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Antibiotic-producing bacteria from the sea surface microlayer in the Trondheim fjord, Norway

Thesis for the degree of Philosophiae Doctor

Trondheim, September 2009

Norwegian University of Science and Technology
Faculty of Natural Sciences and Technology
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Trondheim, June 2009
Sigrid Hakvåg

ABSTRACT

The marine environment has so far been poorly utilized in the search for (producers of) novel antimicrobial compounds. Marine bioprospecting might therefore be a promising field of research for the pharmaceutical industry as an alternative to terrestrial sources and synthetic production of pharmaceuticals.

In this project, over 4000 cultivable isolates have been isolated from different locations in the Trondheim fjord and along the coast of Trøndelag, Norway. Over 1000 of these bacteria were isolated from the sea surface microlayer, whereas the rest originated from sediment samples. The diversity of the isolates from the sea surface microlayer have been investigated by studying cultivable bacteria from two sampling locations as 'model-samples'. Whole-cell based antimicrobial assays revealed surprisingly high numbers of isolates displaying antagonistic activity among the assayed streptomycetes. 16S rDNA analyses indicated that several isolates seemed to be closely related, and studies on the PKS type I genes present in these samples revealed that recent horizontal gene transfer might have taken place. The results indicate that de-replication of isolates can not be performed based on 16S rDNA sequences alone, and the identification of unique KS-sequences in some of these isolates further supports this statement.
Two streptomycete isolates from the sea surface microlayer displayed activity against a vancomycin-resistant *Enterococcus sp*. Analysis of the bacterial extracts indicated that this might be due to the production of a novel antibacterial compound.

Plasmids play an important role in the horizontal gene transfer among bacterial species. The genes involved in the biosynthesis of the antifungal polyene macrolide candicidin were found to be present on a linear plasmid in one on the isolated strains. Production of candicidin was found to be widely distributed among *Streptomyces*

bacteria isolated from the Trondheim fjord, and it is thought that the plasmid might be involved in spreading the gene cluster in the marine environment.

A Gram-negative strain in the isolate collection showing antibacterial activity was show to be a new strain of the genus *Collimonas*. The *Collimonas* CT (Coast of Trøndelag) produces the blue pigmented compound violacein, and genome scanning identified genes for biosynthesis of this compound, as well as several other gene clusters for the production of secondary metabolites of potential industrial interest.

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Mar Drugs. 6:620-35

Paper II

Hanne Jørgensen, Espen Fjærvik, Sigrid Hakvåg, Per Bruheim, Harald Bredholt, Geir Klinkenberg, Trond E. Ellingsen and Sergey B. Zotchev. 2009. Candicidin biosynthetic gene cluster is widely distributed among *Streptomyces spp*. isolated from the sediments and the neuston layer of the Trondheim fjord, Norway.
Appl Environ Microbiol. 75:3296-303

Paper III

Sigrid Hakvåg, Espen Fjærvik, Geir Klinkenberg, Sven Even F. Borgos, Kjell D. Josefsen, Trond E. Ellingsen and Sergey B. Zotchev. 2009. Violacein-producing *Collimonas sp*. from the sea surface microlayer of coastal waters in Trøndelag, Norway: assessment of potential for biosynthesis of secondary metabolites through genome mining. *Manuscript in preparation*

ABBREVIATIONS

A	Adenylation domain
ACP	Acyl carrier protein
AHL	N-acyl-homoserine-lactones
AT	Acyltransferase
ATCC	American Tissue and Culture Collection
BLAST	Basic local alignment search tool
C	Condensation domain
CIL	Cluster identification library
CLF	Chain length factor
CoA	Coenzyme A
DH	Dehydratase domain
CCUG	Culture collection Gothenburg University
CT	Coast of Trøndelag
DAD	Diode array detector
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	Dictionary of Natural Products
E	Epimerisation domain
ER	Enoyl reductase domain
G+	Gram-positive bacteria
G-	Gram-negative bacteria
GSL	Genome sampling library
HGT/LGT	Horizontal/lateral gene transfer
HPLC	High Performance Liquid Chromatography
KR	Ketoreductase domain
KS	Ketosynthase domain

LC-MS	Liquid Chromatography /Mass Spectrometry
MAT	Malonyl-CoA:ACP transacylase
NMR	Nuclear Magnetic Resonance
NRPS	Non-Ribosomal Peptide Synthase
ORF	Open reading frame
PCP	Peptidyl carrier protein
PCR	Polymerase Chain Reaction
PKS	Polyketide Synthase
ppm	parts per million
RNA	Ribonucleic acid
T	Thiolation domain
TE	Thioesterase
TOF	Time-Of-Flight
UV	Ultraviolet light

1 INTRODUCTION

Over the last 20 years few genuinely novel antibiotics have been released on the market, while the number of bacteria becoming resistant to the ones already in use is increasing. The need for new antibiotics is obvious, but different parties (pharmaceutical companies and researchers) have different views on how this problem should be solved.

In the recent years, many pharmaceutical companies have focused on synthetic and semi-synthetic antibiotics. This approach is cheaper, faster and safer than getting involved in the search for new antibiotics from natural sources. The semi-synthetic derivatives approach mostly uses structural scaffolds from already published compounds as starting material for chemical modification [109]. Research communities, however, see the vast potential of the natural environment in terms of discovery of novel drug leads, but are dependent of financial support from external resources. Several advantages can bee seen from utilizing natural products in search for antibiotic, for example their chemical diversity, specificity and excellent starting point for chemical modification.

1.1 MARINE BIOPROSPECTING

Bioprospecting is a goal-oriented, systematic search for elements, bioactive compounds or genes in marine organisms, with the intent of developing products of commercial or social value (http://www.forskningsradet.no/en/Newsarticle/New+impetus+for+bioprospecting/1231229970484).

1.1.1 Why marine bioprospecting

Up until recently, the search for new compounds has mainly been focused on terrestrial environments. This is mainly because this is the human habitat. It has been thought that organisms living under the same conditions as us are more likely to produce compounds that can be utilized by humankind. Another reason is the low costs of sampling compared to the marine environment, and the lack of available techniques and equipment for culturing of marine organisms. Over 70 % of the earth surface is covered by water, and it seems likely that there one could find novel compounds with unique properties.

Over the last 30-40 years the search for novel compounds in the marine environment has intensified. Promising antibiotic [84], anticancer [44, 51], antiparasitic [84], and antiviral [36, 102] compounds have been isolated from different marine sources. Potent producers are plants and algae [36, 51, 102], molluscs [84], bacteria [44], and especially bacteria living in close relationship with marine macroorganisms (e.g. sponges). Actually, natural products initially thought to be produced by marine sponges have recently been shown to be produced by bacteria living symbiotic with the sponge [as reviewed in 105].

1.1.2 Drugs from marine natural compounds

The marine environment in general, and especially the marine bacteria, seems to be very promising as a source of new therapeutics. During 2005 and 2006 over 180 bioactive compounds from marine sources have been reported [as reviewed in 84] (compounds with antitumor and cytotoxic activity not included), whereof over 40 % exhibited anthelmintic, antibacterial, anticoagulant, antifungal, antimalarial, antiprotozoal, antituberculosis, or antiviral activities. The reported number of compounds exhibiting antibacterial or antifungal activity showed an increase from the previous years, suggesting an intensified effort in the search for new compounds to

compete in the race against bacterial and fungal antibiotic resistance. Compounds with potential therapeutic uses recently isolated from marine bacteria are listed in Table 1.1.

Table 1.1 Current status of potential marine bacterial therapeutics (adapted from [135])

Compound	Biological target	Therapeutic use	Current status*¤
Cyanobacteria			
Apratoxin A	STAT3	Oncology	
Apratoxin D	Antiproliferative	Oncology	
Coibamide A	Antiproliferative	Oncology	
Curacin A	Antimitotic	Oncology	
Cryptophycins	Tubilin polymerization	Oncology	
Largazole	Histone deacetylase	Oncology, mood stabilizer, anti-epileptics, neurological disorders	
Dolastatin 10 [79]	Tubulin assembly	Oncology	Phase II (ended)
Actinomycetes			
Marinomycin	Cytotoxic	Oncology	Not suited for clinical trials
Abyssomicin	*p*-Aminobenzoic acid	Infectious disease	
Proximicin	Antiproliferative	Oncology	
SS-228 Y	Antibacterial	Infectious disease	
Thiocoraline	DNA polymerase	Oncology, infectious disease	
Salinosporamide A	Proteasome inhibitor	Oncology	Phase I
Proteobacteria			
Bryostatin	Protein kinase C	Oncology	Orphan status §
Yondelis	Tubulin	Oncology	On market

* Current: May 2009

¤ www.cancer.gov

§ "Orphan drugs" = medicinal products intended for the diagnosis, prevention or treatment of life-threatening rare medicinal conditions (http://ec.europa.eu/)

Several compounds isolated from marine bacteria in the recent years have shown potential as therapeutic agents. None of these has so far reached the market/been approved by the FDA, but it has been speculated that the anticancer compound trabectedin (ET743) sold under the name Yondelis®, might be produced by a yet

uncultered symbiont of, rather than the tunicate *Ecteinascidia turbinata* it was isolated from, and in fact the patent describes the endosymbiont *Endoecteinascidia frumentensis* as the apparent producer [42].
Dolostatin-10 was first isolated from the sea hare *Dolabella auricularia,* but in 2001 its isolation was also reported from a marine cyanobacterium *Symploca* species VP642 [79]. It entered phase II clinical trials as an anticancer drug, but these trials are now ended or closed. Another anticancer drug, Bryostatin-1 was initially thougt to be produced by the bryozoan *Bugula neritina*, but the symbiont bacteria "*Candidatus* Endobugula sertula" was later shown to be responsible for its biosynthesis [33]. Bryostatin-1 is now in phase I trials for combination therapy (www.cancer.gov), and was granted orphan drug status by FDA in 2001 for use in combination with palitaxel in the treatment of esophageal cancer (www.fda.gov), and by the EC in 2002 (http://ec.europa.eu).

Over 70 % of the marine bacterial compounds isolated between 1997 and 2008 are produced by cyanobacteria and actinobacteria [135]. The proteasome inhibitor salinosporamide A was first isolated from the actinobacteria *Salinispora tropica* in 2003 [44]. It is now (by May 2009) in four different phase I clinical trials as an anticancer agent (www.cancer.gov), where its activity is tested against solid tumors, refractory lymphoma, multiple myeloma, non-small cell lung cancer, pancreatic cancer and melanoma. Other promising compounds from marine actinomycetes include abyssomicin, proximicin, SS-228 Y and thiocoraline [as reviewed in 135].

Antagonistic activity among marine microorganisms in organically rich environments, which are abundant in the mesotrophic and eutrophic waters or during phytoplankton blooms, has been reported [120]. Bacteria exhibiting such activities can be either free-living or attached to the organic particles dispersed in the seawater. New data indicate that antibiotic producers are frequently found among both groups, accounting for 40-66 % of all cultivable isolates [78]. These results underline the new opportunities for marine biotechnology screening programs aimed at finding novel anti-infective agent

producers in the sea, which, until recently, have mainly focused on microorganisms associated with sediments and sea animals.

1.1.3 Norwegian coastal waters as a habitat for antibiotic-producing bacteria

Norway is one of the world's northernmost countries. It has a long coast line with many fjords. The climate varies from temperate to arctic, with the warm currents of the Gulf Stream affecting the borders of the arctic zone. Considering the rather unique climate of Norway one might expect to find novel bacteria dwelling in this environment and/or novel antimicrobial compounds produced by the bacteria.
Recent results show that the polar oceans, the arctic, subarctic and the Antarctic, have a higher diversity of organisms than earlier assumed [17, press release: 27, 73]. These areas have been considered 'deserts', but one must now change this view.

1.1.4 The sea surface microlayer

Below the air/water interface the aquatic surface layer contains a series of sublayers [54]. These sublayers include the thin surface nanolayer (<~1 μm), enriched in surface active compounds; the surface microlayer (<~1 mm), with high densities of particles and microorganisms; and the surface millilayer (<~10 mm), inhabited primarily by small animals, eggs and larvae of fish and invertebrates.
The life forms in the surface layer of oceans and lakes are collectively named Neuston, and can be divided into epineuston and hyponeuston. Epineuston organisms live on top of the water surface, and are naturally dependent on the surface tension of the water. Hyponeuston organisms live in the top few centimeters of the water column. High densities of metabolically active bacteria (bacterioneuston), are found in the surface microlayer [121, 10, 122, 25]. The reported enrichment of viable bacteria per unit volume in the surface microlayer relative to the underlying water varies from no enrichment to >1000 times, but is often 10-100 times [10, 122, 39, 94, 65]. Also, total

count studies after acridine orange staining have yielded enrichment factors in the same range [25, 55]. Several authors [10, 122, 65, 96] have suggested that the actual environment for the bacterioneuston is a very thin film (≤10 μm), and the variation in the enrichment factor may partly be due to the thickness of the sampled layer which may vary from less than ten micrometers to a few hundred micrometers depending on the method employed [65, 96]. There is clear evidence that the microbial community of the surface microlayer differs from that of the underlying water [96, 55, 60]. In contrast to this another study suggested that a stable and abundant neustonic bacterial community is not a common trait of coastal marine environments, but still had evidence suggesting that the sea-surface layer may represent a rich source of new microorganisms [1].

High densities of metabolically active bacteria will most likely yield a competitive environment, and properties such as production of antibiotics may give organisms an advantage. There are no reports on systematic screening of the marine surface microlayer for antibiotic-producing organisms, although a number of antibiotic producers have been isolated from the marine environment [63, 106, 107]. Experimental results indicate that production of antibiotics could play an important role in the competitive relationship within marine bacterial populations [71]. In a study on antagonistic interactions among marine pelagic bacteria it has been found that more than a half of the isolates expressed antagonistic activity, and this trait was more common with particle-associated than with free-living bacteria [78]. Particles often tend to accumulate at the sea surface, and one could therefore expect that bacteria living at the sea surface might produce more antimicrobial compounds than other marine (i.e. pelagic) bacteria.

1.1.5 Concerns

Although the marine environment offers exiting new opportunities, this “blue treasure” needs to be protected. Concerns have been raised about affecting the biodiversity of the sea. Habitats need to be protected and the benefits must be shared.

Critical voices use the word 'biopiracy', referring to the uncontrolled utilization of common resources.

In 1992, the Convention on Biological Diversity (CBD) was opened for signature (www.cbd.int, accessed February 2009). The objective of the convention is 'the conservation of biological diversity, the sustainable use of its components and the fair and equitable sharing of the benefits arising out of the utilization of genetic resources…' In 2003 the Cartagena Protocol on Biosafety (CPB), a supplementary agreement to the CBD, was entered into force. The protocol governs movements of living modified organisms between countries. By now, (February 2009), the convention has 191 parties, and the CPB has 151 parties (Figure 1.1), both including Norway. The United States of America has signed the CBD, but has not ratified the agreement. Effort is also put into the work of assessing and explaining the diversity, distribution, and abundance of life in the oceans, including the marine microbes (Census of marine life, www.coml.org).

Yet another problem is the fact that most of the world's water masses and seabeds are in the international waters. These areas are not controlled by any national laws. Exploiting the diversity of these areas might leave traces that potentially could ruin the uniqueness of the special environments. An international understanding and agreement on how to preserve these resources are therefore a matter of necessity.

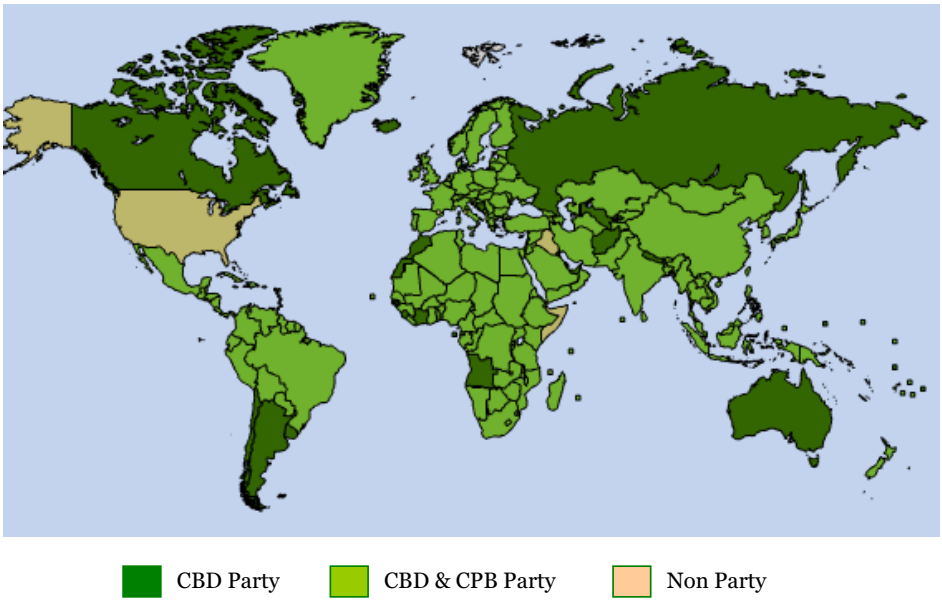


Figure 1.1. Parties of the Convention on biological diversity (CBD) and Cartagena protocol on biosafety (CPB) (www.cbd.int)

1.2 Antibiotics and their production in bacteria

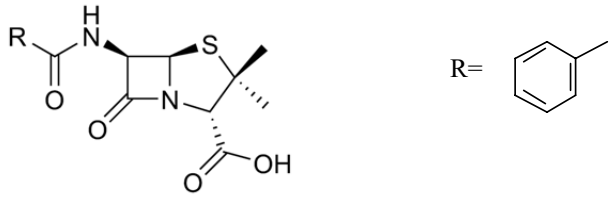
Infectious diseases in humans have been described as far back as in the Old and the New Testaments [81]. The ancient Egyptians and Greeks used different plant extracts, tree resins and spices as antiseptics to inhibit the degenerative effect of bacteria, but still the means to efficiently control and fight infectious diseases remained undiscovered for many years.

1.2.1 The history of antibiotics

The words 'antibiosis' ('against life') and 'antibiote' were first used by the French biologist Vuillemin to describe the activity of one organism inhibiting another, and the drug killing the bacteria (a phenomenon earlier observed by Pasteur and Joubert in 1887). The definition of 'antibiotic' was made by Selman Waksman in 1941, stating: 'an

antibiotic is a chemical substance produced by a microorganism that has the capacity to inhibit the growth and even destroy bacteria and other microorganisms'.

However, the very first antibiotics have not originated from microorganisms, but were chemically synthesized. The first successful antibiotic was the Salvarsan (Arsphenamine) [2]. By chemically modifying a compound that originally had been used as a dye in the textile industry, Paul Ehrlich produced a compound for the treatment of syphilis in 1909. Another successful antibiotic was the sulphonamide antibacterial Prontosil. It was synthesized by Domagk in 1932, and showed activity against *Streptococcus* and *Staphylococcus*. The compound could easily not have been detected as it was not active in the test tube, and rather had to be metabolized by the host to yield the active constituent.
Despite the success of these compounds, the sulphonamides were only bacteriostatic, and their finding was overshadowed by the later discovery of penicillins [81]. Scottish microbiologist Alexander Fleming was not the first to study the biology of the penicillium moulds, but their properties were not paid much attention to until Fleming's discovery of the efficient activity of *Penicillium notatum* against strains of *Staphylococcus aureus* in 1928. In 1945 Fleming received the Nobel Price in Medicine for the discovery of penicillin (Figure 1.2) together with Howard Florey and Ernst Chain, who successfully purified the compound [2]. During the 30 years following the discovery of penicillins, most of the main natural antibiotic chemical classes had been discovered.



A **B**

Figure 1.2. Penicillin core structure (A), and penicillin G (B)

1.2.2 Main classes of antibiotics

As listed in Table 1.2, there exist several classes of antibiotics acting against bacteria and fungi. Antibacterial compounds have several targets for their inhibiting action, as roughly sketched in Figure 1.3.

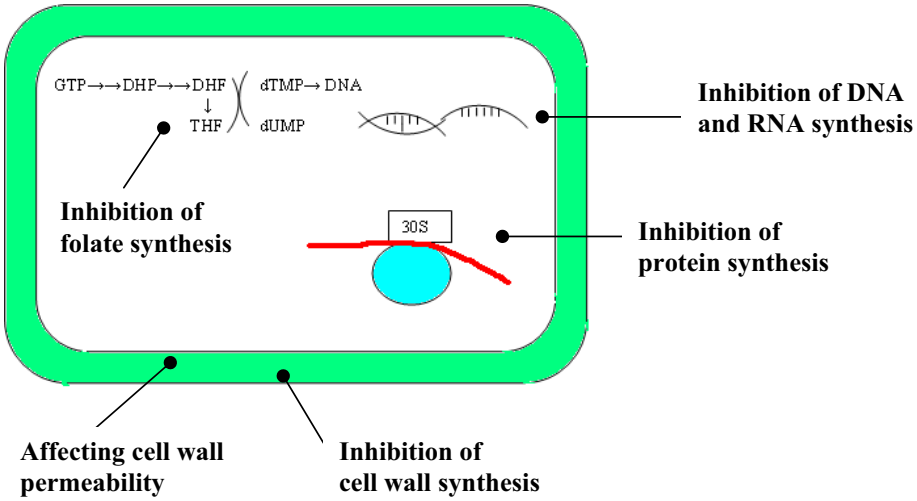


Figure 1.3. Schematic presentation of antibiotic targets in the bacterial cell.

Most antibiotics display their activity either by inhibiting cell wall synthesis (e.g. β-lactams), or protein synthesis (e.g. macrolides). In addition, inhibiting activity can be observed from compounds acting on DNA or RNA synthesis (e.g. quinolones and rifampicin), folate synthesis (e.g. trimethoprim) or by affecting the cell wall permeability (e.g. daptomycin).

