402434.1
79	E1B1	VPB	Bacillus pumilus strain AUES82 16S ribosomal RNA gene, partial sequence	2599	2842	90%	0.0	100%	HM585067.1
80	E1B1	VPB	Paenibacillus amylolyticus strain MLL-8 16S ribosomal RNA gene, partial sequence	2553	2792	90%	0.0	99%	JQ956529.1
84	E1B2	VPB	Pseudomonas sp. L1-8 partial 16S rRNA gene, isolate L1-8	2532	2727	89%	0.0	99%	HF536514.1
86	E1B2	VPB	Psychrobacter sp. DY9-2 16S ribosomal RNA gene, partial sequence	2564	2751	89%	0.0	99%	AY383045.1
87	E1B5	VPB	Paenibacillus amylolyticus strain NRRL NRS-290 16S ribosomal RNA, partial sequence	2588	2588	99%	0.0	99%	NR_025882.1
88	T1B3	VPB	Paenibacillus sp. MOLA 507 partial 16S rRNA gene, culture collection MOLA:507	2569	2569	100%	0.0	100%	AM990732.1
89	Y1B1	VPB	Mycobacterium neglectum gene for 16S rRNA, partial sequence, strain: GMC129	2484	2484	100%	0.0	99%	AB741461.1
90	Y1B1	VPB	Rhodococcus yunnanensis partial 16S rRNA gene, strain R-36475	2510	2510	100%	0.0	99%	FR682691.1
91	E1B4	VPB	Psychrobacter maritimus strain 3ps 16S ribosomal RNA gene, partial sequence	2562	2562	99%	0.0	99%	FJ039851.1
93	T1B1	VPB	Psychrobacter sp. MV2 16S ribosomal RNA gene, partial sequence	2484	2484	100%	0.0	99%	HQ610925.1
94	Y1B3	VPB	Bacillus licheniformis strain ZML-1 16S ribosomal RNA gene, partial sequence	2590	2590	100%	0.0	100%	KC513425.1
98	Y1B3	VPB	Micrococcus sp. 20.9 KSS partial 16S rRNA gene, strain 20.9 KSS	2532	2532	100%	0.0	100%	HE575933.1
102	Y1B4	VPB	Chryseobacterium sp. ARS145-11 16S ribosomal RNA gene, partial sequence	2375	2375	100%	0.0	98%	JX827616.1
114	Y1B5	VPB	Bacillus sp. SS9.15 16S ribosomal RNA gene, partial sequence	2457	2457	100%	0.0	98%	KC160674.1
115	Y1B5	VPB	Paenibacillus sp. C7 16S ribosomal RNA gene, partial sequence	2455	2455	100%	0.0	98%	AY920751.1
117	Y1B1	IM6	Rhodococcus sp. SS12.38 16S ribosomal RNA gene, partial sequence	1020	1020	100%	0.0	100%	KC160926.1
118	Y1B1	IM6	Arthrobacter psychrochitiniphilus gene for 16S rRNA, partial sequence, strain: JCM 13874	2545	2545	100%	0.0	99%	AB588633.1
136	W1B4	VPB	Micrococcus sp. 20.9 KSS partial 16S rRNA gene, strain 20.9 KSS	2523	2523	100%	0.0	99%	EU071593.1
138	W1B4	VPB	Bacillus sp. SS9.15 16S ribosomal RNA gene, partial sequence	2604	2604	100%	0.0	99%	NR_044255.1
139	E1B6	VPB	Micrococcus luteus strain EHFS1_S04Ha 16S ribosomal RNA gene, partial sequence	2540	2540	99%	0.0	100%	NR_044255.1
142	T1B4	VPB	Granulosicoccus antarcticus strain IMCC3135 16S ribosomal RNA, partial sequence	2508	2508	100%	0.0	99%	NR_044255.1

143	T1B4	VPB	Granulosicoccus antarcticus strain IMCC3135 16S ribosomal RNA, partial sequence	2508	2508	100%	0.0	99%	HM563047.1
144	T1B4	VPB	Granulosicoccus antarcticus strain IMCC3135 16S ribosomal RNA, partial sequence	2529	2529	100%	0.0	99%	NR_044255.1