Table 1.2. Main classes of anti-bacterial and–fungal antibiotics [2, 46]

Antibiotic class	Example drug	Source	Antimicrobial use £	Target
β-lactams	Penicillin G Cephalosporin Amoxicillin	*P. notatum* *A. chrysogenum*¤ Semisynthetic	G+ G+, (G-) G+, G-	Cell wall synthesis
β-lactamase inhibitors	Clavulanic acid	*S. clavuligerus*		Inhibitors of β-lactamase
Glycopeptides	Vancomycin Teicoplanin	*S. orientalis* *A. teichomyceticus*	G+ G+	Cell wall synthesis
Polypeptides	Polymyxin B and E	*B. polymyxa*	G-	Cell wall permeability
Aminoglygosides	Streptomycin	*S. griseus*	G+, G-	Protein synthesis
Macrolides	Erythromycin	*Sac. Erythraea**	G+, G-	Protein synthesis
Streptogramins Lincomycins	Virginiamycin [30] Linomycin Clindamycin	*S. virigniae* *S. lincolnesis* Semisynthetic	G+, some G- G+	Protein synthesis Protein synthesis
Tetracyclins	Tetracycline Doxycycline	*S. aureofaciens* Semisynthetic	G+, G-	Protein synthesis
Chloramphenicol	Chloramphenicol	*S. venezuelae*	G+, G-	Protein synthesis
Rifamycins	Rifampicin	*Amycolatopsis rifamycinica*§	G+, G-	RNA synthesis
Sulphonamides	Sulphanilamide	Synthetic	G+, G-	Folic acid synthesis
Trimethoprim	Trimethoprim	Synthetic	G+, G-	Folic acid synthesis
Quinolones	Nalidixic acid Ciprofloxacin	Synthetic	G+, G-	DNA replication
Lipopeptides	Daptomycin [90]	*S. roseosporus*	G+	Ca^{2+} dependent, cell wall permeabilizing
Azoles	Fluconazole Metronidazole	Synthetic	Fungi Anaerobic Fungi	Ergosterol biosynthesis DNA replication and transcription
Polyenes	Nystatin [15] Amphotericin [22]	*S. noursei* *S. nodosus*	Fungi	Ergosterol, cell membrane permeabilization
Pyrimidin analogs	Flucytosine	Synthetic	Fungi	DNA replication
Echinocandins (lipopeptides)	Caspofungin [37]	Semisynthetic (*Glarea lozoyensis*)	Fungi	Fungal cell wall
Allylamines	Terbinafine	Synthetic	Fungi	Ergosterol biosynthesis

£ G +: Gram-positive bacteria, G-: Gram-negative bacteria

¤ Formerly known as *Cephalosporium acremonium*

* Formerly known as *Streptomyces erythraeus*

§ Formerly known as *Streptomyces mediterranei*

1.2.3 Bacterial antibiotic biosynthetic genes

Bacteria produce a wealth of metabolites with secondary roles in their life cycles. These secondary metabolites are not essential for the survival of the bacteria, and typical examples are antibiotics and pigments [134]. Production of secondary metabolites is often under the control of quorum sensing. In Gram-negative bacteria the signalling molecules are typically N-acyl-homoserine-lactones (AHL) whereas Gram-positive bacteria utilize processed oligopeptides [8]. Quorum sensing among marine bacteria is well known [40]. Secondary metabolism is induced at different stages of the bacterial development and in different environmental conditions [134]. Polyketide synthases (PKSs) and/or non-ribosomal peptide synthetases (NRPSs) or a combination of these, are involved in production of many antimicrobial secondary metabolites in *Streptomyces* and other bacteria, fungi, and plants.

Polyketides have a remarkable diversity in structure and function, possessing pharmacologically interesting properties. They are synthesized sequentially by reactions catalyzed by PKSs. The PKSs can be considered as collections of enzyme activities with coordinated groups of active sites. The synthesis of polyketides resembles the synthesis of fatty acids [62]. Both syntheses are initiated by a Claisen condensation between a starter carboxylic acid and a dicarboxylic acid.

The polyketide synthases are usually classified according to their mode of synthesis (sequential or iterative) and whether they consist of a single or multiple proteins.
At least three different types of PKSs, named types I, II and III, have been described, and representatives of all three classes can be found in bacteria. Type I PKSs are single proteins that act either as modules performing sequential condensations, or iteratively as a single module. The multi-modular type PKS Is are usually found in bacteria, while the iterative type is more typical for fungi. These single proteins have one (iterative) or several (multi-modular) modules. The PKS type IIs are protein complexes consisting of several subunits each possessing a single mono-functional active site. More detailed

description of PKS types I and II is provided below. The last type of PKSs, type III, can also be found in plants [118] and are single proteins with multiple active sites functioning in an iterative manner [6]. PKS type IIIs enzymes consist of a homodimer, and unlike type I and type II PKSs, these systems do not contain acyl carrier protein components.

PKS type I

The PKSs type I resemble animal type fatty acid synthases (FAS), but unlike the FAS-system, these PKSs contain multiple modules, each having at least the so-called minimal PKS type I with or without other catalytic domains [89]. The minimal PKS type I module consists of a β-keto synthase (KS), an acyl transferase (AT) and acyl carrier protein (ACP) domains.

The number of modules in type I PKS systems reflects the number of ketide units in the synthesized polyketide [40]. Each module is responsible for one condensation cycle during the synthesis of a polyketide chain. The primer substrate is bound to the KS active site, and condensed with the chain extender substrate [124]. Each module contains a dedicated AT, resulting in the possibility of different extender units being utilized at each elongation step. These AT domains do, however, typically have high specificity for malonyl or methylmalonyl-CoA. In general, the AT domain loading the starter unit has a more relaxed substrate specificity (e.g. can accept 2, 3 or 4 C-atom starters).

Before the next elongation step, the β-ketoacyl product can be subjected to different β-carbon processing by the dehydratase (DH), enoylreductase (ER) and the β-ketoreductase (KR) [89]. The degree of β-carbon processing can vary in each elongation step, adding to the diversity of the PKS products. The anchors in the elongation of the polyketide chain are the ACP domains. They translocate the growing chain both within (through condensation and β-carbon-processing) and between the modules. Each module has one ACP domain. The final release from the

phosphopantetheine thiol, and cyclization of the chain is facilitated by the thioesterase (TE).
Actinomycete bacteria usually contain more than one PKS gene cluster [21]. Search for and/or mapping of PKS type I sequences within a bacterial genome would therefore display some of the diversity of the antibiotic producing potential of these bacteria. Over 10000 PKSs has so far been identified [124].

PKS type II

Bacterial aromatic polyketides are synthesized by PKS type II (aromatic) polyketide synthases. The minimal PKS type II consists of two β-ketoacyl synthase subunits, KSα and KSβ, an acyl carrier protein (ACP) and a malonyl-CoA:ACP transacylase (MAT) [88, 26]. The latter KS is often designated CLF (chain length factor), and it has been suggested that the polyketide chain length in bacterial aromatic PKSs is controlled by a substrate-binding pocket in the KS-CLF dimer interface [20]. CLF is involved in formation of acetyl-ACP (from the decarboxylation of malonyl-ACP) [11]. The two KS-units catalyze the decarboxylative condensation of the malonyl building blocks delivered by the MAT, and the acyl carrier protein (ACP) acts as an anchor for the polyketide chain during the various biochemical manipulations.
Cyclases, aromatases and ketoreductases are required to fold and cyclise the chain. The β-keto acid can be processed by β-ketoacyl-reductase (KR), β-hydroxy-acyl-dehydratase (DH) and enoyl-reductase (ER) domains [24]. A combination of these enzyme activities will determine the structure of the final polyketide.

NRPS (Non-ribosomal peptide synthases)

NRPSs somewhat resemble modular PKSs in their product assembly and their organization of modules [83]. The peptides are assembled from amino acids instead of acyl units as for the polyketides. Following the initiation module, the condensation domains (C) play a central role in the NRPS-catalysed peptide synthesis. Together with the adenylation domain (A) for substrate recognition and activation, thiolation or

peptidyl carrier protein domain (T or PCP) that holds the activated substrate, they (C, A and PCP) constitute the minimal elongation module of the NPRS required for the incorporation of one amino acid into the growing peptide. In contrast to the ribosomal peptide synthesis, which is restricted to 20 amino acids as building blocks, NRPSs can utilize several hundred substrates [45]. In addition to the minimal elongation module, tailoring enzymes may also be involved in maturation of the peptide. NRPSs are often large proteins, and in bacteria syringomycin synthethase E from *Pseudomonas syringae* is the largest NRPS known [117].

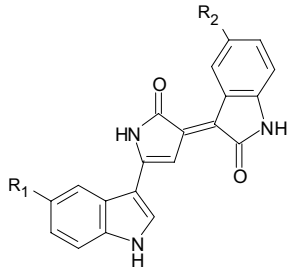
In conjunction with the similarities between NRPS and PKS, it is not surprising that hybrids of these enzyme complexes with mixed modules can be found. Rapamycin [119] and epothilone [128] are examples of compounds synthesized by the hybrid NRPS/PKS enzyme complexes.

1.2.4 Violacein and candicidin

Violacein is an indole-derived blue pigment dye that has first been reported in literature in 1882, with the chemical name 3-[1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ylidene]-1,3-dihydro-2H-indol-2-one. The characteristic colour has made it easy to observe and study in its producers, whereof *Chromobacterium violaceum* is the most prominent one [108]. Other producers include the Gram-negative *Janthinobacterium lividum, Collimonas sp.* and several strains of the genus *Pseudoalteromonas* [100, 92, 136]. The violacein-producing strains have been isolated from several different habitats, including both water and soil in arctic, subtropical and tropical regions, rivers, lakes and springs and from seawater at a depth of 320 m outside Japan. Violacein has been shown to act antibacterial (both G+ and G-) [76, 95, 111] in addition to displaying anti-protozoan, anticancer, anti-viral, and antioxidant [66] activities.

Sequencing of the violacein gene cluster from *Ch. violaceum* and eDNA [5, 14] among others, has given insight into the biosynthesis of this pigment. The violacein clusters from *C. violaceum* and eDNA, spanning 8 kb and 6.7 kb, were reported to contain the

genes *vio*A-D responsible for the production of violacein and deoxyviolacein (Figure 1.4 A and B). A fifth gene (*vio*E) was later described as being essential for the violacein biosynthesis [111].



A: Violacein: R_1 = OH, R_2 = H
B: Deoxyviolacein: R_1 = R_2 = H

Figure 1.4. Chemical structure of violacein (A) and deoxyviolacein (B)

Candicidin (Figure 1.5) is a polyene (heptaene) macrolide antibiotic produced by *Streptomyces griseus* IMRU 3570 [23]. It has first been named antibiotic C135 by its discoverers, but was later renamed candicidin due to its strong activity against *Candida* species. As other glycosylated polyenes, it disrupts the membranes containing sterols (e.g. as in fungi) by forming organized channels, thereby inducing ion leakage [53].

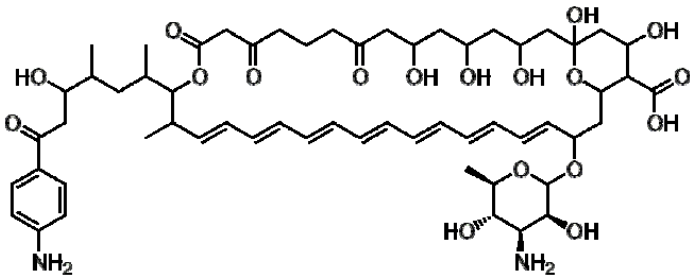


Figure 1.5. Chemical structure of candicidin

Candicidin is produced by a number of *Streptomyces* strains, including *S. coelicolor* JI2159, *S. coelicolor* JI1157, *S. griseus* JI2212 and *S. albus* G, originating from different sources [47]. The biosynthetic gene cluster for the production of candicidin

spans approximately 140 kb, and consists of four PKS type I genes and 11 more genes putatively involved in the candicidin biosynthesis [23].

1.2.5 Horizontal gene transfer

Many different bacterial species have been shown to produce the same antibiotics, suggesting that antibiotic biosynthesis genes can be transferred from one species to another. Horizontal or lateral gene transfer (HGT/LGT) is a process in which genetic material is transferred from one organism to another without reproduction, and thus can be involved in the transfer of antibiotic biosynthesis gene clusters. Multiple mechanisms for the physical transfer of DNA from one species to another are known, and the recombination mechanisms that can absorb this DNA are ubiquitous [127].

Cross-species gene transfer involves insertion of smaller genetic regions such as genes and parts of genes, and endosymbiotic fusion (transfer of genes from endosymbiont to host). Typical mechanisms for genetic transfer are transformation, transduction and bacterial conjugation [80]. Horizontal gene transfer is a major driving force in the evolution of many bacterial pathogens [143]. Virulence factors and genes involved in antibiotic resistance are commonly transferred between strains, but will also experience intrastrain genetic- (and phenotypic) variations due to point mutations, deletions, and pathoadaptive mutations, among other factors.

Due to horizontal gene transfer, species phylogenies derived from comparisons of single genes are rarely consistent with each other [41]. Within prokaryotes the 16S rRNA gene has been most commonly used for construction of phylogenetic relationships, because its sequence is thought to be conserved during evolution. However, differences in the 16S rRNA gene sequences within a single *Heliobacter* species have been reported [132]. Based on this finding, it has been suggested that taxonomic analysis should be supported by other phylogenetically informative macromolecules, e.g. 23S rRNA, gyrase and other “housekeeping genes” when other credible phenotypic and genotypic data deviate from 16S rRNA analyses [38].

1.3 Activity-based discovery of new antibiotics

Screening microbes from natural sources in general and the marine environment in particular is a promising strategy for discovery of new antibiotic leads. Due to their antibiotic producing potential, actinomycete bacteria have been of special interest in this search. At least 10 000 natural products are characterized from actinomycetes, whereof ~70% are produced by S*treptomyces* [16]. Traditionally, this search has been performed with whole cell assays with the extracts prepared from actinobacterial cultures.

A typical flow chart describing the initial processes of discovering the antimicrobial activity of bacteria from different natural sources is shown in Figure 1.6. For many years, the environmental source from which to isolate the bacteria has mainly been terrestrial. The focus on isolation of bacteria from marine environments has increased during the last 20 years. The microbial diversity in this environment is thought to be different from what has been reported for soil communities, and some of the compounds they produce are novel and/or have new and interesting properties [33, 79, 135].

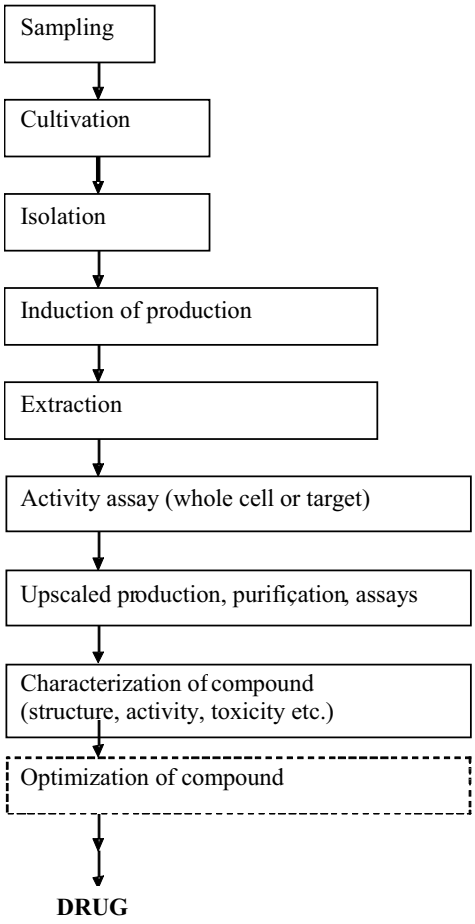


Figure 1.6. Drug discovery from microbial sources

Isolation of marine bacteria has been performed both from sediment [47], pelagic waters (both biofilm/particle associated and free-living) [78], sponges, plants, invertebrates and from the sea surface [19, 3, 142]. Most research on marine bacteria has been done on isolates from sediment and surface-associated bacteria. Collection of sediment samples is performed at depths varying from the intertidal zone [47, 142] to 6500 m [74]. Traditional methods for cultivation of the isolated marine bacteria have involved use of minimal media, dilution to extinction and use of sea water in the

growth medium [3, 47, 69, 16]. When searching for producers of antimicrobial compounds, samples with macroorganism– or sediment-associated bacteria are often subjected to selective treatments prior to plating. These pre-treatments facilitate growth of certain types of bacteria compared to others and include among other methods, drying (of sediment), heat- and/or phenol treatment [47, 16].

Isolation and characterization of diverse bacteria and the increasing number of bacterial genomes being sequenced helps us to understand more about the different cultivation requirements of so far uncultivable bacteria. Use of diffusion chambers and encapsulation of cells in gel microdroplets (OneCell System) for cultivation of bacteria, intended to simulate natural conditions with respect to nutrients, physical environment and symbiosis, have been reported and patented [61, 72, 13, 139, 68, 12]. Up to 70% of the bacteria isolated from both Petri dishes and after cultivation in diffusion chambers were obtained exclusively from diffusion-chamber derived material [61].

After cultivation and isolation of the bacteria, it is important to optimize the conditions for production of secondary metabolites. These metabolites are not essential for growth [134], and might therefore be not constitutively synthesized.
Obtaining production of interesting compounds from bacteria grown as pure cultures in the laboratory might therefore require some kind of induction. Results showing that marine particle-associated bacteria are more likely to produce inhibitory molecules than free-living bacteria [78] indicate that the antagonist molecule production might be triggered by the competitive environment. It has been shown that the antimicrobial activity of surface-associated marine bacteria is induced by the presence of terrestrial bacteria, or culture supernatants [91, 19]. It is thought that the production of antimicrobial compounds might be triggered by the presence of signalling molecules (AHLs and processed oligopeptides) from the ‘competing’ bacteria.

When screening the bacteria for compounds of interest, the assay design and choice of assay method will affect the type of compounds that can be identified. The two most widely used methods are cell-based and target-based assays. The former method is the direct search for compounds inhibiting growth of or killing the living cell/bacteria. The target-based method requires prior knowledge on the function of interest, availability of the target (usually enzyme), and a robust assay suitable for high-throughput screening. Positive candidates from the assays are usually inhibitors of a defined target. Both approaches have their own benefits and shortcomings, as listed in Table 1.3.

Table 1.3. Pro's and con's of two different screening strategies [110, 29]

Cell-based assays		Target based assays	
Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
High chances of finding novel compounds/compounds with novel targets	Insensitive	More sensitive (can detect poorly/not penetrating compounds)	The drug may not penetrate the cell- (not effective in vivo)
Finding (small) compounds able to penetrate cells	Needs to penetrate the cell	Easy screening – enzyme inhibitors	In vitro drug ≠ in vivo drug
Fast. HTS	Unknown mode of action. (No basis for compound optimization)	Ability to do rational drug design	Genetic validation (i.e. gene knock-out) can be misleading
Eliciting desired phenotype	Mixed mechanisms of action	Can drive search into new areas of biology	
Compounds have acceptable pharmacokinetic profiles	Most active compounds are toxic or non-specific		
Reproducible			

For an antibiotic to be effective as a drug, several criterions need to be fulfilled, whereof it not being toxic to the host is one of the most important ones. In addition, the drug needs to permeate the target cell and avoid its efflux- and detoxification systems [130]. Once inside the cell, the drug must be stable enough to reach its target in high enough concentrations to inhibit its function, resulting in growth arrest or

death of the target cell. By using whole cell assays in the search for new antibiotics several of these criterions are already fulfilled. In addition, by not limiting the assay to predefined targets, the chances of finding compounds with novel targets and/or reaction mechanisms might be increased. On the other hand, the whole-cell based assays are insensitive, and many potential promising leads might be missed.

When considering pathogenic bacteria, typical assay targets are functions essential for growth/survival and pathogenicity. As the culturing conditions in the laboratory are most likely different from the environment in the host, assays targeting functions that are essential for growth and survival might be not be sufficient to detect all compounds inhibiting functions essential *in vivo*. [29].

One combinational approach has been described [75] in which initial cell-based assays are followed up by assays with multicopy suppression of the inhibiting activity. Multicopy plasmids with bacterial genomic fragments are introduced into the cells displaying sensitivity to the drug of interest. The inhibition is quenched either by multicopy expression of the target of inhibition or by expression of a resistance mechanism. This forward chemical genetic method has the potential of both identifying the cellular targets and the resistance mechanisms for the novel antibacterials identified in the initial cell-based assay. Identification of the resistance mechanism will help designing chemical modifications to be made in the antimicrobial compound to make it a broad-spectrum drug. In the study of Li, Zolli-Juran, et al. [75] several compounds have been identified as substrates for efflux, indicating that in standard whole cell assays their activity might be missed.

1.4 Genome mining for new bioactive compounds

With the emergence of new techniques for sequencing, the speed and cost of whole genome sequencing has changed inversely (to each other). More and more sequences

are being accessible to the public through common databases and hence enabling genome-based discovery of secondary metabolites.

1.4.1 Sequencing and automated annotation

The traditional method for sequencing has been the Sanger (chain-termination) method, which was developed in 1975 [112]. This method is restricted to sequence reads of 100-1000 base pairs, and genome sequencing can only be done by chromosome walking or shotgun sequencing [113].

The second generation sequencing techniques produce hundred thousands to a million short reads of 13-300 base pairs [113]. These techniques are faster and less expensive than the traditional methods, but they also generate more information to be processed. Several of these methods are based on an initial emulsion PCR, where beads coated with primers and DNA molecules are contained in aqueous droplets within an oil phase. Companies using this method include 454 Life Sciences and Applied Biosystems. The SOLid sequencing (ABi) is based on ligation of immobilized marked oligo nucleotides to the sequence of interest (http://www3.appliedbiosystems.com/AB_Home/index.htm).

Putative cluster for the production of possibly novel secondary metabolites can be predicted in the genome by sequence similarity to known genes and gene clusters. In particular, knowledge about PKS and NRPS systems allows identification of clusters with potential antimicrobial products. These systems also allow prediction of structures of the enzymes encoded by these genes and, at least partially, their products. This is particularly true for the multi-modular PKS type I and NPRS systems, where the gene organization often reflects the order of biosynthetic steps. However, the products of many such clusters can be difficult to detect in assays, as not all clusters are expressed under the conditions tested, and hence they are named cryptic or ‘orphan’ gene clusters. In fact, a high share of the secondary metabolites

encoded in the bacterial genomes remains to be discovered, as exemplified by the sequencing of the *Streptomyces coelicolor* A3(2) and *Salinospora tropica* CNB-440 genomes which showed that less than 20 % of the gene clusters encoding putative secondary metabolites were earlier identified in these bacteria [9, 131].

Whole genome sequencing is a costly method, although the prices continue to drop,. By screening for PKS and NRPS gene fragments in the DNA of a bacterium of choice it is possible to circumvent the whole genome sequencing. PCR-scanning, using degenerate primers enables the potential identification of orphan gene clusters which can then be further examined.

1.4.2 Genome scanning

Cryptic gene clusters involved in the production of natural products can be detected also without genome sequencing. A genome scanning method has been developed by Zazopoulos et al., and applied to detect the enediyne antitumor antibiotics biosynthesis genes in several actinomycetes [138]. Genomic DNA is fragmented and small fragments (700 bp) are maintained on plasmids in a genome sampling library, GSL, while larger fragments are maintained on cosmid or BAC vectors in a cluster identification library (CIL). Sequencing of the GSL clones allows identification of those harboring genes involved in production of natural products/secondary metabolism. The gene (fragment) of interest can then be used as a probe for identification of CIL clones containing the gene and its surrounding sequences. Sequencing of the positive CIL clone(s) will then help identification of the secondary metabolite gene cluster(s). The sequence information can be used to make computer predictions of the chemical structure [43]. More than 450 secondary metabolite gene clusters have been identified in actinomycetes using this technique, including the biosynthetic gene clusters for the production of the antifungal ECO-02301 produced by *Streptomyces aizunensis* NRRL B-11277 and the antibacterial ECO-0501 produced by *Amycolatopsis orientalis* ATCC 43491 [85, 7]. Examples of natural products isolated after genome mining, and

computer predictions are listed in table 1.4. The genome scanning method can also be a useful tool when searching metagenomic libraries for secondary metabolite gene clusters. The marine microbial diversity is high and it is assumed that only 1-5 % is (so far) cultivable. Marine metagenomics is an emerging field allowing the access to the full biotechnological potential of the marine microbiota via culture independent techniques. Metagenomic libraries have been made from several marine sources, including sediment [57], sponges [115] and water samples from the Sargasso Sea [133].

Table 1.4. Natural products isolated by genome mining and computer prediction approaches [adapted from 70]

Natural product	Class	Source	Activity
Diazepinomicin	Dibenzodiazinone	*Micromonospora* sp.	Anticancer
Coelichelin	Tetrapeptide	*S. coelicolor*	Iron chelator
E-837	Alkenylfuranone	*S. aculeolatus*	Anthelmintic
E-492, E-975	Alkenylfuranones	*S. sp.*	Anthelmintic
ECO-02301	Linear glycosylated polyene	*S. aizunensis*	Antifungal
ECO-3396	Angular polycyclic ketide	*Micromonospora echinospora*	Antibacterial
ECO-7942	Hexadepsipeptide	*S.* sp.	Anticancer
ECO-0501	Octaenoic acid glucuronide	*Amycolatopsis orientalis*	Antibacterial
TLN-4601 (ECO-4601) [86]	Farnesylated dibenzodiazepinone	*Micromonospora* sp., 046Eco-11	Anticancer

1.4.3 Identification and isolation of secondary metabolites encoded by cryptic gene clusters

A number of strategies can be employed to identify and isolate the compounds of interest. The major challenge of all methods, however, is the activation of the relevant gene clusters. Different strategies can involve as simple methods as applying stress, change of growth medium and incubation conditions, or co-culturing with other microorganisms [114]. Allowing growth on/in several different growth mediums designed for the production of secondary metabolites has been shown to be effective [85, 138]. Production of antimicrobial compounds has also been induced by mimicking the natural competitive environment by adding bacteria or culture supernatants [91,

19]. Other methods involve homologous overexpression of a pathway activator [114], or transfer of the cluster to another host to better control the expression [32]. Heterologous expression of putative secondary metabolite gene clusters is also an alternative for Metagenomic libraries prepared from environmental DNA samples.
Several methods for discovering the products of cryptic gene clusters have been employed in the recent years. Tools for the isolation of these natural products have been summarized as follows [28]:

1) In vitro reconstitution
2) Heterologous gene expression/ comparative metabolic profiling
3) Gene knock-out/ comparative metabolic profiling
4) Prediction of physio-chemical properties from genetics
5) Genomisotopic approach

As mentioned, the structure of the polyketides synthesized by PKS type I systems can be predicted based on the primary sequence of the gene cluster. This was done by McAlpine et al. [85] and Banskota et al. [7] when discovering the antifungal ECO-02301 and the antibacterial ECO-0501 respectively. The gene clusters putatively encoding the biosynthetic genes of the novel compounds were detected in the genome and predictions of the structure and physiochemical properties of the product allowed the detection of the metabolite in the fermentation extracts.

In vitro reconstitution involves prediction of the precursors, purification of the enzymes involved and mixing of the components for in vitro biosynthesis.
This method might be most suitable for smaller clusters as the procedure is more laborious than the others. On the other hand, the biosynthesis of the compound of interest can be studied in a more controlled manner. The two-component lantibiotic haloduracin is encoded by the genome of *Bacillus halodurans* C-125 [87]. Haloduracin was produced by overexpression of the prepeptides and the modifying enzymes in *E. coli* followed by purification and *in vitro* reconstitution.

Also the genomisotopic approach relies on the prediction of the precursor. The latter approach is based on labeling putative precursors of the metabolic product with isotopes, feeding the precursor to the bacteria, followed by isotope-guided fractionation by NMR [50]. In the study by Gross et al. a cryptic gene cluster containing a NRPS responsible for the production of an antimicrobial lipopeptide encoded in the *Pseudomonas fluorescens* Pf-5 genome was identified. The amino acid precursors predicted from the NRPS sequence were labeled and aided the identification of the cyclic lipopeptide orfamide A.

Several of the listed techniques rely on credible prediction of the precursors. In some cases, as for the iterative PKS type II systems, substrate specificity can not easily be predicted. The comparative metabolic profiling techniques (number 2 and 3) circumvent this problem by comparing production versus no production of a biologically or analytically detectable compound.

2 AIMS OF THE STUDY

The overall aim of this project was to isolate bacteria from the sea surface microlayer that produce valuable products, in particular antibiotics, and might have an industrial potential. However, the culture collections built up in this project will be a good starting point for later bioprospecting for other products as well. The project aims at the utilization of Norwegian marine resources for the development of national industry, by building a national culture collection of marine microorganisms from the sea surface microlayer. The Norwegian marine ecosystems have developed in a rather cold and severe climate, suggesting that selective pressure on the microorganisms comprising part of such systems must have been quite unique (cold seawater environment). Because of this, it might be that these microorganisms have developed antibiotic biosynthesis pathways that differ from those utilized by terrestrial microorganisms.

We wanted to isolate new microbial strains of commercial interest for production of antibiotics by studying marine bacteria from the sea surface microlayer collected in the Trondheim fjord, Norway. The individual isolates would then be screened for antibiotic activity against bacteria and fungi. Secondary screening would aim at selection of the producers of the candidate anti-microbials, and their taxonomical characterization. A further sub-goal has been small-scale production, purification, structural and biological characterization of antimicrobial compounds.

The project has been set to serve two purposes: (i) to evaluate the potential of the microorganisms living in the surface microlayer to produce antibiotics; (ii) to isolate and characterize the producers of novel antimicrobial agents with commercialization potential. Logical continuation of this project might include strain development for commercial production of the new antimicrobials, and cloning and manipulation of the genes for their biosynthesis.

3 SUMMARY OF RESULTS & DISCUSSION

3.1 Construction of a marine isolate collection and detection of antimicrobial activity

A library of over 1000 bacterial isolates recovered from the sea surface microlayer of the Trondheim fjord and the coast of Trøndelag was constructed. Both rich- and minimal media were used in the initial isolation of the bacteria, and the collection contained bacteria that were able to grow on different selective media supporting growth of actinomycetes. To increase bacterial diversity in the collection, isolation was performed on both media with and without seawater added. In parallel, a collection of over 3200 sediment isolates sampled at different depths in the Trondheim fjord was constructed by Harald Bredholt. These samples were subjected to pre-treatments before plating on the selective media.

To screen the library for bacteria producing compounds with antimicrobial activity, a whole-cell-based growth inhibition assay was chosen. By using this simple function-driven method, a larger number of bacteria could be assayed at the same time. In addition, by choosing a cell-based over a target-based assay the chances of finding a novel compound or a compound with a novel action mechanism/target would be optimized, since the assay is not restricted by an already defined target. Preferred qualities of the potential drug(s) (ability to penetrate the target cell, to reach and act upon its target) would also be naturally selected for in these assays. In the initial bioassays isolate extracts were tested for growth-inhibiting activity against several indicator organisms. To maximize the number of possible antimicrobial compounds extracted from the cells, both polar and nonpolar, DMSO was chosen as a solvent. Antagonistic activity in the extracts was assayed in agar diffusion- and liquid culture

assays against representatives of non-filamentous fungi (*Candida albicans* ATCC 10231), Gram-positive (*Micrococcus luteus* ATCC 9341) and Gram-negative bacteria *(Escherichia coli* K12). Liquid culture assays were performed with the indicator organisms *C. albicans* (CCUG 39343), C. g*labrata* (CCUG 39342) and two strains of *Enterococcus faecium* (CCUG 37832 and CTC 492), whereof the former is vancomycin-resistant.

The bacterial sample collection was incubated on several solid growth media prior to extraction to stimulate the production of different antimicrobial compounds. Initial studies were performed by Espen Fjærvik and Harald Bredholt to determine the ability of four different production media to promote the production of antimicrobial compounds in the marine isolates (unpublished results). The tests, performed with over 450 sediment isolates, indicated high frequency of antimicrobial activity among the bacterial extracts tested (~78 %). Of the total antimicrobial activities detected on the four production media, ≥70 % could be recovered using a combination of any of the two media. Cultivating the isolates on a production medium containing dry yeast seemed to increase the number of bacterial extracts having antifungal activity. It is known that the presence of other bacteria and/or culture supernatants thereof might induce the antimicrobial compound production in marine bacteria [78, 19, 91]

3.2 Diversity among *Streptomyces* spp. in the Trondheim fjord surface microlayer (paper I)

Isolates from two sampling sites were selected for studies on the diversity among the *Streptomyces* spp. from the sea surface microlayer in the Trondheim fjord. Total number of bacteria isolated from the two sampling sites where $2.5x10^3$ and $1.2x10^4$ cells/ml seawater, whereof presumed (based on macromorphology) actinomycetes accounted for $9.8x10^2$ and $1.3x10^3$ cells/ml, respectively.

Re-streaks of the bacteria were performed on agar media with and without 50 % seawater. None of the isolates were restricted to growth on seawater-containing media, but the seawater seemed to induce better/faster growth of all the isolates. The seawater was added to mimic natural conditions and to allow isolation of marine actinomycetes. The resulting enhanced growth of the isolates suggests that they might have adapted to the marine environment and may occur naturally in the surface microlayer.

3.2.1 Antimicrobial activity among streptomycete bacteria

Isolated bacteria morphologically similar to streptomycetes were assayed for their antibiotic producing potential. 217 colonies from the two sampling sites (134 and 83 isolates from samples 1 and 2, respectively) were selected for the analysis. Antagonistic activity could be detected against at least one of the indicator organisms for 79% (sample 1) and 85% (sample 2) of the sample isolates, displaying considerably higher numbers than in earlier reports.

Previous studies have reported 44-50 % antibacterial activity among streptomycetes from marine sediments [101] and pelagic bacteria [78], whereas the antifungal activity among *Streptomyces* species isolated from sediment was considerably lower than the antibacterial activity [101, 125]. In our study, over 50% of the isolates from both sample 1 and 2 showed antifungal activity. Among the sediment-associated streptomycetes from the Trondheim fjord (paper II), about 28 % of the isolates were found to display antifungal activity.

Several of the isolates showing antimicrobial activity were active against more than one indicator organism, and this may indicate production of several antimicrobial compounds and/or production of compounds with multiple microbial targets. In particular, 80% of all the antibacterial activity detected was against both Gram-positive and Gram-negative bacteria.

The isolates were grouped and sub-grouped based on colony morphology (colour of substrate and aerial mycelia, pigment production) and the displayed antagonistic activity (Table 2, paper I). Isolates from group G1 dominated the samples, indicating that the sampling/isolation conditions were best suited for these isolates and/or that they are abundant in the surface microlayer.

3.2.2 Phenotypic grouping and molecular taxonomy discrepancy

Analysis of partial 16S rDNA sequences (1351 bases) from 46 assumed non-identical streptomycete isolates distinguished by colony morphology and antimicrobial activity was performed, as shown in Figure 3.1. Several of these isolates displayed 100% identical 16S rDNA sequences, suggesting that they are very closely related. These isolates, however, did not share the same colony morphology or the same antimicrobial inhibition patterns. Some *Streptomyces* strains can have very similar 16S rDNA sequences, and still be classified as different species [67]. It has also been shown that sequencing of 16S rRNA genes can not be used as the only tool for dereplication of bacteria [132].

BLAST searches revealed that all except four isolates displayed ≥99 % identity to 16S rDNA sequences from *Streptomyces* spp isolated from marine sediments and sponges [141, 59, 56, 140], which is in agreement with the fact that they thrive on seawater-containing media.

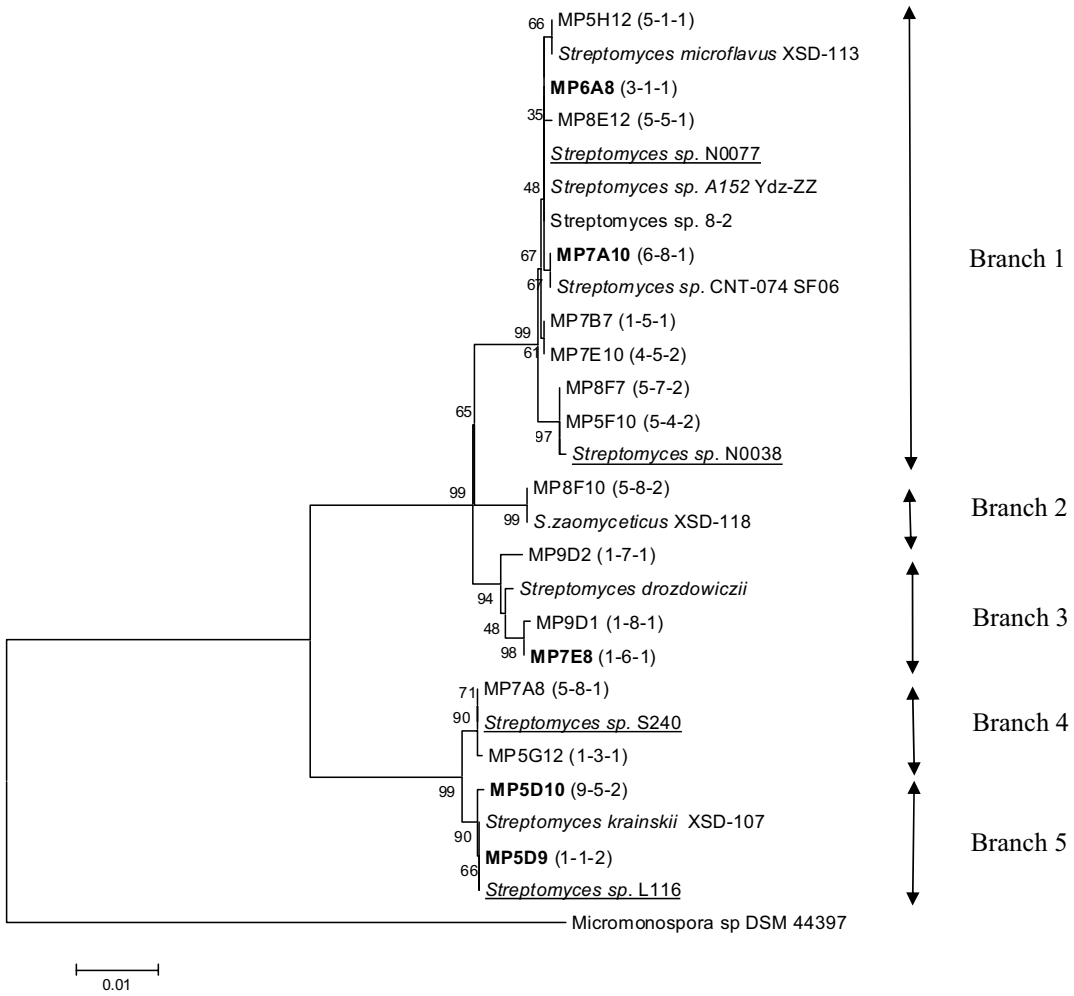


Figure 3.1. Phylogenetic tree constructed for partial 16S rDNA sequences of 46 *Streptomyces* species isolated from the surface microlayer in the Trondheim fjord, Norway. The tree also contains some of the closest matches from BLAST searches. The 16S rDNA sequence from *Micromonospora sp* DSM 44397 is included to root the tree. Numbers in brackets (x-y-z) refers to x: morphology group (Table 1, paper I), y: inhibition pattern (Table 2, paper I), and z: sample number. Arrows indicate the different branches of the tree. Bold font indicates sequences representing several isolates. Strains of marine origin are underlined.

3.2.3 Possible horizontal gene transfer of bacterial type I (modular) PKS genes

Closely related isolates displaying 100 % identical 16S rDNA fragments were subjected to further analysis. The presence of PKS type I (modular) genes were investigated in a group of seven of these isolates displaying different inhibition patterns to possibly elucidate any diversity in their potential to produce polyketide/macrolide compounds (Table 3, paper I). 16 rDNA sequences from the seven isolates are represented by the one from the isolate MP6A8 in branch 1, Figure 3.1.

A total of 13 different sequences were obtained which, upon phylogenetic analysis, formed 6 clades, representing 6 different types of KS domains as shown in Figure 3.2. For each of the isolates one to six unique PKS type I sequences could be identified. The clustering of KS domains in phylogenetic trees are known to be affected both by evolutionary relatedness and substrate specificity [93], indicating that one or more of the KS sequences in the clade might belong to the same PKS type I gene cluster.

Five of the PKS type I sequences from one isolate (isolate MP8E7) displayed 71-83 % identity (at amino acid level) to three PKS's involved in the biosynthesis of the commercially interesting compounds meridamycin (neuroprotectant) [126] (sequences MP8E7 PKSI-1 and -6), filipin (antifungal) [97] (sequences MP8E7 PKSI-2 and -3), and an antibacterial of a new chemical class [7] (sequence MP8E7 PKSI-4). The first two KS sequences (sequence -1 and -6) cluster with two other KS sequences from the same isolate, but not with their closest matches from the BLAST search. These four sequences that are 68-95 % identical might be amplified from the same cluster, potentially encoding a novel polyketide.

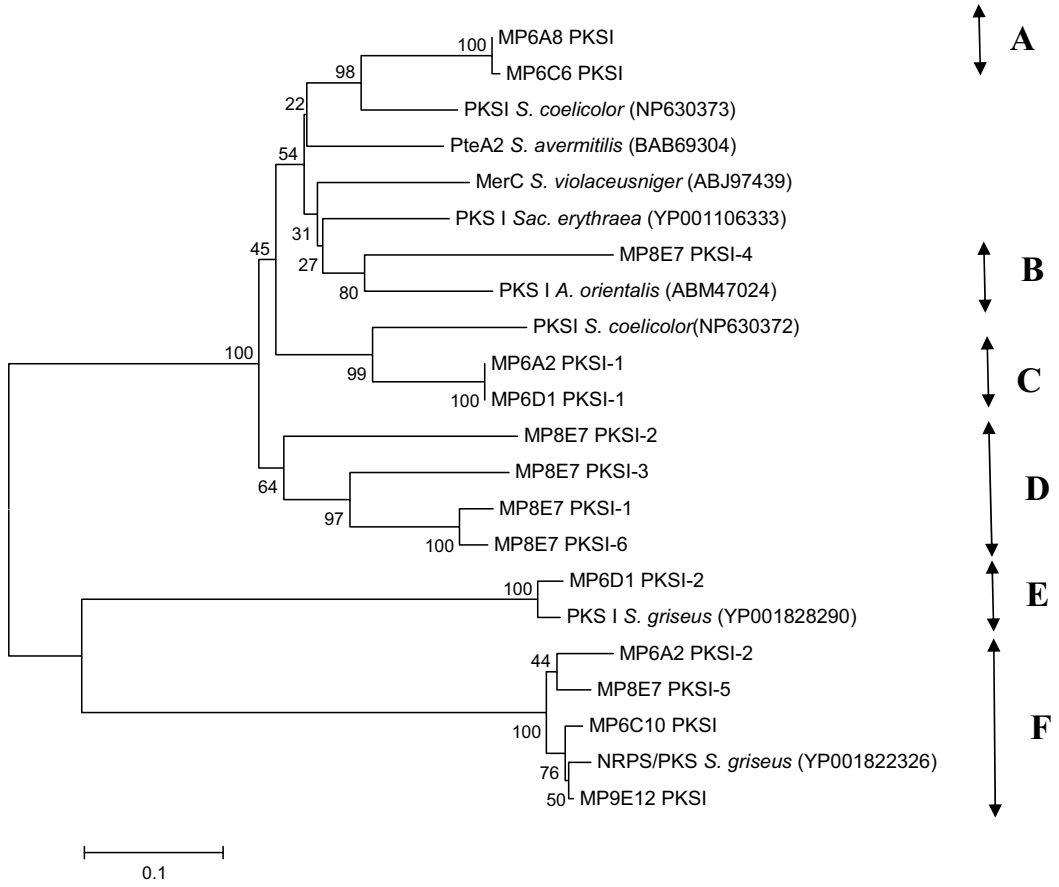


Figure 3.2. Phylogenetic relationship between PKS type I amino acid sequences from Streptomyces isolates with identical partial 16S rDNA sequences. Closest matches from the BLAST searches are also included. Putative distinct KS domain types are indicated with letters (A, B, C etc).

Three of the KS sequences shared by two or more isolates were highly similar (91-100 %) indicating that their genes might have been subject to a relatively recent horizontal gene transfer. In two of these (cluster F and C, Figure 3.2) there is no correlation between the observed antimicrobial activity of the isolates and the presence of the different KS types. This incongruity might have been caused by our inability to detect

the compound produced using the inhibition assays employed, or by the lack of PKS clusters expression under the conditions tested.

Analysis of the KS domains clearly shows that, despite sharing identical 16S rDNA sequences, some of the isolates display unique KS types (cluster B, D and E, Figure 3.2). This further supports the view that de-replication can not be done by 16S rDNA analysis alone.

3.3 Antifungal polyene compounds production by streptomycetes isolated from the Trondheim fjord (paper II)

A high number of *Streptomyces* bacteria isolated from different samples in the Trondheim fjord and assayed for antifungal activity were found to produce polyene compounds. Polyene macrolide antibiotics are naturally occurring, antifungal compounds [52].

Extracts from 3708 isolates from the sediments and sea surface microlayer of the Trondheim fjord were assayed, whereof 28 % displayed antifungal activity. Possible polyene production was detected by the UV/VIS spectrophotometry in 63 % of the isolates displaying antifungal activity, and the majority of those (70 %) presented the same unique heptaene-like UV absorbance spectrum. LC-DAD-MS-TOF analysis performed on the latter isolates identified the same three compounds in extracts from 52 of 62 isolates, with accurate masses corresponding to candicidin D and two candicidin analogues. Two potentially new pentaene macrolides were also detected in the extracts from two polyene-producing isolates in the collection.

3.3.1 The candicidin biosynthetic cluster

A 16S rDNA sequence analysis was performed to determine the molecular taxonomy of the morphologically different candicidin producers sampled at different locations. Seven out of eight isolates analyzed had very similar 16S rDNA sequences, and were also similar to that of the candicidin producer *Streptomyces griseus* IMRU 3570 (99.4-100 %).

The presence of the candicidin cluster in two of the isolates was proven by amplification and sequencing of gene fragments from the *can* biosynthetic cluster. Primers were made based on the DNA sequences of *can*RA (ABC-transporter), *pab*AB (*p*-aminobenzoic acid synthetase) and *can*P3 (polyketide synthase) from *S. griseus*. Sequencing of the partial *pab*AB and *can*P3 amplified from the two isolates demonstrated their 99 % identity to the sequences from *S. griseus*.