149	T1B4	VPB	Granulosicoccus antarcticus strain IMCC3135 16S ribosomal RNA, partial sequence	2361	2361	100%	0.0	99%	NR_044255.1
151	T1B4	VPB	Granulosicoccus antarcticus strain IMCC3135 16S ribosomal RNA, partial sequence	2551	2551	100%	0.0	99%	NR_044255.1
163	Y1B1	IM8	Arthrobacter sp. Cr6-08 16S ribosomal RNA gene, partial sequence	2586	2586	99%	0.0	99%	GU784867.1
165	E1B4	VPB	Paenibacillus sp. IHB B 3415 16S ribosomal RNA gene, partial sequence	2529	2529	99%	0.0	99%	HM563047.1
183	Y1B5	IM8	Micrococcus sp. LJY5 16S ribosomal RNA gene, partial sequence	2484	2484	100%	0.0	99%	EU379020.1
190	T1B6	VPB	Micrococcus sp. A1 partial 16S rRNA gene, strain A1	2226	2226	99%	0.0	99%	AM403127.2
196	E1K	SCN	Pseudomonas sp. R3.12 16S ribosomal RNA gene, partial sequence	2566	2566	100%	0.0	99%	KC433650.1
203	W1B2	VPB	Micrococcus luteus strain EHFS1_S04Ha 16S ribosomal RNA gene, partial sequence	2538	2538	99%	0.0	100%	EU071593.1
210	T1B7	VPB	Granulosicoccus antarcticus strain IMCC3135 16S ribosomal RNA, partial sequence	2545	2545	100%	0.0	99%	NR_044255.1
211	T1B7	VPB	Granulosicoccus antarcticus strain IMCC3135 16S ribosomal RNA, partial sequence	2508	2508	100%	0.0	99%	NR_044255.1
213	T1B7	VPB	Staphylococcus pasteuri strain F77032 16S ribosomal RNA gene, partial sequence	2538	2538	98%	0.0	99%	HQ908742.1
214	T1B7	VPB	Granulosicoccus sp. ZS4-22 16S ribosomal RNA gene, partial sequence	2560	2560	100%	0.0	99%	FJ889674.1
216	T1B7	VPB	Granulosicoccus antarcticus strain IMCC3135 16S ribosomal RNA, partial sequence	2547	2547	100%	0.0	99%	NR_044255.1
218	T1B7	VPB	Granulosicoccus sp. ZS4-22 16S ribosomal RNA gene, partial sequence	2547	2547	100%	0.0	99%	FJ889674.1
219	T1B7	VPB	Granulosicoccus antarcticus strain IMCC3135 16S ribosomal RNA, partial sequence	2549	2549	100%	0.0	99%	NR_044255.1
222	T1B7	VPB	Granulosicoccus antarcticus strain IMCC3135 16S ribosomal RNA, partial sequence	2545	2545	100%	0.0	99%	NR_044255.1
225	T1B7	VPB	Granulosicoccus antarcticus strain IMCC3135 16S ribosomal RNA, partial sequence	2551	2551	100%	0.0	99%	NR_044255.1
229	TIB5	VPB	Granulosicoccus sp. ZS4-22 16S ribosomal RNA gene, partial sequence	2519	2519	100%	0.0	99%	FJ889674.1
230	TIB5	VPB	Granulosicoccus antarcticus strain IMCC3135 16S ribosomal RNA, partial sequence	2547	2547	100%	0.0	99%	NR_044255.1
231	TIB5	VPB	Granulosicoccus antarcticus strain IMCC3135 16S ribosomal RNA, partial sequence	2558	2558	100%	0.0	99%	NR_044255.1
234.1	W2B4	VPB	Bacillus subtilis strain TUST019 16S ribosomal RNA gene, partial sequence	2619	2619	100%	0.0	100%	KC456633.1
234.2	W2B4	VPB	Bacillus subtilis strain TUST019 16S ribosomal RNA gene, partial sequence	2615	2615	100%	0.0	100%	KC456633.1
240	T1B3	VPB	Micrococcus sp. 20.9 KSS partial 16S rRNA gene, strain 20.9 KSS	2551	2551	100%	0.0	100%	HE575933.1