Southern blot analysis with the *pab*AB and *can*P3 gene fragments as probes shown hybridization (for both probes) to the total DNA from *S. griseus*. No hybridization with the *pab*AB was detected to total DNA from a candicidin non-producer and an isolate producing another polyene macrolide, indicating that this probe is specific for the candicidin gene cluster. The *can*P3 probe was less specific, hybridizing to the PKS-cluster of the isolate producing polyene other than candicidin. Both probes hybridized to total-DNA from the eight candicidin producers, confirming that candicidin biosynthetic genes are present in all of them.

Seven candicidin producers with similar 16S rDNA sequences showed hybridization patterns with the *pab*AB probe similar to that of *S. griseus*, indicating that the region containing this fragment is conserved among these isolates. The isolate showing a more distinct 16S rDNA sequence did also have a different hybridization pattern, suggesting a different organization of the *pab*AB region in this strain.

3.3.2 The candicidin biosynthetic genes are located on a linear plasmid in one of the isolates

Due to the close phylogenetic relationship between seven of the eight candicidin producing isolates it seemed possible that the *can* genes might have originated from a common ancestor or that they might have been transferred by a mobile genetic element among the isolates. The *can* gene cluster spans ca 140 kb, and due to its large size a giant linear plasmid could be a possible vehicle for transferring it.

Linear plasmids were identified in six out of the eight candicidin producers analyzed by pulsed-field gel electrophoresis (PFGE) of their total DNA. The plasmids ranged from 50 to 820 kb in size. No plasmid could be detected in the candicidin non-producing strain, the isolate producing another polyene, or *S. griseus* IMRU 3570.
Only the 250-kb plasmid from one of the isolates hybridized to both the *pab*AB- and *can*P3-probe, whereas the other candicidin producers demonstrated hybridization to the chromosome, indicating chromosomal location of the *can* genes, or integration of the plasmid into the chromosome. Integration of giant linear plasmids into the chromosome has earlier been reported [49, 64].

3.3.3 Transfer of the *can* cluster-containing plasmid

In order to demonstrate the transfer of the plasmid carrying *can* genes, mating experiments were performed with both *S. lividans* TK64 (pSET152) and a closely related non-producing strain (identical 16S rDNA) as recipients, but plasmid transfer could not be demonstrated. Attempts were therefore made to “cure” the *can*-plasmid containing strain.

Incubating the strain at elevated temperature (37 °C) resulted in the loss of the plasmid, as earlier reported for *Streptomyces* [99]. A Rif^R mutant of the plasmid-free strain was used as a recipient in a new mating experiment, and the mating resulted in

restoration of candicidin production in the recipient. The result was confirmed by hybridization of the total DNA to the *pab*AB probe and candicidin accurate mass determination by LC-MS-TOF. It is assumed that the inability to demonstrate the transfer of the *can*-plasmid to other strains might be due to plasmid instability, or that the recipient stains are unable to produce candicidin and are therefore not recognized during the selection.

3.4 A putative novel antibacterial compound produced by a *Streptomyces* sp. isolated from the water surface microlayer (unpublished results)

3.4.1 Identification of the bacteria

Two *Streptomyces* bacteria were isolated from the sea surface microlayer at two sampling sites in the Trondheim fjord, Norway. Cultivating the isolates on seawater-containing medium greatly enhanced their growth, giving rise to colonies with red substrate mycelium and gray aerial mycelium. The surface microlayer held the temperatures 4.3 °C and 5.8 °C at the time of sampling, but the growth optimum for the isolates, MPS05-B41 and MPS06-B66, was found to be between 20 and 25 °C, as for most of the other streptomycetes isolated from the Trondheim fjord. Incubation at 30 °C inhibited growth of the isolates when cultivated on solid medium.

3.4.2 Detection of antimicrobial activity

Antimicrobial assays were performed both on solid media and in liquid media. In solid medium-based assays, activity against *M. luteus* and *E. coli*, but not *C. albicans* could be detected. Lack of antifungal activity against *C. albicans* was confirmed in liquid medium-based assays, but week activity against *C. glabrata* could be detected. LC-DAD analysis of the extracts showed them being quite complex, and the demonstrated antifungal activity might be caused by another component in the extracts than the

compound(s) exhibiting the antibacterial activity. Activity against Gram-positive bacteria was confirmed in liquid medium-based assays by showing activity against a vancomycin resistant *Enterococcus faecium*. Liquid cultures of MPS05-B41 and MPS06-B66 were pelleted before extraction, and both pellet and supernatant were subjected to analysis. Antimicrobial activity could be found both in pellet and supernatant extracts, indicating that the active compound(s) are secreted into the growth media and suggesting that it might be water-soluble. The inhibiting activity was found to be higher in extracts of the supernatant than in the pellet extracts. The active compound(s) was shown to be poorly soluble in methanol, and attempts to extract with 1-butanol gave extracts with no activity at all. Extraction was therefore performed with methanol and 1-butanol prior to extraction with DMSO, in order to get rid of most of the other metabolites in the extracts.

3.4.3 Identification of antimicrobial compound

Fractionation of bacterial extracts from both MPS05-B41 and MPS06-B66 followed by the bioassay of the fractions were performed. The red pigment was found in fraction 12, whereas the bioactivity was found in two neighbouring fractions 13 and 14, showing that the pigment and the bioactivity are not linked. Fractions 13 and 14 were further subjected to LC-MS-TOF analyses and the ion mass of the compound in the bioactive fraction of bacterial extracts of MPS05-B41 was determined with a deviation of 0.15 ppm between two subsequent rounds of analysis. Corresponding masses were also found in the MPS06-B66 extracts, and the active compound(s) is therefore thought to be identical. Search in the Dictionary of Natural Product (DNP) database with this accurate mass indicated one possible hit. However, the difference between the measured ion mass of the bioactive compound and the ion mass for the compound from DNP was 11 ppm. Considering the minimal deviation between the two rounds of analysis, this indicates that the active compound might be novel. In addition, the DNP hit compound has reportedly better UV-absorbance than measured for MPS05-B41-derived antibacterial, is not reported to have antibacterial activity, and is soluble in

methanol and poorly soluble in H_2O. These characteristics do not correlate with the data obtained for the antibacterial compound in MPS05-B41 and further support the assumption that the active compound produced by MPS05-B51 might be a different, presumably new, compound.

A genome cosmid library of *Streptomyces* TF MPS05-B41 has been constructed with the aim of identification of genes involved in the biosynthesis of this presumably novel antibacterial compound.

3.5 A violacein producing *Collimonas sp*. and its secondary metabolite producing potential *(*paper III)

3.5.1 Isolation, identification and detection of antimicrobial activity in *Collimonas* CT

A new strain belonging to the genus *Collimonas* was identified within the isolate collection. Other *Collimonas* spp. have earlier been isolated from terrestrial sources [35, 58], stream water, and submarine ikaite coloumns in Finland and Greenland, respectively [92, 116]. The *Collimonas* CT (Coast of Trøndelag) displayed highest growth rates at 20-25 °C. A blue pigment synthesized by the bacteria was not produced when bacteria was incubated at 30 °C. No growth could be observed when the bacterium was incubated at 37 °C. These observations are in accordance with other reports on *Collimonas* bacteria [35, 92]

Collimonas CT isolates grew slower or displayed no growth on media containing seawater (50 %), indicating that they might be of terrestrial origin, and have been washed off shore. Antagonistic activity only against *Micrococcus luteus* could be detected in bacterial extracts when assayed against *M. luteus, Candida albicans*,

Escherichia coli, and *Enterococcus faecium*. The antibacterial activity of violacein against *E. coli* is reported to be low, even at high concentrations [95, 34].

3.5.2 Identification and characterization of antimicrobial compound and pigment

Extracts from *Collimonas* CT showing antibacterial activity were analyzed by LC-MS. The UV (DAD) absorbance plot shown in Figure 3.5 displayed four peaks. The main compound in the sample had an m/z = 342.0882 (retention time 12.5 min), whereas a compound with similar UV profile had an m/z value of 326.0938 (retention time 15.5 min), resulting in the stoichiometric formulas ([M-H]$^-$ ion) of $C_{20}H_{12}N_3O_3$ and $C_{20}H_{12}N_3O_2$. Based on the MS-analysis, colour of the substrate and the UV-profile similar to that of violacein, the two main compounds were assumed to be violacein ($C_{20}H_{12}N_3O_3$) and deoxyviolacein ($C_{20}H_{12}N_3O_2$).

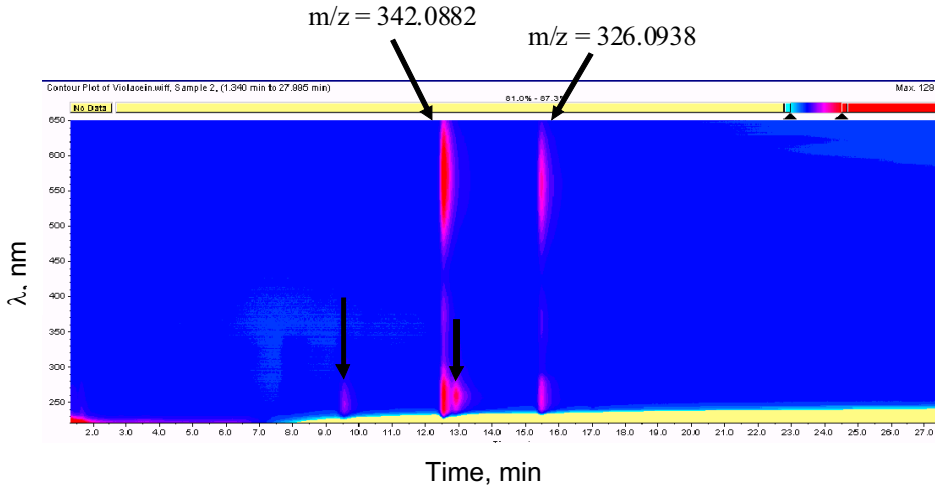


Figure 3.5. Diode array UV absorbance isoplot of *Collimonas* CT extract. The four main eluting peaks have retention times of 9.5, 12.5, 13 and 15.5 min, respectively, and are marked with arrows. Exact masses m/z (M-H$^-$ ion) of the putative violacein and deoxyviolacein are given.

Colourless colonies of *Collimonas* CT were at one point observed in restreaks and when incubated at high temperatures (30 ˚C) as reported for other violacein producers [31, 137]. It has been suggested that this phenomenon might be due to interclonal polymorphism [77]. pH and agitation are also known to affect violacein production [137]. No antimicrobial activity could be detected in extracts from these colonies, and the UV-profile confirmed that no violacein was present in the samples. In support to these findings, antimicrobial assays performed with fractionated bacterial extracts revealed that the antibacterial activity was found in the same fractions as the blue pigment. These results indicate that pigment production is not essential for growth, and that the colour and antimicrobial activity are linked. Violacein is also not essential for growth in other violacein producers [123]. The reported antibacterial activity of violacein is mostly against Gram-positive bacteria, which corresponds with the observed activity against *M. luteus*. These findings confirm that the observed antibacterial activity of the *Collimonas* CT isolates most probably is not caused by several compounds, and that the main bioactive compound is violacein.

Violacein has earlier been found in bacteria isolated from marine environment. As the *Collimonas* CT displays inhibited growth on seawater containing media, this might suggest that they are growing as biofilm in the tidal zone of brackish water, or in soils/fresh water and had been washed out into the sea. Several of the violacein producers seem to prefer sessile microbial communities as they produce more violacein during growth in biofilm than during planktonic growth [82].

3.5.3 The violacein biosynthetic gene cluster in *Collimonas* CT

In order to assess the ability to synthesize secondary metabolites, draft sequencing of the *Collimonas* CT genome was performed. The 5.8 million base pair genome was assembled to 257 contigs. As expected, the genes constituting the violacein biosynthetic cluster could be identified in the draft genome. The genes *vioA, vioB, vioC, vioD and vioE*, are arranged in a cluster spanning 7.3 kb. Among the

known violacein producers, 16S rDNA from *Collimonas* CT shows highest similarity to *Janthinobacterium lividum* (94 %), and *Chromobacterium violaceum* (88 %). This is also the case with the VioA/B/C/D/E proteins; displaying ~81 and ~64 % identity to the corresponding proteins from the two strains, respectively.

The flanking sequences of the violacein cluster *Ch. violaceum* ATCC12472, *J. lividum* DSM 1522 and *Pseudoalteromonas tunicata* D2 were compared to those of *Collimonas* CT. 42, 43 and 27 kb long sequences (including the *vio*-clusters) from the violacein producers were studied. No direct resemblance to the predicted proteins from *Collimonas* CT could be seen in the flanking sequences of *Ch. violaceum*.

Downstream the *vio*-cluster in *Collimonas* CT an ABC-transporter system (ORF10-13) can be found. Genes involved in drug transport are also encoded in the flanking sequences of *J. lividum* DSM 1522 and *P. tunicata*. As the violacein is associated with the outer membrane and accumulates in the periplasma in *P. tunicata*, it is assumed that it functions as a defence mechanism against predators when growing in a biofilm [82, 129]. It has been speculated that the MATE pump encoded by *P. tunicata* might provide a mechanism for export of violacein [129]. Both *J. lividum* and *Collimonas* CT encode a TonB-dependent receptor upstream the violacein cluster. A role for the TonB-dependent receptor in the biosynthesis of violacein in *J. lividum* has not been determined.

The *vio*-cluster flanking sequences in the violacein producers differ from each other. The flanks in *Collimonas* CT shows highest similarity to the flanks in *J. lividum,* which is the violacein producer most closely related to *Collimonas* CT based on 16S rDNA-sequence analysis. The codon usage in the *Collimonas* CT *vio*-cluster and the rest of the genome (predicted) are similar, indicating that violacein biosynthesis is an old characteristic of *Collimonas* CT bacteria.

3.5.4 Putative other secondary metabolite biosynthesis genes in *Collimonas* CT

Searching the *Collimonas* CT genome revealed (in addition to violacein) genes and gene clusters possibly involved in the production of secondary metabolites, as summarized in table 3.1 (Table 5, paper III).

Table 3.1. Gene clusters identified in the *Collimonas* CT genome, putatively involved in the production of secondary metabolites.

Cluster no.	ORFs no.	Size (Kb)	Genes identified (putative)	Putative function
1	04929-04931	3.1	Phytoene synthases and dehydrogenase	Production of lycopene/ carotenoid biosynthesis
2	05750-05752	4.8	Cyanophycin synthetase	Cyanophycin synthesis
3	05144-05156	26.6	NRPS (siderophore), and transport	Siderophore biosynthesis and transport
4	03557	4.0	NRPS, adenylation domain	Unknown
5	03015-03020	25.3	Acetyltransferase, NRPS (18.8 kb), tailoring enzymes, thioesterase and a transcription regulator	
6	05502-05505	12.5*	Partial NRPS* (10 kb) related to arthrofactin/ syringomycin synthethase C module and a thioesterase superfamily protein	Syringomycin, syringopeptin and arthrofactin -related peptide biosynthesis
7	05573+05575	3.7*	Partial NRPS* related to arthofactin/syringomycin synthethase C module	
8	01844	2.2*	Partial NRPS*, syringopeptin synthetase C related	
9	02224-02232	7.1	Minimal PKS type II, modifying and post-PKS modifying enzymes	Aromatic polyketide biosynthesis
10	03611-03629	16.8	Minimal PKS type II, modifying and post-PKS modifying enzymes	Aromatic polyketide biosynthesis

* Encoding partial genes, due to end of sequencing contigs.

Cluster number 1 is putatively involved in carotenoid biosynthesis. The genes encode putative phytoene synthase and putative squalene/phytoene dehydrogenase, enabling the production of lycopene [103]. The reaction catalyzed by the phytoen synthase is considered the first reaction unique to carotenoid biosynthesis [4].

Two genes encoding putative cyanophycin synthetase was identified in gene cluster number 2. Cyanophycin (multi-L-arginyl-poly-L-aspartic acid), accumulates in the cytoplasma in cyanobacteria, and is thought to function as a nitrogen reserve [104]. Production of a water-soluble polymer, similar to cyanophycin in amino acid composition and chemical structure has been reported for non-cyanobacterial eubacteria [144]. These polymers are produced from strains harbouring genes with considerable homology to *cph*A, encoding cyanophycin synthetase. Cyanophycin is of potential industrial interest as a source of (poly) aspartic acid.

A gene cluster (number 3) putatively involved in the biosynthesis of a siderophore was identified. The cluster contained genes involved in the transport of siderophore/iron (Fe^{3+}) compounds and a putatively siderophore-related NRPS followed by a monooxygenase and a TonB-dependent siderophore receptor. Another NRPS (cluster number 4) putatively encode an amino acid adenylation domain, but a potential product is not known.

Four clusters (number 5 to 8) harboured ORFs encoding non-ribosomal peptide synthases (NRPS) putatively related to biosynthesis of arthrofactin/ syringomycin. Syringopeptins and syringomycin are related phytotoxic lipodepsipeptides. Three of the ORFs only encode partial NRPS genes due to the gaps in the genome sequence. Two ORFs situated at the ends of their contigs are probably associated with each other. They both encode partial genes putatively related to arthrofactin/syringomycin synthethase C module. In the case of *Pseudomonas syringae pv. syringae* it is thought that the gene clusters for syringomycin and syringopeptin form genetic islands [117]. One might therefore speculate that the clusters identified in *Collimonas* CT might be

linked. The clusters are most likely involved in the production of a peptide related to syringomycin, syringopeptin and arthrofactin.

Two putative aromatic polyketide biosynthesis gene clusters are encoded in the *Collimonas* CT genome (clusters number 9 and 10). The clusters could not be associated with the production of any known natural products. Bacterial aromatic polyketides are synthesized by PKS type II (iterative) polyketide synthases. A minimal PKS type II is encoded in cluster number 9, followed by genes encoding proteins for modification and post-PKS modification of the polyketide chain. However, both ketosynthases contain conserved domain of initiating KAS III.

A minimal PKS type II was also identified for the second putative aromatic PKS cluster. A putative 4'-phosphopantetheinyltransferase is thought to modify and activate the acyl carrier proteins. Several genes are found within the minimal PKS, including genes involved in modification and post-PKS modification of the polyketide chain. Despite not being common in type II PKS systems, a putative thioesterase superfamily protein was identified. It has been suggested that they are involved in chain release, or function as an esterase for the hydrolysis of the ester intermediates [98].

Even though genes for several secondary metabolites with potential antimicrobial activity could be found in the genome, only activity against *M. luteus* could be detected in the inhibition assays. This activity is thought to be associated with the production of violacein. Production of the other secondary metabolites might not be induced under the conditions tested, or their activity is not detected in the assays employed. However, the genome analysis demonstrates that *Collimonas* CT might have an industrial potential for production of diverse secondary metabolites.

4 CONCLUSIONS

The sea surface microlayer in the Trondheim fjord is rich in streptomycetes with antimicrobial activity. 16S rDNA analyses indicated that several of these isolates are closely related. Sequencing and phylogenetic analysis of PSK type I fragments suggested that horizontal gene transfer between closely related species might have taken place. Identification of unique PKS genes in assumed identical isolates (based on partial 16S rDNA sequences) implies that de-replication can not be performed based solely on the 16S rDNA sequences.

Production of the antifungal polyene macrolide candicidin is widely distributed among *Streptomyces* bacteria isolated from/inhabiting different environmental niches in the Trondheim fjord. It was shown that the genes involved in the biosynthesis of candicidin are present on a linear plasmid in one of the strains. Reintroduction of the genes after curing the strain of the plasmid restored candicidin production, indicating that the plasmid might be responsible for the spreading of the candicidin biosynthetic gene cluster in the marine environment.

Two *Streptomyces* isolates displaying antibacterial activity against a vancomycin-resistant *Enterococcus sp*. have been identified. LCMS-TOF-analysis of the bioactive fraction of the bacterial extracts revealed that the compound might be a novel antibacterial antibiotic. Construction of a genome cosmid library of this isolate shall allow identification, cloning and manipulation of the genes involved in the biosynthesis of this potentially novel compound.

A new strain of the genus *Collimonas* was isolated from the sea surface microlayer. The strain produced the blue pigmented compound violacein. Genome scanning identified several genes for the production of secondary metabolites of potential

industrial interest, indicating that Gram-negative bacteria should not be excluded from isolate collections when screening for bioactive secondary metabolites.

The results obtained in this study clearly suggest that bacteria inhabiting the sea surface layer along the coast of Trøndelag might be a considerable resource of new bioactive secondary metabolites. With only 1-5 % of the marine bacteria so far being cultivable and only 10 % of the chemical potential of the already cultivated bacteria revealed, it is clear that a large amount of natural products still remain to be discovered. Currently, the technical issues are limiting the efficient exploration/exploitation of the marine microbial resources. The main challenges will be defining new ways of scanning the environment and the genomes of the microbes living there, in addition to designing assays optimized for the detection of a wide range of biologically active compounds, and activation of the gene clusters involved in their production.

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Article

Characterization of *Streptomyces* spp. Isolated from the Sea Surface Microlayer in the Trondheim Fjord, Norway

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Abstract: The water surface microlayer is still poorly explored, although it has been shown to contain a high density of metabolically active bacteria, often called bacterioneuston. Actinomycetes from the surface microlayer in the Trondheim fjord, Norway, have been isolated and characterized. A total of 217 isolates from two separate samples morphologically resembling the genus *Streptomyces* have been further investigated in this study. Antimicrobial assays showed that about 80% of the isolates exhibited antagonistic activity against non-filamentous fungus, Gram-negative, and Gram-positive bacteria. Based on the macroscopic analyses and inhibition patterns from the antimicrobial assays, the sub-grouping of isolates was performed. Partial 16S rDNAs from the candidates from each subgroup were sequenced and phylogenetic analysis performed. 7 isolates with identical 16S rDNA sequences were further studied for the presence of PKS type I genes. Sequencing and phylogenetic analysis of the PKS gene fragments revealed that horizontal gene transfer between closely related species might have taken place. Identification of unique PKS genes in these isolates implies that de-replication can not be performed based solely on the 16S rDNA sequences. The results obtained in this study suggest that streptomycetes from the neuston population may be an interesting source for discovery of new antimicrobial agents.

Keywords: Sea surface microlayer, streptomycetes, antimicrobial activity, phylogenetic analysis.

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1. Introduction

Search for new biologically active microbial secondary metabolites is important in order to meet the increasing demand for new antibiotics. Actinomycetes, especially those belonging to the genus *Streptomyces*, are known to produce a wide variety of biologically active compounds. *Streptomyces* bacteria were reported to produce ~70% of the currently characterized actinomycete natural products [1]. However, most of the *Streptomyces* characterized up to date were isolated from the terrestrial environment, while those originating from marine sources still remain poorly explored.

In an environment with high densities of metabolically active bacteria, competition is likely to be fierce, and properties such as production of antibiotics may give organisms an advantage. A number of antibiotic producers have been isolated from the marine environments [2, 3], and experimental data indicate that production of antibiotics could play an important role in the competitive relationship within the marine bacterial populations [4]. Antagonistic interactions among soil-living microorganisms are well documented, and are attributed to the production of antibiotics by certain bacteria and fungi in environments rich in organic material [5, 6]. Recently, the same trend has been discovered for marine microorganisms, which are abundant in mesotrophic and eutrophic waters or during phytoplankton blooms [7].

In a study of antagonistic interactions among marine pelagic bacteria it was found that more than half of the isolates expressed antagonistic activity, and this trait was more common among particle-associated (66%) than free-living bacteria (40%) [8]. Particles often tend to accumulate at the sea surface, and the aquatic surface layer contains a series of sub layers [9]. Neuston is a collective name for the life forms in the surface layer of oceans and lakes, and can be divided into epineuston and hyponeuston. Epineuston organisms live on the top of the water surface, and are naturally dependent on the surface tension of the water. Hyponeuston organisms live in the top few centimetres of the water column. High densities of metabolically active bacteria, often called bacterioneuston, are found in the surface microlayer [10-13].

Norwegian marine ecosystems have developed in a rather cold and severe climate, suggesting that the selective pressure on microorganisms comprising parts of such systems must have been quite unique (cold seawater environment). Because of this, it seems likely that these microorganisms have developed antibiotic biosynthesis pathways that differ from those utilized by terrestrial microorganisms. Even though the diversity of microorganisms in the marine environment is high, only a minor fraction (less than 1 %) can be cultivated in the laboratory, presumably because of failure to mimic the natural growth conditions [14]. In this work we isolated *Streptomyces* bacteria from the surface microlayer in the Trondheim fjord (Norway). The isolates were characterized using molecular taxonomy, assays for antimicrobial activity and presence of polyketide synthase genes.

2. Results and Discussion

2.1. A large proportion of cultivable neuston actinomycetes produce antimicrobial compounds

Bacteria morphologically similar to streptomycetes were isolated from surface microlayer collected at Steinvikholmen (a small islet) and in the Åsen fjord in the Trondheim fjord, Norway. The water

temperatures during sampling were 4.3 and 5.8 °C, respectively, and the air temperature was 3 °C in both cases. Water was sampled from two sites, both to increase the number of isolates and to possibly detect any spatial variations in diversity. Collecting water samples close to the shore increases the risk of cultivating terrestrial bacteria that have been washed into the sea. Initial isolation of the bacteria was therefore performed on agar media with 50 % seawater to increase the chance of isolating bacteria adapted to the marine environment.

Total numbers of bacteria isolated on Actinomycete isolation seawater agar with cycloheximide and nalidixic acid added to inhibit the growth of fungi and Gram-negative bacteria, were $2.5x10^3$ and $1.2x10^4$ cells/ml seawater from the two sites, respectively. Presumed actinomycetes (based on colony morphology) accounted for $9.8x10^2$ and $1.3x10^3$ cells/ml, respectively. From these, a total of 217 colonies from samples 1 and 2 represented by 134 and 83 isolates, respectively, were selected for further analyses.

Previously, it has been reported that bacteria isolated from the surface microlayer at coastal stations in the north-western Mediterranean Sea, contained an average of Gram-positive cultivable bacteria ranging from $2.3x10^3$ (France) to $3.0x10^4$ (Spain) ml^{-1} [15], indicating that the cell number can vary considerably depending on the sampling site. Based on these reports, the total number of isolates in the samples collected in this study is assumed to reflect at least some of the diversity in the Trondheim fjord.

Based on the colony morphology (colour of substrate and aerial mycelia, pigment production), the isolates could be divided into 10 groups, shown in Table 1.

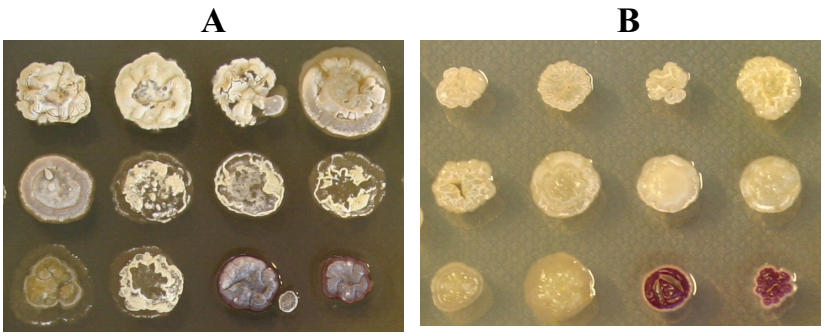
Table 1. Characteristics of the different isolate groups, when grown on ½ISP2 medium with 50% seawater for up to 14 days. SM = substrate mycelium, AM = aerial mycelium

Group	**Characteristics**		
	SM	AM	Other
1	Colourless	White	
2	Colourless	White	Produces yellow metabolite diffusing in solid media
3	Colourless	Greenish-white	
4	Colourless	Greenish-white	Produces yellow metabolite diffusing in solid media
5	Light brown	Light grey	
6	Brown /greenish	Grey	
7	Colourless /light brown	Light purple	
8	Red	None	
9	Red	White	
10	Yellow	None	Flaky

Cultivation of the isolates on agar medium with and without seawater, showed that they all grew better/faster on media with 50 % seawater added, as exemplified on Figure 1. Actinomycetes isolated from marine sediments have earlier been analyzed for their seawater requirement for growth [16]. The detected requirement has been interpreted as indication of marine origin or marine adaptation. None of the isolates in this study demonstrated inhibited growth on salt-containing media, suggesting that all

isolates are marine bacteria or terrestrial bacteria adapted to the marine environment, and presumably occur naturally in the surface microlayer.

Figure 1. Growth of isolated actinomycetes after 7 days of incubation at 30 ˚C on ½ ISP2 agar with (A) and without (B) 50% seawater.



In order to explore the potential of the isolates to produce antimicrobial compounds, extracts from the colonies grown on three different solid agar media were tested in microbial inhibition assays. After an appropriate incubation time (depending on the growth rate), the plates with cells were dried, and extracted with DMSO. The extracts were tested in agar diffusion assays for antimicrobial activity. The initial assays were performed with *Micrococcus luteus* ATCC 9341*, Candida albicans* ATCC 10231 and *Escherichia coli* K12 as indicator organisms. The antimicrobial activity, presented in Table 2, is the total combined activity displayed by the isolates when grown on any of the 3 agar media. As expected, a high share of the isolates exhibited antimicrobial activity. In particular, 79% of the sample 1 isolates, and 85% of the sample 2 isolates showed antagonistic activity against at least one of the indicator organisms. Several of the isolates showing antimicrobial activity were active against more than one indicator organism, as shown in Table 2. This was particularly evident for the isolates with antibacterial activity, where around 80% of the isolates inhibiting Gram-negative bacteria also inhibited Gram-positive bacteria, and vice versa. Table 2 shows how the different inhibition patterns are distributed among the isolates from different morphological groups. The fact that some isolates displayed activity against more than one indicator organism may indicate production of several antimicrobial compounds and/or production of compounds with multiple microbial targets.

The percentage of neuston actinomycete isolates displaying antimicrobial activity was found to be considerably higher than those reported previously. In the earlier studies, about 50% of isolated marine pelagic bacteria exhibited antagonistic properties against other pelagic bacteria [8], and only 44 % of streptomycetes from the marine sediments have shown antibacterial activity [17]. In the latter study, 17% of the isolates displayed antifungal activity. A noticeably lower degree of antifungal compared to antibacterial activity among *Streptomyces* species isolated from marine sediments has also been reported by [18]. In our study, about 40% of the assumed (based on morphology and inhibition patterns) non-identical isolates from both sample 1 and 2 showed antifungal activities.

Table 2. Total number of streptomycete-like isolates from bacterioneuston, sample 1 and 2, grouped and sub-grouped according to antimicrobial activity and colony morphology. DMSO-extracts from all strains were tested for activity against *C. albicans* (C), *M. luteus* (M) and *E. coli* (E). Samples 1 and 2 contain 134 and 83 isolates, respectively S1 and S2 indicate sample 1 and sample 2, and G1-G10 indicate morphology groups 1- 10, (see Table 1). The percentages (S1 and S2 combined) of antifungal, antibacterial and no activity in each of the groups, G1-G10, are also given.

				Group (G1-10) and sample number (S1, S2)																			
Inhibition				G1		G2		G3		G4		G5		G6		G7		G8		G9		G10		
Nr	C	M	E	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	Total
1	x	x	x	6	7	0	1	5	0	2	0	1	0	0	0	0	0	1	4	0	2	0	0	29
2	x	x		6	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	8
3	x		x	9	4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	14
4	x			28	14	4	0	0	2	0	0	4	3	3	3	0	0	0	1	0	0	0	0	62
5		x	x	4	7	1	3	2	2	7	2	1	0	0	1	0	0	2	8	3	1	1	0	45
6			x	5	2	0	0	0	1	0	0	0	0	0	0	2	0	0	0	0	0	1	0	11
7		x		2	0	0	0	0	0	4	0	1	1	0	0	0	0	0	0	0	0	0	0	8
8				12	7	2	0	1	0	0	0	5	4	7	2	0	0	0	0	0	0	0	0	40
Sum, no of isolates				72	42	7	4	8	5	13	2	13	8	11	6	2	0	3	13	3	3	2	0	217
Sum, no of isolates				114		11		13		15		21		17		2		16		6		2		
Antifungal (%)				66		45		54		13		43		41		0		38		33		0		
Antibacterial (%)				46		45		77		100		24		12		100		94		100		100		
No activity (%)				17		18		8		0		43		53		0		0		0		0		

The methods chosen for surface sampling and cultivation may facilitate isolation of some types of bacteria over others. This will affect both the quantity and the diversity of the samples. Isolates from group G1 were frequently found in both samples. This may be due both to the fact that the selected growth conditions were best suited for the G1 isolates, and that they were in fact abundant in the surface microlayer. The diversities of streptomycete-like bacteria within the samples 1 and 2, at least as judged from colony morphology and inhibition patterns, were quite similar. This is probably not surprising, considering that the currents in the fjord continuously mix the water, thereby homogenizing the content of bacterioneuston to some extent.

Groups G5 and G6 had the highest share of isolates without any detectable antimicrobial activity under the conditions used. About half of these isolates displayed neither antifungal nor antibacterial activity. In both groups, one third of the isolates showed antifungal activity. Similarity in inhibition patterns was also noticeable between the G8 and G9 isolates. In these two groups, all isolates exhibited antimicrobial activity, whereof two thirds showed activity against both *M. luteus* and *E. coli*, and the rest also had activity against *C. albicans*. In addition G3 and G4 isolates showed a high degree of antibacterial activity, 77 % and 100 %, respectively. In total these results display a weak connection between morphology and antimicrobial activity to some extent.

Analysis of the 16S rDNA from the isolated bacteria reveals discrepancy between phenotypic grouping and the molecular taxonomy.

In order to reveal the diversity among isolated actinomycetes, a limited analysis of 1351 nt 16S rDNA gene fragments was performed. In total, 16S rDNA fragments from 46 isolates representing

different groups distinguishable by morphology and inhibition patterns were amplified and sequenced. Alignment of the sequences showed a relatively high degree of homology within the candidate collection, suggesting replication of some isolates. However, several of the isolates representing potential replicates based on the 16S rDNA sequence, displayed unique inhibition patterns, indicating that they are not identical.

BLAST searches for the obtained sequences showed that the 16S rDNAs from all except 4 isolates had at least 99 % identity to sequences from *Streptomyces* spp isolated from marine sediments and sponges [19-22]. A widespread distribution of these bacteria in marine environments is consistent with the fact that they thrive on the salt-containing media.

A phylogenetic analysis of the partial 16S rDNA sequences, displayed in Figure 2, was performed to reveal the taxonomic relationship between the different subgroups. In cases where several isolates had identical sequences, only one sequence was included in the analysis, without regard to differences in the morphology and inhibition patterns. As noted above, at least 10 morphologically different groups could be distinguished among the isolates. No clustering of these groups was observed in the phylogenetic analysis. Only minor grouping of isolates sharing the same inhibition patterns could be found. However, some clustering of isolates displaying either antifungal or antibacterial activity could be identified. In several cases, the "closest match" strains were reported to have antimicrobial activity that may be interesting from a commercial point of view.

Sequence for the isolate MP7A10 represents a group of six isolates, whereof five appear to have the same morphology (group G6). These isolates display different inhibition patterns, strongly suggesting that 16S rDNA gene sequences alone can not be used for dereplication of isolates.

Sequence for the isolate MP6A8 represents ten isolates with varying morphology (group G1-G5 and G10), of which eight were shown to have antibacterial activity. Including the remaining isolates in branch 1, a total of 17 out of 23 isolates in this branch displayed antibacterial activity, of which 15 were active against Gram–positive, and 2 against Gram-negative bacteria.

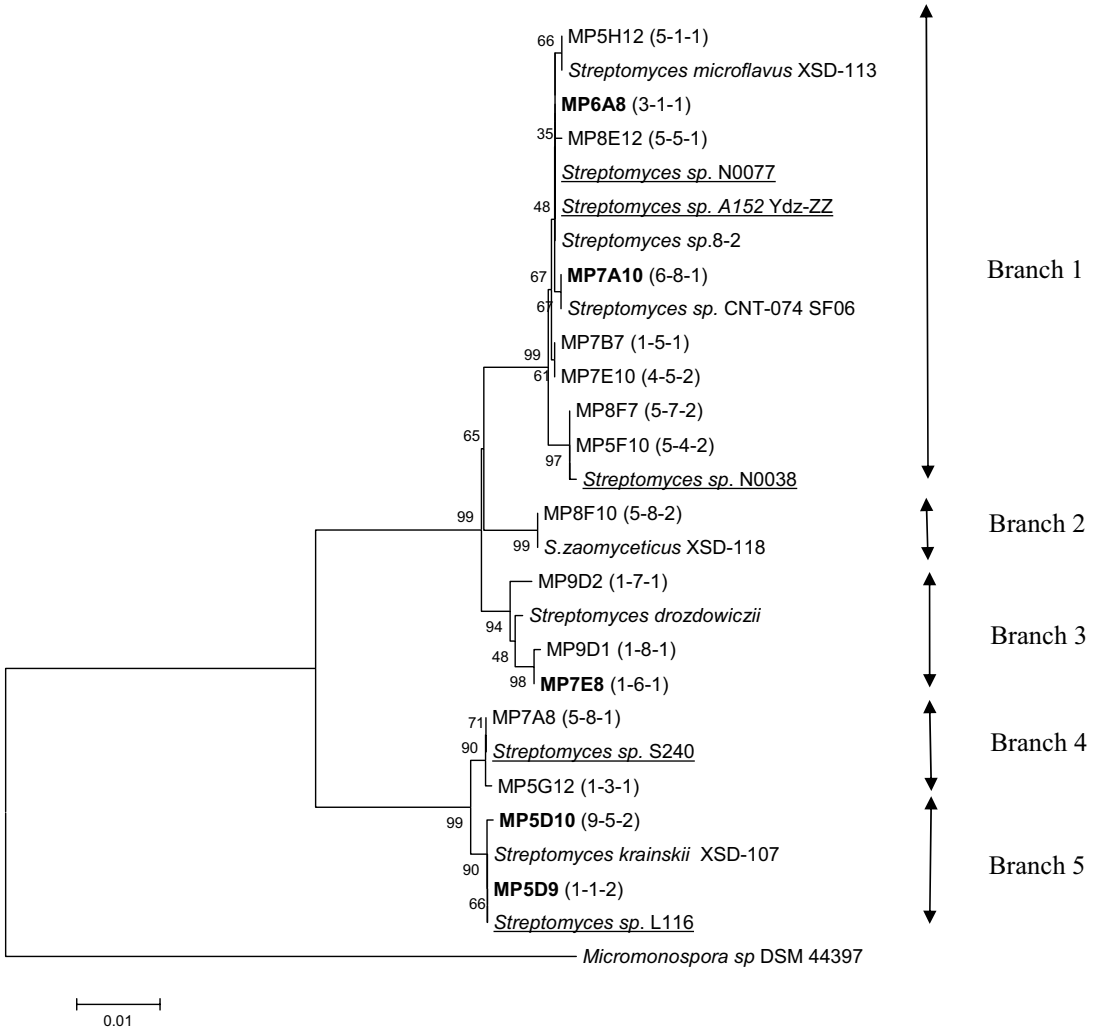
Three out of four isolates in branch 3 displayed antibacterial activity. The deviating isolate did not show any antimicrobial activity under the conditions tested. Sequence for isolate MP7E8 represents an additional isolate with the same inhibition pattern. Branch 4 and 5 consisted of isolates displaying both varying morphology and inhibition patterns. Isolate MP5D9 represents a group of 13 isolates whereof 8 displayed antifungal activity. There was a considerable variation in morphology among these isolates.

Several of the isolates with antibacterial activity (MP8F7, MP7E10, MP7B7, MP7E8 and MP9C8) showed 99 and 100% identity to *Streptomyces* species isolated from coastal sediments [19], but no antimicrobial activity has been reported for these species.

The sequence from isolate MP5F10 having antifungal activity showed 99% identity to *Streptomyces olivoviridis* and *Streptomyces sp.* N0028. The cultural appearance of *S. olivoviridis* agrees with that of MP5F10. This strain has been shown to produce a new antitumor antibiotic, thioviridamide, stimulating apoptosis signalling [23].

16S rDNA from the isolate MP9D2, which inhibits growth of Gram-positive bacteria, was 100% identical to that of *S. drozdowiczii* and *Streptomyces sp*. WL-2 (1351 bases). *S. drozdowiczii* NRRL B-24297 was reported to have cellulolytic activity [24], while *Streptomyces sp.* WL-2 produces xylanase [25].

Figure 2. Phylogenetic tree constructed for partial 16S rDNA sequences (1351 bp) of 46 streptomycetes isolated from the surface microlayer in the Trondheim fjord, Norway. The tree also contains some of the closest matches from BLAST searches. The 16S rDNA sequence from *Micromonospora sp* DSM 44397 is included to root the tree. Numbers in brackets (x-y-z) refers to x: morphology group, y: inhibition pattern (see table 2), and z: sample number. Arrows indicate the different branches of the tree. Bold font indicates sequences representing several isolates. Strains of marine origin are underlined.



16S rDNA from the isolate MP8F10 was 99% identical to *S. zaomyceticus* XSD-118. Different *S. zaomyceticus* strains have been shown to produce foroxymithine, narbomycin, picromycin and methymycin. Foroxymithine is an inhibitor of angiotensin-converting enzyme produced by actinomycetes, and may be of interest for medical use [26]. 16S rDNA from the isolate MP5H12 shows 99 % similarity to *S. microflavus* 173958.

2.3. Analysis of PKS gene fragments from selected streptomycetes *isolates suggests horizontal gene transfer between closely related species*

Polyketide synthases (PKSs) and/or non-ribosomal peptide synthetases (NRPSs) or a combination of these, are involved in production of many antimicrobial secondary metabolites in *Streptomycetes* and other bacteria, fungi, and plants. Screening for and analysis of PKS-I, PKS-II and NRPS genes in marine metagenomic libraries as well as soil samples have earlier been reported [27-30]. These analyses have been performed both to elucidate diversity and to pre-screen soil samples for identifying the ones most likely to contain producers of novel bioactive molecules.

Based solely on the 16S rDNA sequence analysis of our isolates, several of them seemed to be very closely related (i.e. had 100 % identical 16S rDNA fragments). At the same time, they showed different inhibition patterns in addition to displaying different morphology (Table 3). This fact prompted us to investigate the presence of PKS type I genes in a selected group of such isolates, which were chosen without considering morphology. In the phylogenetic analysis of 16S rDNA sequences (Figure 2), these isolates are represented by the isolate MP6A8 in branch 1.

Table 3. Names and inhibition patterns of isolates selected for PKS analysis. Activity is shown against *C. albicans* (C), *M. luteus* (M) and *E. coli* (E). Morphology group and sample number are indicated.

Isolate	Morphology group	Sample number	Inhibition		
			C	M	E
MP6A2	G4	S1		x	x
MP6A8	G3	S1	x	x	x
MP6C6	G4	S1		x	
MP6C10	G1	S1	x	x	
MP6D1	G2	S1	x		
MP8E7	G10	S1			x
MP9E12	G1	S2			

Bacterial type I (modular) PKS gene fragments were amplified with the degenerate primers KSMA-F and KSMB-R [31], which can be used to amplify β-ketoacyl synthase (KS) domain encoding fragments of ca 700 bp. PCR with these primers resulted in amplification of fragments of expected size from all isolates, indicating their potential for production of polyketide secondary metabolites. Since PKS type I genes encode modular enzymes, and actinomycete strains usually contain more than one PKS gene cluster [28], it was expected that PCR products obtained with the KS-specific primers would represent mixtures of the KS-coding sequences. Therefore, sequencing of these gene fragments would be required for a better understanding of the diversity within the selected group of isolates and their dereplication.