244	T1B3	VPB	Virgibacillus halodenitrificans gene for 16S rRNA, partial sequence, strain: NSW13-2	2651	2651	100%	0.0	100%	AB697714.1
263	Y1B2	VPB	Rhodococcus luteus partial 16S rRNA gene, isolate 7Y	2549	2549	100%	0.0	99%	AJ576249.1
264	Y1B2	VPB	Rhodococcus luteus partial 16S rRNA gene, isolate 7Y	2549	2549	100%	0.0	99%	AJ576249.1
267	W2B6	VPB	Micrococcus flavus strain HME8781 16S ribosomal RNA gene, partial sequence	2519	2519	99%	0.0	99%	KC134360.1
268	W2B6	VPB	Micrococcus sp. PA-E028 16S ribosomal RNA gene, partial sequence	2553	2553	100%	0.0	99%	FJ233852.1
277	Y1B7	IM7	Agreia sp. CJ-G-R2A4 16S ribosomal RNA gene, partial sequence	2566	2566	100%	0.0	100%	HM584293.1
280	T1B7	IM7	Erythrobacter sp. R14 16S ribosomal RNA gene, partial sequence	2495	2495	100%	0.0	99%	EF177676.1
282	Y1B7	IM8	Arthrobacter sp. Cr6-08 16S ribosomal RNA gene, partial sequence	2551	2551	100%	0.0	99%	GU784867.1
286	Y1B1	WA	Methylobacterium adhaesivum gene for 16S ribosomal RNA, partial sequence, strain: 99c	2457	2457	100%	0.0	99%	AB698697.1
288	Y1B1	WA	Methylobacterium adhaesivum gene for 16S ribosomal RNA, partial sequence, strain: 21e	2462	2462	100%	0.0	99%	AB698702.1
293	W1B1	VPB	Rhodococcus sp. FI 1022 16S ribosomal RNA gene, partial sequence	1452	1452	97%	0.0	99%	JQ691546.1
296	T1B1	VPB	Granulosicoccus sp. ZS4-22 16S ribosomal RNA gene, partial sequence	2545	2545	100%	0.0	99%	FJ889674.1
297	T1B1	VPB	Micrococcus sp. LJY5 16S ribosomal RNA gene, partial sequence	2555	2555	99%	0.0	99%	EU379020.1
298	T1B1	VPB	Micrococcus luteus strain EHFS1_S04Ha 16S ribosomal RNA gene, partial sequence	2540	2540	99%	0.0	100%	EU071593.1
304	T1B4	SCN	Erythrobacter sp. PaH2.09b 16S ribosomal RNA gene, partial sequence	2429	2429	99%	0.0	99%	GQ391951.1
306	T1B4	IM8	Salinibacterium sp. KJF1-8 16S ribosomal RNA gene, partial sequence	2571	2571	100%	0.0	100%	JQ799993.1
308	T1B4	IM8	Agreia sp. CJ-G-R2A4 16S ribosomal RNA gene, partial sequence	2566	2566	100%	0.0	100%	HM584293.1
311	Y1B7	IM6	Arthrobacter psychrochitiniphilus gene for 16S rRNA, partial sequence, strain: JCM 13874	2588	2588	100%	0.0	99%	AB588633.1
313	Y1B7	IM6	Arthrobacter psychrochitiniphilus gene for 16S rRNA, partial sequence, strain: JCM 13874	2579	2579	100%	0.0	99%	AB588633.1
315	Y1B7	IM6	Rhodococcus sp. FI 1030 16S ribosomal RNA gene, partial sequence	1384	1384	98%	0.0	95%	JQ691549.1
318	Y1B3	VPB	Rhodococcus sp. ZS351 16S ribosomal RNA gene, partial sequence	2543	2543	100%	0.0	99%	JX428883.1
339	E1	IM7/NBC+SW	Brevibacterium sp. EP11 partial 16S rRNA gene, strain EP11	2577	2577	100%	0.0	99%	AM398220.1

340	E1	IM6/NBC+SW	Arthrobacter rhombi strain F98.3HR69 16S ribosomal RNA, partial sequence	2549	2549	99%	0.0	99%	NR_026448.1