PKS type I PCR fragments were cloned in *Escherichia coli* vector, and for each isolate 12 clones were sequenced. A total of 13 different sequences were obtained from 7 selected isolates. Six different KS-encoding sequences were amplified from the total DNA of the isolate MP8E7. BLAST search of

the corresponding amino acid sequences revealed that fragments PKSI-1 and PKSI-6 from this isolate encode 95% identical KS domains showing 83% identity to the KS domain of MerC, a PKS involved in biosynthesis of the neuroprotectant meridamycin in *S. violaceusniger* [32]. The amino acid sequences for PKSI-2 and -3 fragments displayed 72% and 71% identity, respectively, to the PKS from *Saccharopolyspora erythraea* (unknown product) [33] and PteA2 PKS responsible for biosynthesis of antifungal polyene macrolide filipin in *S. avermitilis* [34]. Amino acid sequences of the PKSI-1, 2, 3 and -6 products were 68% to 95% identical, and might have been amplified from the same PKS gene cluster. The PKSI-4 and -5 fragments amplified from MP8E7 were quite different from each other and from the rest of the PKS sequences from this isolate. The amino acid sequence for PKSI-4 displayed 72% identity to the PKS from *Amycolatopsis orientalis* involved in the biosynthesis of the antibacterial compound ECO-0501 of a new chemical class [35]. The amino acid sequence for PKSI-5 displayed 96% identity to the PKS part of the NRPS-PKS fusion protein from *Streptomyces griseus* subsp. *griseus* NBRC 13350 (NC_010572.1)

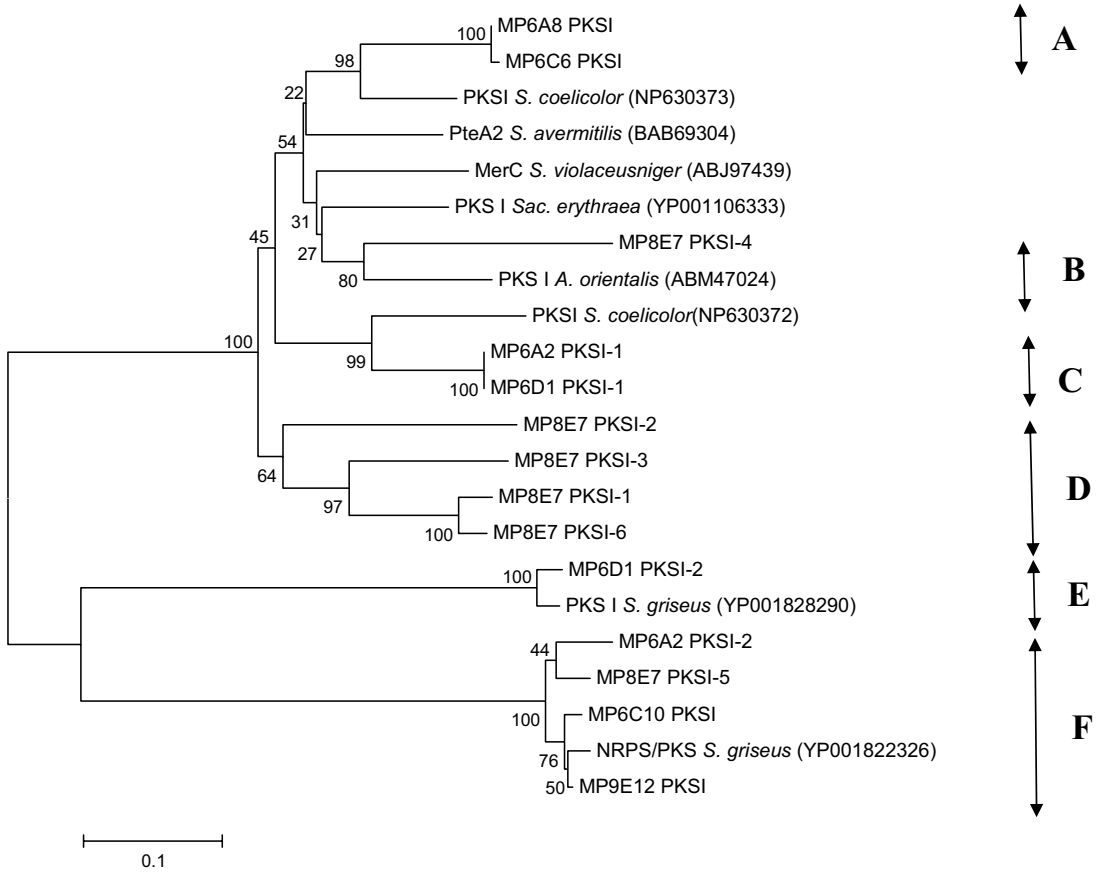
The isolates MP6A2 and MP6D1 yielded 2 PKS sequences each. Both on the nucleotide and amino acid levels, MP6A2 PKSI-1 and MP6D1 PKSI-1 sequences were 100% identical to each other, and shared 83% identity with *S. coelicolor* cryptic PKS type I [36] The identity of the sequences suggests recent horizontal gene transfer between the two isolates, which has involved a PKS gene cluster. Two other PKS sequences amplified from these isolates, MP6A2 PKSI-2 and MP6D1 PKSI-2, encoded KS domains showing 94% and 96% identity, respectively, to the PKS and NRPS-PKS proteins encoded by two different gene clusters in *Streptomyces griseus* subsp. *griseus* NBRC 13350 [37]. Interestingly, the MPS06-A2 PKSI-2 sequence displayed 94% identity to the MP8E7 PKSI-5 sequence, also suggesting a relatively recent transfer of the corresponding gene between MP6A2 and MP8E7 isolates.

Isolates MP6A8, MP6C6, MP6C10 and MP9E12 each yielded one distinct KS domain encoding sequence. MP6A8 and MP6C6 PKSI sequences were 98% identical, suggesting recent horizontal gene transfer, and displayed 83% identity to the PKS type I from the cryptic gene cluster from *S. coelicolor*. The latter gene was different from the one showing closest match to the MP6A2/D1 PKS-1 sequences (see above), although apparently belonged to the same PKS cluster.

Both MP6C10 and MP9E12 PKS sequences were closely related (96% and 98% identity, respectively), to the NRPS-PKS fusion protein from *Streptomyces griseus* subsp. *griseus* NBRC 13350 [37]. They were also very similar to the PKS sequences MP6A2 PKSI-2 and MP8E7 PKSI-5, showing 93% and 96% identity, respectively.

In order to visualize taxonomic relationship between the amino acid sequences encoded by the PCR-amplified fragments, a phylogenetic tree was constructed, which also included sequences from the best matches according to the BLAST search. The architecture of the tree, presented in Figure 3, clearly shows some discrepancy between the BLAST search and the phylogenetic analysis. For example, the PKSI-1,-2,-3 and -6 sequences from the isolate MP8E7 do not cluster with the corresponding best matches from the BLAST search, and form a separate branch on the tree. This suggests that the PKS gene cluster represented by these sequences might have evolved separately, and potentially can encode a novel polyketide metabolite.

Figure 3. Phylogenetic relationship between PKS type I amino acid sequences from streptomycete isolates with identical partial 16S rDNA sequences. Closest matches from the BLAST searches are also included. Putative distinct KS domain types are indicated with letters (A, B, C etc). Numbers at tree nodes represent the number of times the topology to the right of the node was recovered in 1000 bootstrap re-samplings. Accession numbers for the sequences are given in parentheses. Scale bar represents the number of changes per amino acid position.



Both BLAST and phylogenetic analyses suggest that we have been able to identify 6 distinct types of KS domains in 7 closely related (according to the 16S rDNA sequences) isolates. This does not necessarily mean that each type represents a distinct PKS gene cluster. In phylogenetic analysis, the KS domains are known to cluster not only according to their evolutionary relatedness, but also according to their substrate specificity [38]. Interestingly, one of the KS types belonging to the NRPS-PKS fusion protein (F) seems to be shared by at least 4 isolates and its coding DNA might have been subject to a relatively recent horizontal gene transfer. There is, however, no correlation between the presence of this KS type and antimicrobial activity profiles of the four isolates (Table 3). The latter suggests that the corresponding PKS cluster might be either not expressed in the conditions tested, or governs biosynthesis of a compound which is not detectable by the assays employed. The same might

be true for the KS type C represented by the sequences MP6A2 PKSI-1 and MP6D1 PKSI-1, since the corresponding isolates have different inhibition patterns (see Table 3).

The analyses of the KS domains from selected isolates with identical 16S rDNA sequences do not enable solid conclusions about the nature of the compounds that may potentially be produced. However, the fact that unique KS types, such as B, D and E, seem to be isolate-specific, further supports the notion that streptomycete isolates can not be distinguished on the basis of 16S rDNA sequences alone. A more complex approach that includes PCR-based PKS and NRPS genome "scanning", inoculation in a wide range of growth/production media, metabolite profiling and diverse biological assays with fractionated extracts is required to reveal the true potential for production of medically useful secondary metabolites.

3. Experimental Section

3.1. Sampling and isolation of Streptomycete bacteria from sea surface microlayer (Sampling sites and sample collection)

Samples were collected on the 22nd of March 2004 at two sites (63°32,511 N, 010°48,797 E and 63º56,009 N, 010º91,020 E) in the Trondheim fjord, Norway. Steinvikholmen (sample 1) is a small islet situated approximately 200 m from the mainland, whereas the other sampling point was close to shore. The surface microlayer was collected using Teflon plates as earlier described [39]. The plates were immersed in water, gently lifted through the water surface, and the bacterioneuston scraped off using a rubber edge. Both samples were collected early in the morning during low tide, and 2 to 3 meters from the shoreline.

Samples were plated on selective agar plates (2% w/v), within 24 hours after collection, and was incubated at 20 ºC. Three different media was used; ½ ISP2; Malt extract (5 g), yeast extract (2 g), glucose (2 g), natural sea water (0.5 L) and distilled water (0.5 L), Kusters streptomycete isolation medium (modified); Glycerol (10 g), Casein (0.3 g), KNO_3 (2 g), $FeSO_4$*7 H_2O (0.25 mg), H_2SO_4 (0.5 mg), natural sea water (0.5 L) and distilled water (0.5 L) and Actinomycete isolation medium without $MgSO_4$ [40]. The pH of the isolation media was adjusted to pH 8.2. All media contained 50% sea water and was supplied with Cycloheximide (50 µl/ml) and Nalidixic acid (30 µl/ml). Selected isolates were transferred to ½ ISP2 agar to ensure pure colonies, and incubated for 16 days before storing as glycerol stock in micro well plates at -80 °C.

3.2. Extraction and antimicrobial assay

The selected strains were transferred to microwell filter plates (Nunc Silent screen nr 256073, Loprodyne 3.0 µm) with 80 µl of three different 1% agarose media (production media) to facilitate production of secondary metabolites. The production media (PM) were: PM2; Mannitol (20 g), soya bean flour (20 g), Clerol (antifoam, 0.5 g), dry yeast (3.4 g), agarose (10.0 g), tap water (1 L), PM3; Oatmeal (20 g), glycerol (2.5 g), $FeSO_4 \cdot 7H_2O$ (0.1 mg), $MnCl_2 \cdot 4H_2O$ (0.1 mg), $ZnSO_4 \cdot 7H_2O$ (0.01 mg), H_2SO_4 (0.1 mg), agarose (10 g), tap water (1 L), PM4; glucose (0.5 g), glycerol (2.5 g), oatmeal (5.0 g), soybean meal (5.0 g), yeast extract (1 g), casaminoacids (2.0 g), $CaCO_3$ (1.0 g), Clerol (0.2 g), agarose (10 g) and tap water (1 L).

After an appropriate incubation time, 3 mm glass beads were added to the plates, and the strains were dried in the dark over night before extraction with 150 µl DMSO. The plates were shaken for 2 h at 1000 rpm before vacuum filtration (Event 4160, Eppendorf). These extracts were stored at -20 °C, and tested in agar diffusion assays for content of antagonistic compounds active against *Micrococcus luteus* (ATCC 9341)*, Candida albicans* (ATCC 10231) and *Escherichia coli* K12 .

A variant of Burkholder agar diffusion assay [41] was used when screening for antimicrobial activity. Indicator agarose was prepared by mixing 1% agarose medium with 0.5-1% v/v indicator organism culture (OD_{600} = 3,6 $_{M. luteus}$, 5,0 $_{C. albicans,}$ 3,0 $_{E. coli}$.), and poured into Petri dishes. LB agarose medium was used for *E. coli*, M19 for *C. albicans* and M1 for *M. luteus*. The media contained: M1; peptone (6.0 g), trypton (4.0 g), yeast extract (3.0 g), beef extract (1.5 g), dextrose (1.0 g), agarose (10 g) and tap water (1 L), pH 6,6. M19; beef extract (2.4 g), yeast extract (4.7 g), peptone (9.4 g), dextrose (10.0 g), NaCl (10.0 g), agarose (10 g) and tap water (1 L), pH 6,1.

DMSO-extracts were stamped manually from microwell plates onto the indicator agarose with the selected indicator organism. Approximately 2 µl of each extract was applied onto plates with 1.3 cm thick indicator agarose. The plates were preincubated for 3 to 4h at 4 °C, before incubating at 30 ºC over night. Extracts were defined as inhibiting if inhibition zones were ≥ 2mm larger than the diameter of the applied sample.

3.3 Cloning, sequencing and phylogenetic analysis

Based on morphology and inhibition patterns from the antimicrobial assays, subgrouping was performed, and candidates from each subgroup sequenced. PCR was performed directly on colonies or with isolated total-DNA as template. Total-DNA of the bacteria was isolated using DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's protocol.

The primers BP_F27: 5'-AGA GTT TGA TCM TGG CTC AG-3' and BP_R1492: 5'-TAC GGY TAC CTT GTT ACG ACT T-3' [42], were used to amplify 1,5 kb of the 16S rRNA gene. The PCR was performed using initial denaturation at 94 °C for 4 minutes, followed by 35 cycles of 94 °C for 45 seconds, 55 °C for 20 seconds and 66 °C for 2 minutes. A final extension was performed at 70 °C for 5 minutes. PCR products were purified directly or after excision from agarose gel, using QIAquick Spin Kits according to the manufacturer's instructions (Qiagen). Purified PCR-products were transformed into *E. coli* EZ competent cells after ligation into the pDrive cloning vector using the QIAGEN PCR-cloning Kit (Qiagen).

The 16S rRNA fragments were sequenced either from the pDrive-clones or directly after PCR. The primer M13 reverse: 5'-AACAGCTATGACCATG-3' described in the Qiagen PCR Cloning Handbook (04/2001) was used for the pDrive-clones. Sequencing directly on the PCR products were performed with the same primers as for the PCR. The sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing program consisted of a initial step at 96 °C for 3 minutes, and 25 cycles of 96 °C for 30 seconds, 50 °C for 20 seconds and 60 °C for 4 minutes.

The phylogenetic analyses of the cloned sequences were performed using MEGA 4. A phylogenetic tree was constructed using neighbour-joining with 500 bootstrap replicates. Comparisons of the sequences with other available 16S rDNA sequences were done by BLAST searches to determine

strain homology. The 16S rDNA sequence from *Micromonospora* sp DSM 44397 was included to root the tree.

3.4 PCR amplification of PKS and NRPS-genes

Bacterial modular type I PKS genes were amplified with the degenerate primers KSMA-F (5'-TS GCS ATG GAC CCS CAG CAG-3') and KSMB-R (5'-CC SGT SCC GTG SGC CTC SAC-3') [31]. PCR with these primers, amplifying the β-ketoacyl synthase (KS) domain (~700 bp), was performed using initial denaturation at 96 ˚C for 5 min, 35 cycles of 95 ºC for 1 min, 60 ºC for 1 min and 72ºC for 2 min. Final extension was performed at 72 ºC for 5 min.

For each reaction 200 µM dNTPs 20-40 ng total-DNA and 200 nM of each primer were used. Cloning of the fragments was performed as described for 16S rDNA Sequencing was performed by Eurofins MWG Operon.

3.5 Nucleotide sequence accession numbers

DNA sequences reported in this study have been deposited to GenBank under accession numbers FJ190540-FJ190569

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Paper II

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Paper III

Violacein-producing *Collimonas sp.* from the sea surface microlayer of coastal waters in Trøndelag, Norway: assessment of potential for biosynthesis of secondary metabolites through genome mining

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Abstract

A new strain belonging to the genus *Collimonas* was isolated from the sea surface microlayer off the coast of Trøndelag, Norway. The bacterium, designated *Collimonas* CT, produced a blue pigment with antibacterial activity identified as violacein. Draft genome sequencing for this bacterium followed by genome mining allowed identification of several gene clusters potentially governing biosynthesis of secondary metabolites, including the complete gene cluster for the biosynthesis of violacein (*vio*). These findings prompt further studies on *Collimonas* CT, which may reveal its full potential as a producer of biologically active secondary metabolites.

Introduction

The genus *Collimonas* was described for the first time in 2004 [14]. These bacteria were isolated from slightly acidic dune soils in the Netherlands, and are strictly aerobic, Gram-negative rods. *Collimonas fungivorans gen. nov., sp. nov*., are chitinolytic and able to grow on living hyphae of several soil fungi. Based on 16S rDNA sequences, the most closely related genera are *Herbaspirillum* and *Janthinobacterium*.

Violacein (3-[1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ylidene]-1,3-dihydro-2H-indol-2-one) is a blue-black indole-derived pigment described already in 1882. The violacein carbon skeleton is produced from two molecules of $_{L}$-tryptophan, and molecular oxygen is required for production of the pigment [16, 38, 15]. The role of

violacein production in the bacteria is not understood, but it has been suggested that it gives a survival advantage in the competition with other microorganisms in the environment [34]. Other suggestions include involvement in protection against visible radiation [4] and regulation of tryptophan production, which is toxic for bacteria at high concentrations [17].

Violacein is produced by several bacterial species, including the Gram-negative species *Chromobacterium violaceum*, *Janthinobacterium lividum*, *Pseudoalteromonas luteoviolacea*, *Ps. sp* 520P1 and *Ps. sp.* 710P1 [45, 42, 56]. These strains have been isolated from water and soil in tropical and subtropical regions, rivers, lakes and springs and from seawater at a depth of 320 m outside Japan.

The gene cluster for violacein biosynthesis has been sequenced from several of the violacein producers, including *Ch. violaceum* and environmental DNA [6, 8]. The 8 kb and 6.7 kb violacein clusters have been reported to contain four genes (*vio*A-D) responsible for the production of violacein and deoxyviolacein [15] (Fig. 1). A fifth gene (*vio*E) has later been described as being essential for violacein biosynthesis [47].

Violacein has shown anti-protozoan [33, 30], anticancer [20, 26], anti-viral [1], antibacterial (both G+ and G-) [31, 39, 47] and antioxidant [28] activities. The antibacterial activity includes inhibition of *Staphylococcus aureus, Neisseria meningitidis, Streptococcus spp., Bacillus spp., Mycobacterium* and *Pseudomonas,* among others.

Based on these properties, violacein would seem to be commercially interesting for therapeutic purposes and it has in fact been proposed for dermatological purposes [4].

It has been suggested that violacein should be considered an in vitro genotoxic compound to mammalian cells, (due to its toxicity in VERO and FRhK-4cells), but further

investigations are needed before drawing any conclusions on violacein's future pharmaceutical potential [2].

Up until now *Ch. violaceum*, which is the best studied violacein producer, has not been widely utilized for commercial purposes. One reason might be that it can act as an opportunistic pathogen in humans [46]. New producers of violacein would therefore be interesting to isolate.

In this study, a new strain of the genus *Collimonas* has been isolated, and examined for its antimicrobial potential. The production of a characteristic blue pigment and the demonstrated antibacterial activity seems to be ascribed to the violacein cluster. Other genes for the production of secondary metabolites have also been identified in the *Collimonas* CT genome. These findings suggest that this bacterium might have a potential as a producer of biologically active compounds of interest to the biotechnological industry, and prompt further studies.

Materials and methods

Sampling sites and sample collection

The *Collimonas* CT strains were isolated from the sea surface microlayer at two locations along the coast of Trøndelag, Norway. Water samples were collected on 18th of June 2004 in Snillfjord (63º 23,755 N, 009º 29,327) and 1th of July 2004 at Sula (63º 50,595 N, 008º 27,552 E). The water temperatures were 12.4 and 13.8 ºC respectively, and the salinity corresponded to 18.5 and 33.1 practical salinity units (psu). The surface microlayer was

collected using teflon plates and the bacteria isolated as earlier described [22]. Table 1 lists the microbial strains and plasmids used in this study.

Both samples were collected around high tide and 2 to 3 meters from the shoreline.

The *Collimonas* CT strains were initially isolated from Kusters streptomycete isolation agar (2 % w/v) (modified); Glycerol (10 g), Casein (0.3 g), KNO_3 (2 g), $FeSO_4 \cdot 7\ H_2O$ (0.25 mg), H_2SO_4 (0.5 mg), natural sea water (0.5 L) and distilled water (0.5 L), pH 8.2, supplied with Cycloheximide (50 µl/ml) and Nalidixic acid (30 µl/ml).

Preparation of bacterial inoculums

A mixture of 1-4 colonies with 5 g glass beads and 2.5 ml 0.9 % NaCl with 0.1 % Tween 20, was whirlmixed for one minute. Whirlmixing was repeated after 15 min. The cell-material was centrifuged and washed twice with sterile water, before resuspension in 1.5 ml sterile water.

Culture conditions for production and extraction of secondary metabolites

The isolates were cultivated on different 1% agarose (production) media to facilitate production of secondary metabolites. Initial cultivation, extraction and antimicrobial assays were performed as described earlier [22]. A fourth production medium (PM1) was included, containing: Malt extract (5 g), yeast extract (2 g), glucose (2 g), agarose (10 g) and tap water (1 L), pH 8.2. PM1 is identical to ½ ISP2 medium used in the initial cultivation of the bacteria, except for the use of tap water, and exchanging the agar (2 %) with agarose (1 %). Cultivation on production media with 50 % seawater was also

performed. To identify the optimal incubation time for production of bioactive compounds, different incubation time points (1 to 9 days) and two incubation temperatures (20 and 30 °C) were tested for each medium. Extraction was performed with both ethyl acetate and methanol in parallel with DMSO.

Upscaled tests, with reduced number of production parameters, were performed by cultivation in flat 6-well Tissue Culture Plates (Sarstedt nr 83.1839.500) with 1.5 ml agarose media in each well, inoculated with 25 µl inoculum. Extraction was performed with 2.5 ml DMSO. Dried cultures on agarose media was crushed, and incubated with DMSO and glass beads on a rotator for 2 hours in the dark.

Cell extracts for liquid assays were prepared from supernatant and pellet of liquid cultures. Initial tests based on results from cultivation on solid media and agar diffusion assays, were performed to determine the optimal incubation time and production medium. Precultures of *Collimonas* CT were prepared by picking colonies from plates, and growing them in PM2 (production medium 2) for 16 hours at 20 ºC. PM2 contained; Mannitol (20 g), soybean flour (20 g), Clerol (antifoam, 0.5 g), dry yeast (3.4 g), and tap water (1 L). Fresh medium (30 ml) was inoculated 3 %, and incubated for three days at 20 ºC. The bacteria were pelleted by centrifugation (10 000 x g, 10 minutes). The pellet and the supernatant were freeze dried separately. Freeze dried material was extracted with equal volumes of DMSO.

Antimicrobial assay

The bacterial extracts were stored at -20 °C, and tested in agar diffusion (a.d.) and liquid assays (l.a.) for antagonistic activity against *Micrococcus luteus* (ATCC 9341)*, Candida*

albicans (ATCC 10231) and *Escherichia coli* K12 (a.d. only), and *Enterococcus faecium* CCUG 37832 and CTC 492 (l.a. only). Agar diffusion- and liquid assays were performed as described earlier [22, 25]. Only DMSO extracts from bacteria grown on PM2 and PM3 were tested in the liquid assays.

Fractionation and LC-MS-TOF analysis of bacterial extracts

Samples of selected DMSO-extracts were fractionated using an Agilent 1100 series HPLC system equipped with a diode array detector (DAD) and a fraction collector. Each sample was fractionated using 2 different types of LC-columns: (1) Agilent ZORBAX Eclipse XDB-C18, 5 um, 4.6 x 150 mm and (2) Agilent SB-CN 3.5 um, 4.6 x 75 mm. For both types of columns, a flow of 1 ml/min of a mixture of 0.005 % formic acid in deionized water and acetonitrile was used as mobile phase. In both cases the concentration of acetonitrile was kept at 25 % the first minute, then increased linearly from 25 to 95 % during the next 11 minutes and kept at a concentration of 95 % for the rest of the run. The fraction collector was used to collect 12 fractions of the eluent from 1 minute until 13 minutes from injection. The samples were first fractionated using LC-column (1). The fractions displaying antibacterial activity (see below) were further fractionated in parallel using conditions (1) and (2).

The samples from LC-fractionation were dried in a vacuum centrifuge (Savant Speed-Vac), dissolved in DMSO and the bioactivity of the fractions determined in an agar diffusion assay using *M. luteus* as indicator organism [22]. Selected samples from the second LC-fractionation that showed bioactivity were further analysed using an Agilent 1100 series

HPLC system connected to a diode array detector (DAD) and a time of flight (TOF) mass spectrometer. The column and buffer conditions used were as described for condition (2) above. Electrospray ionization was performed in negative mode. The DAD plots were used to identify the approximate retention times of the bioactive compounds in the fractionation runs and in the LC-MS-TOF analysis. Molecular masses corresponding to significant peaks identified in bioactive samples from parallel fractionations (C18 and CN columns) were compared and molecular masses common to fractions from the C18 and CN columns were identified. These molecular masses (10 ppm window) were submitted to the online version of the Dictionary of Natural Products (http://dnp.chemnetbase.com/) in order to search for previously characterized compounds with bioactivity.

Bacterial DMSO extracts were purified on a C18 solid-phase extraction column, 55-105 µm (Waters nr. WAT036945), and eluted with methanol. The methanol solutions of the unknown compound were analyzed on an Agilent 1100 HPLC system equipped with a diode array detector (DAD) and an Agilent time-of-flight (TOF) mass spectrometer with an electrospray ion source run in negative mode. For the LC separation, a Bonus-RP column (2.1×50 mm, Agilent Technologies, USA) was used. The mobile phase consisted of 10 mM ammonium acetate pH 4.0 and a linear gradient of acetonitrile from 25 to 90 %.

Cloning, sequencing and phylogenetic analysis

Total-DNA of the bacteria was isolated using DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's protocol. PCR with bacteria specific primers, BP_F27: 5'-

AGA GTT TGA TCM TGG CTC AG-3’ and BP_R1492: 5’-TAC GGY TAC CTT GTT ACG ACT T-3’, was performed to amplify 1.5 kb of the 16S rRNA gene [29]

The PCR was performed using initial denaturation at 94 °C for 4 minutes, followed by 35 cycles of 94 °C for 45 seconds, 55 °C for 20 seconds and 66 °C for 2 minutes. A final extension was performed at 72 °C for 8 minutes. PCR products were purified after excision from agarose gel, using QIAquick Spin Kits according to the manufacturer’s instructions (Qiagen). Purified PCR-products were transformed into *E. coli* EZ competent cells after ligation into the pDrive cloning vector using the QIAGEN PCR-cloning Kit (Qiagen).

The 16S rDNA fragments were sequenced from the pDrive-clones using the primers M13 reverse: 5'-AACAGCTATGACCATG-3' and M13f forward: 5’-GTAAAACGACGGCCAGT-3’ described in the Qiagen PCR Cloning Handbook (04/2001). The sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing program consisted of a initial step at 96 °C for 1 minute, and 25 cycles of 96 °C for 30 seconds, 45 °C (M13r) or 50 °C (M13f) for 15 seconds and 60 °C for 4 minutes.

Degenerate primers for amplification of parts of *vioA* and *vioB* genes were designed based on the VioA and VioB amino acid sequences from different violacein producers, retrieved from the GenBank. For amplification of a ~1.0 kb segment encoding the flavoenzyme VioA, the degenerate primer pair VPA3: 5'-CCRCAGCTSCAYCCGCATTTCCAG-3' and VPA4: 5'-CAGGCYGCCCTCCATCCAGCCRCA-3' were used. Parts of *vioB*, encoding the heme protein VioB was amplified using two primerpairs. The primerpair VPB1: 5'-CTGTTCAATATGTCGACGCCGC-3' and VPB2: 5'-

GCGGATCGCACATCTGCCACATC-3' amplificated a ~900 bp strech, and the degenerate primers VPB3: 5'-CCGGCCGGCCGSCTGCTGC-3', VPB4: 5'-GSCGCGAGCGSCKSAGGCTGC-3' amplificated a ~1.85 kb segment of *vio*B. The 1.0 kb segment of *vioA* and the ~900 bp segment of *vioB* were cloned as described for 16S rDNA–sequences. Sequencing was performed by Eurofins MWG Operon.

The phylogenetic analyses of the cloned sequences were performed using MEGA 4 [53]. Sequences were aligned with their closest hits from BLAST searches, trimmed to the same length, and the phylogenetic trees constructed using neighbour-joining with 2000 bootstrap replicates. Comparing the sequences with other available 16S rDNA and *vio*A/B/C/D/E sequences were done by BLAST searches to determine strain homology and identity.

Sequencing and automated annotation of the Collimonas CT genome

Isolated total-DNA from the *Collimonas* CT isolate MP11E8 was sequenced and *de novo* assembled by Fasteris SA (Switzerland). Sequencing was performed using Solexa technology, in paired-ends channel. The library was sequenced twice, yielding ~ 45 x coverage of the ca 5.8 million base pair genome. *De novo* assembly was performed by Velvet, MAQ and EDENA software. The resulting 257 contigs were combined randomly into a 'pseudogenome' by adding a linker sequence (jcvi.org) that creates stop-codons in all six reading frames. Automated annotation of the "pseudogenome" was performed by BASys: Bacterial annotation system [55]. The annotations are made by BASys using over 40 programs and databases listed on their webpage (http://wishart.biology.ualberta.ca/basys/cgi/submit.pl). ORFs of interest were further

analyzed by nucleotide BLAST and BLASTX online searches at NCBI web pages (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Codon usage in the genome was estimated using the CountCodon program in the CUTG Database (Codon Usage Tabulated from GenBank) (http://www.kazusa.or.jp/codon/). The estimate was based on open reading frames in the submitted genome sequence, with ATG as start codon and TAA, TAG and TGA as stop codons. Codon usage tables for other bacteria of interest were retrieved from the same site [40]. NRPS Domain Search program (www.nii.res.in/nrps-pks.html) was used to predict structural domains encoded by the putative NRPS clusters [3]. DNA sequences reported in this study were deposited to GenBank under accession numbers FJ965828-965838, FJ985255, FJ985256, GQ160908 and GQ160909.

Results and discussion

Isolation of Collimonas CT

In this study, four bacteria producing a blue pigment were isolated from the sea surface microlayer at the coast of Trøndelag, Norway. Sequencing of partial 16S rDNA sequences (1490 bases) from the 4 strains revealed two unique sequences that were 99.3 % identical. Both displayed 98.8 % identity to *Collimonas fungivorans* CTE227. The isolates are therefore named *Collimonas* CT (Coast of Trøndelag) in this article. Other *Collimonas sp*. has been isolated from terrestrial sources, mainly soil [14, 24]. One extremofile *Collimonas sp.* has been isolated from submarine ikaite columns in Greenland [48] and one from stream water in Finland [35]. Initial cultivation of the seawater samples from the coast of

Trøndelag was performed on media containing nalidixic acid, to minimize growth of Gram-negative bacteria. Isolation of the Gram-negative *Collimonas* CT from these samples indicates that the bacteria are able to grow in the presence of this antibiotic at the concentrations used. Resistance to nalidixic acid has been observed *for Janthinobacterium lividum* and *Chromobacterium violaceum* [50, 19]

C. fungivorans strains show high sequence similarity to representatives of the genus *Janthinobacterium* (~95 %) and *Herbaspirillum* (~96 %), and are reported to display the highest growth rates at 20-30 ºC [14]. As for *Collimonas* CT, an increase in incubation temperature from the water temperature at the sampling site (ca 13 ºC) to 20 ºC and 25 ºC did not inhibit growth or pigment production. The CT bacteria did not produce pigment when cultivated at 30 ºC and did not grow at 37 ºC. Loss of pigment production when incubated at 25 ºC or higher has been observed by others [35].

To optimize the conditions for production of antimicrobial compounds, the isolates were cultivated on four different production media, with or without 50 % seawater. Interestingly, the isolates grew slower or displayed no growth on media containing seawater. Some pigment production could be seen in the growing cultures, but probably due to the poor growth the antimicrobial activity was very low in the extracts of such cultures.

Antimicrobial activity was assayed with *Micrococcus luteus* (ATCC 9341)*, Candida albicans* (ATCC 10231), *Escherichia coli* K12, *Enterococcus faecium* CCUG 37832 and *E. faecium* CTC 492 as indicator organisms. Activity could only be detected against *M.luteus* under the production and assay conditions tested.

Identification and characterization of antimicrobial compound and pigment

Extracts from *Collimonas* CT showing antibacterial activity were analyzed by LC-MS. The UV (DAD) absorbance plot shown in Figure 3 displayed four peaks, of which the peaks at 12.5 and 15.5 minutes showed similar UV profiles. The main compound in the sample, eluting at 12.5 minutes, had an m/z = 342.0882, whereas the compound eluting at 15.5 minutes had an m/z value of 326.0938. The measured masses deviates 0.6283 and 0.9188 ppm from the stoichiometric formulas ($[M-H]^-$ ion) of $C_{20}H_{12}N_3O_3$ and $C_{20}H_{12}N_3O_2$. Based on measurements at the UV absorbance maximum of 572 nm, the relative abundance of the latter compound was 49.6 % of the former. Based on the MS-analysis, colour of the substrate and a UV-profile similar to that of violacein (http://dnp.chemnetbase.com/), the two main compounds were assumed to be violacein ($C_{20}H_{12}N_3O_3$) and deoxyviolacein ($C_{20}H_{12}N_3O_2$).

The compounds eluting at 9.5 minutes and 13 minutes had m/z values deviating less than 1.4 ppm from the molecular ion stoichiometries of $C_{15}H_9O_4$ and $C_{15}H_9O_5$, respectively. Relative amounts of these two compounds could not be estimated from the UV data as their extinction coefficients are not known.

As previously mentioned, *Collimonas* CT isolates did not produce pigment when grown at 30 ˚C. Temperature, agitation and pH also affect violacein production in *Ch. violaceum* [as cited by 57]. Colourless colonies were also at one point observed in re-streaks of the CT-isolates. This phenomenon is not unknown among violacein producers [12, 57]. Sequencing of 16S rDNA, and PCR with degenerate primers for *vio*A- and *vio*B- biosynthesis genes confirmed that these isolates were in fact potential violacein producing *Collimonas sp*.

Inhibition assays revealed that antimicrobial activity was lost in extracts from the white colonies, indicating that colour and antimicrobial activity might be linked. The UV-profile of extracts from the colourless mutants confirmed that no violacein was present in the sample, and that the production of violacein was lost. These experiments show that violacein is not essential for growth as also reported earlier for other violacein producers [51]

Fractionation of the bacterial extracts followed by antimicrobial assays revealed that the antibacterial activity was found in the same fractions as the blue pigment, indicating that the activity found in *Collimonas* CT extracts is due to the blue pigment. The reported antibacterial activity of violacein is mostly against Gram-positive bacteria, which is in accordance with the observed activity against *M. luteus* and lack of activity against *E. coli*. These results confirm that the observed antibacterial activity of the *Collimonas* CT isolates most probably is not caused by several compounds, and that the main bioactive compound is violacein.

As earlier described, *Collimonas* CT 16S rDNA is highly similar to *Janthinobacterium* and *Herbaspirillum*. Production of violacein is a characteristic of *Janthinobacterium* [52]. Despite the close relationship, *C. fungivorans* has not been reported to produce violacein even though assumed violacein producing strains of *Collimonas* has been described [35]. Violacein non-producing strains of both *Janthinobacterium* and *Chromobacterium violaceum* have also been described [32, 51]. The phylogenetic relationship between the partial 16S rDNA sequences from the aforementioned species is shown in Figure 2.

Despite that the *Collimonas* CT bacteria were isolated from marine samples, the isolates show inhibited growth on seawater-containing media. Violacein has earlier been found in bacteria isolated from marine environment, and this might suggest that the *Collimonas* CT are growing as biofilm in the tidal zone of brackish water, or in soils/fresh water and had been washed out into the sea not long before sampling.

The violacein biosynthetic gene cluster in Collimonas CT

Initial screening for *vio*A and *vio*B sequences was performed to substantiate the assumption that the produced pigment was in fact violacein. *vio*A- and *vio*B-fragments were obtained by PCR with degenerate primers. It is reported that disruption of *vioA* or *vioB* would completely abrogate the biosynthesis of violacein [6]. Attempts to inactivate *vio*B by homologous recombination in this study gave rise to white colonies without antimicrobial activity. Results from the following Southern blotting with the *vioB* fragment as probe were inconclusive, probably due to large deletion in the mutants.

The genome was sequenced in order to identify the full secondary metabolite biosynthesis potential of the bacterium. The 5.8 million base pair genome of *Collimonas* CT has a GC-content of ~60 %, and automated annotation of the genome yielded 5831 ORFs. All the genes for biosynthesis of violacein, *vioA*, *vioB*, *vioC*, *vioD* and *vioE* could be found in the genome and the cluster spanned 7.3 kb. Phylogenetic analyses of amino acid sequences of *vio*A-E genes from different violacein producers were performed. The resulting trees were very similar, suggesting that the *vio* genes in the different strains share the same evolutionary history. Only the tree for the *vioB* genes is shown in Figure 4. A comparison

of the similarity of the proteins in the violacein cluster from different violacein producers to violacein genes from *Collimonas* CT is shown in Table 2. ORFs1-13 and *vio*A-E are numbered ORFs05301-05318 in the annotated sequences submitted to GenBank.

16S rDNA of *Collimonas* shows higher degree of identity to *Janthinobacterium lividum* (94 %) (formerly *Chromobacterium lividum* [52]), than to *Chromobacterium violaceum* (88 %). This is also the case with the proteins in the violacein cluster. In average the proteins displayed ~81 % identity to VioA/B/C/D/E from *J. lividum* and ~64 % identity to VioA/B/C/D/E from *C. violaceum*.

The carbon skeleton of violacein is produced from two molecules of tryptophan, and in *Ch. violaceum* the genes for tryptophan biosynthesis are not organized in an operon [4] The organization of genes for the biosynthesis of tryptophan varies from whole-pathway operons to dispersed genes, among different species [37].

As for *Ch. violaceum* the *trp*-genes in *Collimonas* CT are not assembled in one operon, and the organization of the genes resembles that of *Janthinobacterium sp* (GI:152979768), and is somewhat similar to what is found among other betaproteobacteria [37], including *Ch. violaceum*. Also for these genes (*trp*A/B/C/D/E/F) *Collimonas* CT show higher similarity to *J. lividum* (~82 %) than to *Ch. violaceum* (~76 %).

The codon usage in the violacein cluster in *Collimonas* CT does not differ substantially from the predicted codon usage in the rest of the genome of this bacterium, Table 3. The codon bias (main codon) in the related species *Collimonas fungivorans* (not violacein producer) and *J. lividum* is somewhat similar to that of *Collimonas* CT whereas the more distantly related *Ch. violaceum* and *Pseudoalteromonas tunicata* display an increasing

difference in codon-bias (main codon). This indicates that the violacein cluster is unlikely to have been recently transferred to the isolates. In view of this, it was interesting to study the sequences flanking the violacein cluster. The organization of the violacein cluster in *Collimonas* CT including flanking sequences is shown in Figure 5. Analysis of the surrounding open reading frames (ORF's) was also performed, and predicted gene products are listed in Table 4.

The flanking sequences of the violacein cluster in *Chromobacterium violaceum* ATCC12472 (GI:34105712), *Janthinobacterium lividum* DSM 1522 (GI:71726055) and *Pseudoalteromonas tunicata* D2 (GI:88821000) are known. The predicted genes CV_3292→3275 and CV_3269→3256, spanning 42 kb including the violacein cluster, in *Ch. violaceum* display no direct resemblance to the predicted proteins in the flanking sequences in *Collimonas* CT. This is also the case for the flanking genes PTD2_09264→09314 and PTD2_19467→19607, spanning 43 kb including the violacein cluster in the *P. tunicata* genome. As for the 27 kb (including the violacein cluster) flanking sequences in *J. lividum*, some similarities with the *Collimonas* CT sequence can be seen. Genes encoding amino acid transport proteins can be found upstream the violacein cluster in *J. lividum* and *Collimonas* CT. In addition, both *J. lividum* and *Collimonas* CT encode a TonB-dependent receptor upstream the violacein cluster. TonB-dependent receptors lie in the outer membrane of Gram-negative bacteria and transmit signals (energy dependent) to the cytoplasm, resulting in transcriptional activation of target genes [27]. A role for the TonB-dependent receptor in the biosynthesis of violacein in *J. lividum* has not been determined.

Both *J. lividum* and *P. tunicata* encode proteins for drug transport in their flanking sequences. A putative transmembrane drug/metabolite transporter is situated (4.5 kb) downstream the violacein cluster in *J. lividum*, while a Multi-Antimicrobial and Toxic compound Extrusion (MATE) family efflux pump is encoded directly upstream of the cluster in *P. tunicata*. It has been shown for *P. tunicata* that violacein is associated with the outer membrane and accumulate in periplasma [54, 34], assumingly as a defense mechanism against predators when growing in a biofilm. It has been speculated that the MATE pump encoded by *P. tunicata* might provide a mechanism for export of violacein [54]. In the flanking sequences of *Collimonas* CT, genes encoding a putative ABC-transporter system (ORF10-13) can be found.

Downstream of the violacein cluster in both *J. lividum* and *Ch. violaceum* (8 kb and 15 kb downstream) a gene for a LysR transcription regulator can be found. The genome of *Ch. violaceum* contains a large number of transcriptional activators that may interact with alternative sigma factors involved in bacterial stress response, such as LysR [23]. It has been suggested that the bacterium produces violacein as a response to stress [4]. A similar transcription regulator was not found in *Collimonas* CT. The variation of genes in the flanking sequences among the different violacein producers might indicate the biosynthesis of violacein being an old characteristic of *Collimonas* CT.

Putative secondary metabolite biosynthesis genes in Collimonas CT

In addition to the genes in the violacein biosynthetic cluster, also genes that might be involved in the biosynthesis of other secondary metabolites were identified in the draft genome, as summarized in Table 5.

NRPS-related metabolites*;* Seven putative NRPS gene clusters were identified. A graphic representation of six of these is shown in Figure 6. Four contigs harbouring genes putatively related to the production of syringomycin, syringopeptin and/or arthofactin were identified in the *Collimonas* CT genome. An 18.8 kb-long non-ribosomal peptide synthetase (NRPS) gene (ORF03016 in cluster 5) with 65 % identity to putative syringomycin synthetase from *Burkholderia pseudomallei Pasteur* was identified, presumably containing six modules. Immediately upstream of the NRPS gene, an ORF encoding a putative acetyltransferase was found. The two ORFs downstream the NRPS gene encodes proteins with 68 % and 85 % identity to carbamoyltransferase and penicillin amidase, respectively. Further downstream, two ORFs encoding a putative thioesterase and a two component transcription regulator of the LuxR family were identified. The two aforementioned proteins display 58 and 44 % identity, respectively to the corresponding homologues encoded by *Burkholderia phytofirmans* and *Pseudomonas entomophila* genomes. Syringopeptins and syringomycin are related phytotoxic lipodepsipeptides. Production of both syringomycin and syringopeptins has been reported from isolates of *Pseudomonas syringae pv. syringae* [18]. Syringomycin biosynthesis is not reported from *Burkholderia pseudomallei*.

Partial sequence of ORF05502 (10 kb) on another contig (cluster 6) was found to encode an apparent NRPS with 48 % identity to a protein from *Ralstonia solanacearum*. This NRPS apparently contain at least 3 modules. The downstream ORF05503 encodes a protein with 40 % identity to a putative NRPS domain from a *Bradyrhizobium sp*., and ORF05505 encodes a thioesterase superfamily protein. The synthetase in ORF05502 is probably associated with ORFs05573-05575, also situated at the end of a contig. ORFs05573 and 05575 in cluster 7 constituting one module, encode parts of a single gene (probably one base missing due to sequencing errors). Both ORFs05502 and 05573 + 05575 are putatively arthofactin/syringomycin synthethase C module related. ORF01844 in cluster 8 encodes adenylation domain of a NRPS that might be related to syringopeptin synthetase C (57 % identity). Based on these similarities, it is likely to assume that *Collimonas* CT can produce a peptide related to syringomycin, syringopeptin and arthofactin. It is conceivable that these clusters might be linked, as in the case of *P. syringae pv. syringae*, where syringomycin and syringopeptin gene clusters apparently form a genomic island [49].

Yet another putative NPRS is encoded by ORF03557 (cluster 4), deduced product of which displays 69 % identity to the amino acid adenylation domain from *Dechloromonas aromatica* RCB3534. A potential product of this NRPS is not known.

Cluster 3 (ORFs05144-05156) span 26.6 kb, and presumably represent a siderophore biosynthetic cluster. MbtH-like domains as encoded in ORF05144 are often found in antibiotic synthesis gene clusters (http://pfam.sanger.ac.uk/). Putative dioxygenases can also be identified, as Taurine catabolism dioxygenase encoded by ORF05145. The downstream ORFs05146-05151 encode proteins involved in transport of siderophore/iron

(Fe^{3+}) compounds. ORFs05152 to 05154 most likely encode one NRPS, putatively siderophore-related. This NRPS conatins at least four modules, as illustrated in Figure 6. The last two ORFs encode, L-ornithin-5-monooxygenase and a TonB-dependent siderophore receptor. All ORFs in this cluster display 61-85 % identity to proteins from different *Burkholderia* strains.

A putative cyanophycin synthetase was found to be encoded by ORFs05750-05752 (cluster 2), displaying 75 and 45 % identity to the corresponding synthetases from *Cupriavidus taiwanensis*. The ORFs05750 and 05751 probably represent one gene, appearing as two, due to a sequencing error. Cyanophycin (multi-L-arginyl-poly-L-aspartic acid) is a non-ribosomically synthesized peptide, which in cyanobacteria accumulate in the cytoplasma as granules during stationary phase or starvation conditions (except shortage of nitrogen). [44]. It is thought to function as a nitrogen reserve for the bacteria. Cyanophycin is of potential industrial interest as a source of (poly) aspartic acid.

Carotenoid biosynthesis; Cluster 1 (ORFs04929-04931), spanning ca 3.1 kb encodes genes presumably involved in carotenoid biosynthesis. ORF04931 and 04930 encode putative phytoene synthases, and their products display 70-83 % identity to corresponding proteins from *Herminiimonas arsenicoxydans*. Product of ORF04929 displays 63 % identity to a putative squalene/phytoene dehydrogenase; carotene 7,8-desaturase from the same bacterium. Phytoen synthase catalyzes the condensation of two molecules of geranyl geranyl pyrophosphate to C40 phytoene which is considered the first reaction unique to carotenoid biosynthesis, whereas the phytoene desaturase catalyzes the desaturation steps

from phytoene to lycopene [5, 43]. Clustering of carotenoid biosynthetsis genes is not a universal rule, as they are not necessarily confined to a single cluster in cyanobacteria [13].

Putative aromatic polyketides; Two putative aromatic polyketide biosynthesis gene clusters were identified in the genome, shown in Figure 7. Cluster 9 (ORFs02224-02232) and cluster 10 (ORFs03611-03629) spans 7.1 and 16.8 kb respectively inside larger contigs. Based on the bioinformatics analysis, we could not associate these clusters with the production of any known natural products. Bacterial aromatic polyketides are synthesized by PKS type II (iterative) polyketide synthases. A typical minimal PKS type II consist of two β-ketoacyl synthase subunits, KS_{α} and KS_{β}, an acyl carrier protein (ACP) and a malonyl-CoA:ACP transacylase (MAT) [36, 11]. The latter KS is often designated CLF (chain length factor), and it has been suggested that the control of chain length in bacterial aromatic PKSs is controlled by a substrate-binding pocket in the KS-CLF dimer interface [9]. CLF is involved in formation of acetyl ACP (from the decarboxylation of malonyl-ACP) [7]. The two KS-units catalyze the decarboxylative condensation of the malonyl building blocks delivered by the ACP and the acyl carrier protein (ACP) acts as an anchor for the polyketide chain during the various biochemical manipulations.

In order to fold and cyclize the chain genes as cyclases, aromatases and ketoreductases are required. The β-keto acid can be processed by β-ketoacyl-reductase (KR) domains, β-hydroxy-acyl-dehydratase (DH) domains and enoyl-reductase (ER) domains [10]. A combination of these genes will determine the structure of the new polyketide.

ORFs02224-02227 in cluster 9 encode a putative minimal PKS type II. However, the ketosynthases in ORFs02224 and 02227 both contain conserved domain of initiating KAS

III. They display 59 % identity to a β-ketoacyl-acyl-carrier-protein synthase I from *Methylobacterium extorquens* and 58 % identity to 3-oxoacyl-(acyl-carrier protein) synthase from *Burkholderia xenovorans*, respectively. The putative acyl carrier protein of ORF02226 displays 47 % identity to the protein from *Burkholderia phytofirmans*. The downstream ORFs (02228-02232) encode proteins for modification of the polyketide chain, including a reductase, acetyltransferase of the hexapeptide transferase family, hydroxylating dioxygenase related iron-sulphur cluster-binding protein (Rieske family), and a 3-deoxy-D-manno-oct-2-ulosonic acid transferase homologue, belonging to the glycosyl transferase 1 family. All ORFs display around 50 % identity to known proteins. ORFs03611-03629 constitutes a 16.8 kb putative PKS II gene cluster, cluster 10. The putative aromatic minimal PKS is encoded in ORF03612 (β-ketoacyl synthase II), ORF03615 (β-ketoacyl synthase I/II) and ORF03627 + 03628 (ACP). Both ketosynthases contain the conserved domain of elongating, condensing KAS I/II. ORF03611 encode a putative 4'-phosphopantetheinyltransferase required for the post-translational modification of ACP and the resulting activation of the acyl carrier domains (thioester) in ORFs03627 and 03628. [21]. ORFs for a β-ketoacyl reductase and a β-hydroxyacyl-(ACP)-dehydratase were also identified. Genes for modification of the polyketide were also identified, including a monooxygenase and glycosyl tranferases, as illustrated in Figure 6. ORF03621 displays 56 % identity to a thioesterase superfamily protein from *Ralstonia metallodurans*. Thioesterases are not common in type II PKS systems, but at least five genes associated with such gene clusters have been reported [41]. It was suggested that the thioesterease could be involved in chain release, or function as an esterase for the hydrolysis of ester

intermediates. The phospholipid/glycerol acyltransferase encoded by ORF03629 presumably borders the cluster.

Only activity against Gram-positive bacteria could be detected in extracts from *Collimonas* CT. This activity is thought to be due to the production of violacein. In addition to the gene cluster for violacein biosynthesis, genes for several other secondary metabolites with potential antimicrobial activity could be identified. The inability to detect the activity of these compounds in the antimicrobial assays might be due to that their production was not induced under the conditions tested.

Conclusion

A new violacein producer has been isolated from the sea surface microlayer at the coast of Trøndelag, Norway. The bacterium is a new strain belonging to the genus *Collimonas.* A limited analysis of the genome of *Collimonas* CT indicates that the violacein biosynthesis is an old characteristic of the *Collimonas* CT isolates. Genes for the production of other interesting secondary metabolites have also been identified in the *Collimonas* CT genome. A further study of the bacterium and its genome is necessary to fully elucidate its biotechnological potential.

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TABLE 1. Bacterial strains and plasmid used in this study

Strain or plasmid	Description	Source or reference
Candida albicans	Cyh^r AmB^s, Hp^s, Nys^s †	ATCC• (strain 10231)
Micrococcus luteus	$Amox^s$, Amp^s, Cm^s, Ccl^s, Nb^s, Ole^s, Pen^s, Pcn^s, Rif^s, Ty^s £	ATCC• (strain 9341)
E. coli K12		
Enterococcus faecium	Amp^r, Ctc^r, Ery^r,Lcm^r, Vcm^r, Am^r, Bac^r, Cs^r, Sp^r §	CCUG* (strain 37832)
Enterococcus faecium	Am^r, Bac^r, Cs^r, Sp^r	CTC (strain 492)
Collimonas CT		This study

• The American type Culture Collection

† Cyh: cycloheximide, AmB: amphotericin B, Hp: haloprogin, Nys: nystatin

£ Amox: amoxicillin, Amp: ampicillin, Cm: clindamycin, Ccl: cyclacillin, Nb: novobiocin, Ole: oleandomycin, Pen: penicillamine, Pcn: penicillin, Rif: rifamycin, Ty: tylosin

§ Amp: ampicillin, Ctc: chlortetracyclin, Ery: erythromycin, Lcm: lincomycin, Vcm: vancomycin, Am:apramycin, Bac: bacitracin, Cs: cycloserine, Sp: spectinomycin

* Culture Collection, Gothenburg University.

TABLE 2. Identity of the proteins from the violacein cluster in different violacein producers, to the violacein-proteins from *Collimonas* CT

	% identity at amino acid-level					
	VioA	VioB	VioC	VioD	VioE	Average
Janthinobacterium lividum¤	76	83	84-87	84-86	74-76	80-82
Uncultured bacterium	76	78	83	81	NA*	80
Chromobacterium violaceum‡	54	62	73	70	61	64
Pseudoalteromonas tunicata†	40	51	61	55	50	51

¤ GI:71726055 and GI:118161378

‡ ATCC 12472

† *Pseudoalteromonas tunicata* D2

* Not available

TABLE 3. Estimated codon usage in the *Collimonas* CT genome, based on 1934710 condons. Frequency is given in numbers per thousand.

Triplet	Frequency	Triplet	Frequency	Triplet	Frequency	Triplet	Frequency
UUU	13.4	UCU	8.8	UAU	7.2	UGU	10.0
UUC	16.0	UCC	13.1	UAC	7.0	UGC	25.4
UUA	4.5	UCA	14.6	UAA	4.5	UGA	14.4
UUG	16.1	UCG	21.8	UAG	4.9	UGG	20.2
CUU	13.9	CCU	14.2	CAU	16.2	CGU	14.0
CUC	8.5	CCC	9.9	CAC	13.2	CGC	37.5
CUA	4.9	CCA	20.5	CAA	16.3	CGA	21.6
CUG	24.6	CCG	29.5	CAG	24.8	CGG	29.4
AUU	11.2	ACU	7.3	AAU	11.2	AGU	7.3
AUC	18.3	ACC	15.0	AAC	11.0	AGC	22.3
AUA	7.6	ACA	10.4	AAA	13.5	AGA	9.0
AUG	16.3	ACG	14.2	AAG	13.7	AGG	13.9
GUU	11.1	GCU	22.5	GAU	18.2	GGU	14.7
GUC	15.0	GCC	35.2	GAC	14.9	GGC	34.9
GUA	6.9	GCA	25.4	GAA	15.8	GGA	13.0
GUG	13.2	GCG	37.9	GAG	8.4	GGG	9.9

TABLE 4. Predicted proteins encoded by the genes in the violacein cluster and the flanking sequences.

ORF number	Strand	Predicted protein	Id[§] to known sequences
1	+	putative N-acyl-D-amino-acid deacylase	54 %
2	-	Mercuric reductase, putative pyruvate/2-oxoglutarate dehydrogenase complex	69 % and 67 %
3	+	TonB-dependent receptor	48 %
4	+	acyl CoA thioester hydrolase	77 %
5	-	amino acid transporter, periplasmic ligand binding protein	62 %
6	-	Putative acetyltransferase	41 %
VioA-E	+		
7	-	Putative acetyltransferase	57 %
8	-	SPFH domain / Band 7 family protein	31 %
9	+	Fatty acid desaturase	58 %
10	+	substrate binding transport protein, ABC type	74 %
11	+	an integral membrane subunit, ABC type	64 %
12	+	ATP-binding protein, ABC type	68 %
13	+	periplasmic part of phosphate /phosphonate transport system, ABC type	46 % and 63 %

§ Identity

TABLE 5. Gene clusters identified in the *Collimonas* CT genome, putatively involved in the production of secondary metabolites.

Cluster no.	ORFs no.	Size (Kb)	Genes identified (putative)	Putative function
1	04929-04931	3.1	Phytoene synthases and dehydrogenase	Production of lycopene/ carotenoid biosynthesis
2	05750-05752	4.8	Cyanophycin synthetase	Cyanophycin synthesis
3	05144-05156	26.6	NRPS (siderophore), and transport	Siderophore biosynthesis and transport
4	03557	4.0	NRPS, adenylation domain	Unknown
5	03015-03020	25.3	Acetyltransferase, NRPS (18.8 kb), tailoring enzymes, thioesterase and a transcription regulator	
6	05502-05505	12.5*	Partial NRPS* (10 kb) related to arthrofactin/ syringomycin synthethase C module and a thioesterase superfamily protein	Syringomycin, syringopeptin and arthrofactin -related peptide biosynthesis
7	05573+05575	3.7*	Partial NRPS* related to arthofactin/syringomycin synthethase C module	
8	01844	2.2*	Partial NRPS*, syringopeptin synthetase C related	
9	02224-02232	7.1	Minimal PKS type II, modifying and post-PKS modifying enzymes	Aromatic polyketide biosynthesis
10	03611-03629	16.8	Minimal PKS type II, modifying and post-PKS modifying enzymes	Aromatic polyketide biosynthesis

* Encoding partial genes, due to end of sequencing contigs.

Figure legends

Figure 1. Chemical structures of violacein (**A**) and deoxyviolacein (**B**)

Figure 2. Phylogenetic relationship between 16S rDNA sequences (1368 bp) of known violacein producers, using the neighbor-joining method with 2000 bootstrap replicates. Closest matches from the BLAST search, *Collimonas fungivorans* CTE227, and *Herbaspirillum* sp. Hg1 (both not known to produce violacein) are also included in the tree. Two *Collimonas sp* from this study are displayed, while *Pseudomonas aeruginosa* NGKCTS is included as an out-root.

Figure 3. Diode array UV absorbance isoplot of bacterial extract. UV absorbance is shown in heat-map style as function of HPLC retention time and wavelength. The HPLC injection peak (void peak) at far left has been omitted for clarity of visualisation. The four main eluting peaks have retention times of 9.5, 12.5, 13 and 15.5 minutes, respectively, and are marked with arrows. Exact masses m/z ($M-H^-$ ion) of the putative violacein and deoxyviolacein are also given.

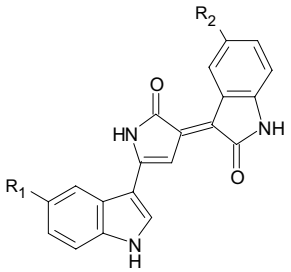
Figure 4. Phylogenetic tree constructed of the amino acid sequence (1013 amino acids) of *vio*B genes from known violacein producers. The analysis is performed using the neighbor-joining method with 1000 bootstrap replicates**.**

Figure 5. Organization of the violacein cluster in *Collimonas* CT **(A)** and *Janthinobacterium lividum* (GI:71726055) **(B)**. Open reading frames (ORF's), in the flanking sequences are also shown, and a total of 23 kb (**A**) and 25.5 kb (**B**) is shown. (t) = TonB-dependent receptor, (a) = genes for amino acid transport, (e) = efflux encoding

genes. *vio*-genes are marked with dark blue arrows, and homologous genes in the flanking sequences are marked with green arrows. The genes in the two bacteria are numbered sequentially, not by function.

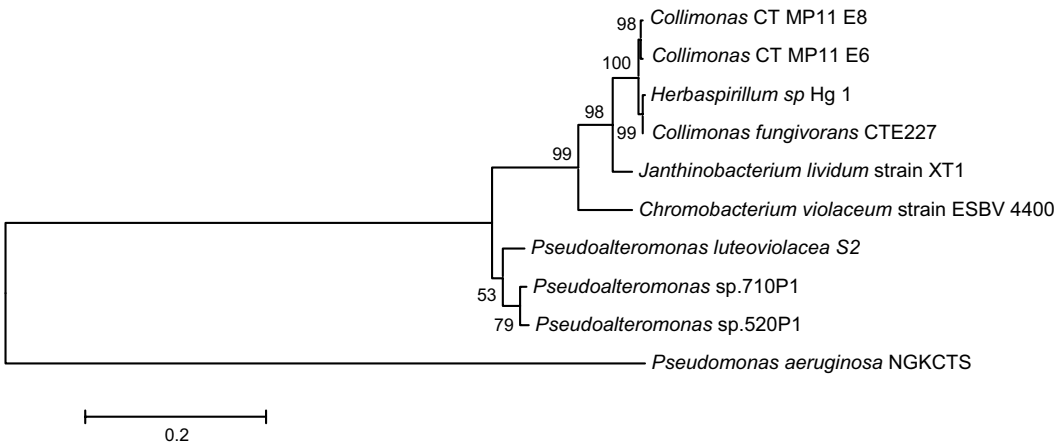
Figure 6. Prediction of functional elements of NRPSs encoded in the *Collimonas* CT genome. A: Adenylation domain, C: condensation domain, T: Thiolation domain, E: epimerisation domain, TE: Thioesterase domain. Asterisk indicates partial sequence due to end of sequencing contig. (**A**): Putative NRPS (ORF03016) in cluster 5. (**B**): Putative partial NRPS(s) (ORF05502 and 05503) in cluster 6. (**C**): Putative partial NPRS (ORF05573+05575) in cluster 7. (**D**): Putative partial NRPS (ORF01844) in cluster 8. (**E**): Putative part of NRPS (ORF03557) in cluster 4. (**F**): Putative NPRS (ORFs 05152-05154) in cluster 3.

Figure 7. Organization of putative aromatic polyketide gene clusters, cluster 9 (**A**) and 10 (**B**), in *Collimonas* CT. KS: β-Ketoacyl synthase. ACP: Acyl carrier protein. (**A**) KR: oxioreductase, short chain dehydrogenase/reductase. AT: Acetyl transferase. Hyp: Hypothetical protein. (**B**) PT: Phosphopantetheniyl transferase. KR: β-Ketoacyl reductase. DH: dehydratase. 16: Hypothetical protein. 17: Monooxygenase FAD-bind. 18+26: Transmembrane protein. 20: outer membrane lipoprotein carrier protein, LolA. TE: Thioesterase. GT: Glycosyl transferase. 25: AMP dependent synthetase and ligase. AT: Phospholipid/glycerol acyltransferase. Numbering of genes refers to last two numbers in ORF numbering (16 = ORF03616).



A: Violacein: R_1 = OH, R_2 = H
B: Deoxyviolacein: R_1 = R_2 = H

Figure 1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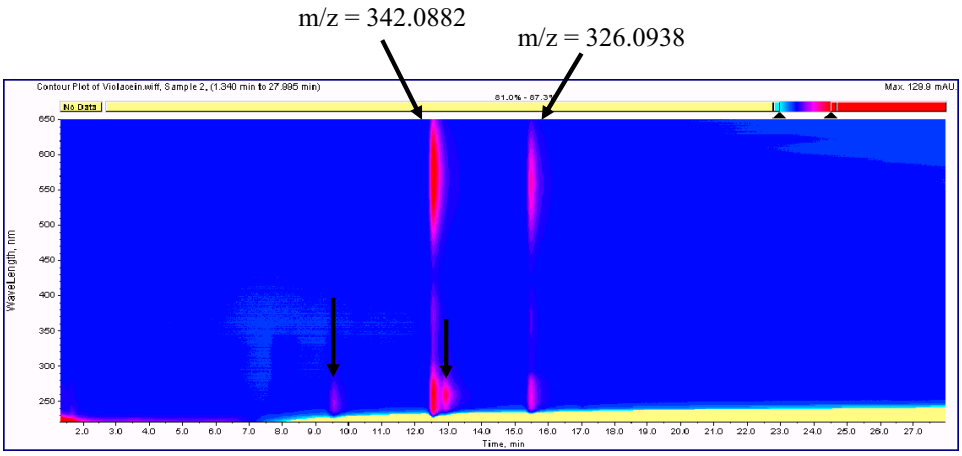
Collimonas CT MP11 E8
100
Collimonas CT MP11 E6
Herbaspirillum sp Hg 1
98
99
Collimonas fungivorans CTE227
99
Janthinobacterium lividum strain XT1
Chromobacterium violaceum strain ESBV 4400
Pseudoalteromonas luteoviolacea S2
53
Pseudoalteromonas sp.710P1
79
Pseudoalteromonas sp.520P1
Pseudomonas aeruginosa NGKCTS
0.2

Figure 2


m/z = 342.0882
m/z = 326.0938
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Max. 129.8 mAU
No Data
Wavelength, nm
Time, min

Figure 3

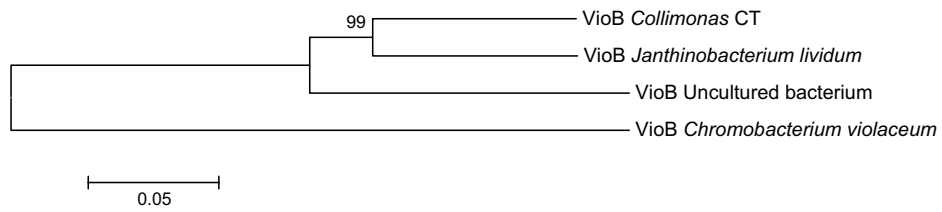
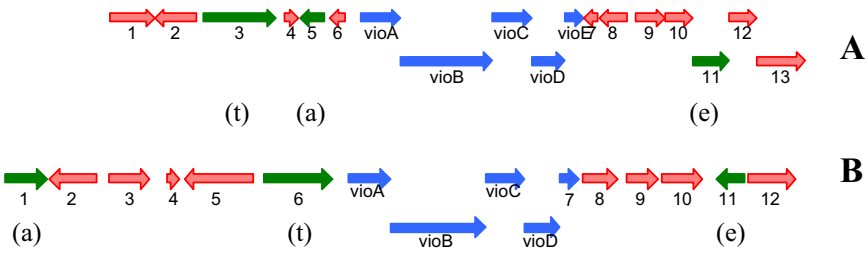

99
VioB *Collimonas* CT
VioB *Janthinobacterium lividum*
VioB Uncultured bacterium
VioB *Chromobacterium violaceum*
0.05

Figure 4


1
2
3
4
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vioA
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A
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1
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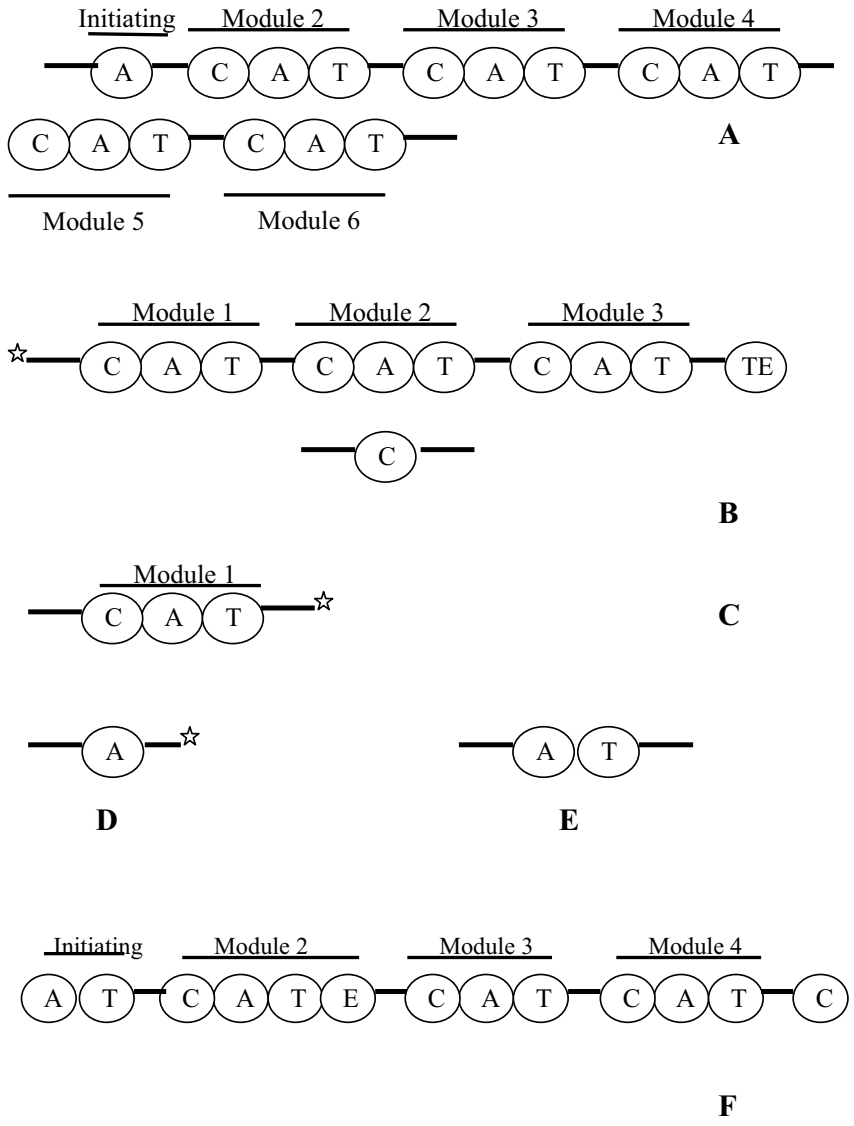
vioB
vioD
(e)

Figure 5


Initiating
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Module 3
Module 4
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C A T C A T
A
Module 5
Module 6
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Module 3
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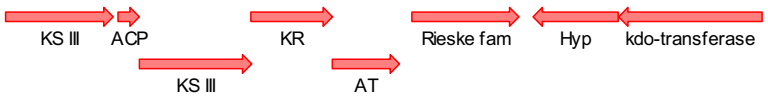
F

Figure 6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ACP
KR
Rieske fam
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kdo-transferase
KS III
AT

A

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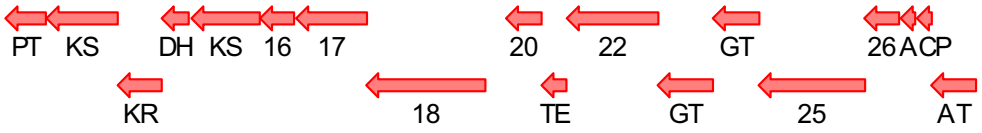
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ACP
KR
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TE
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25
AT

Figure 7