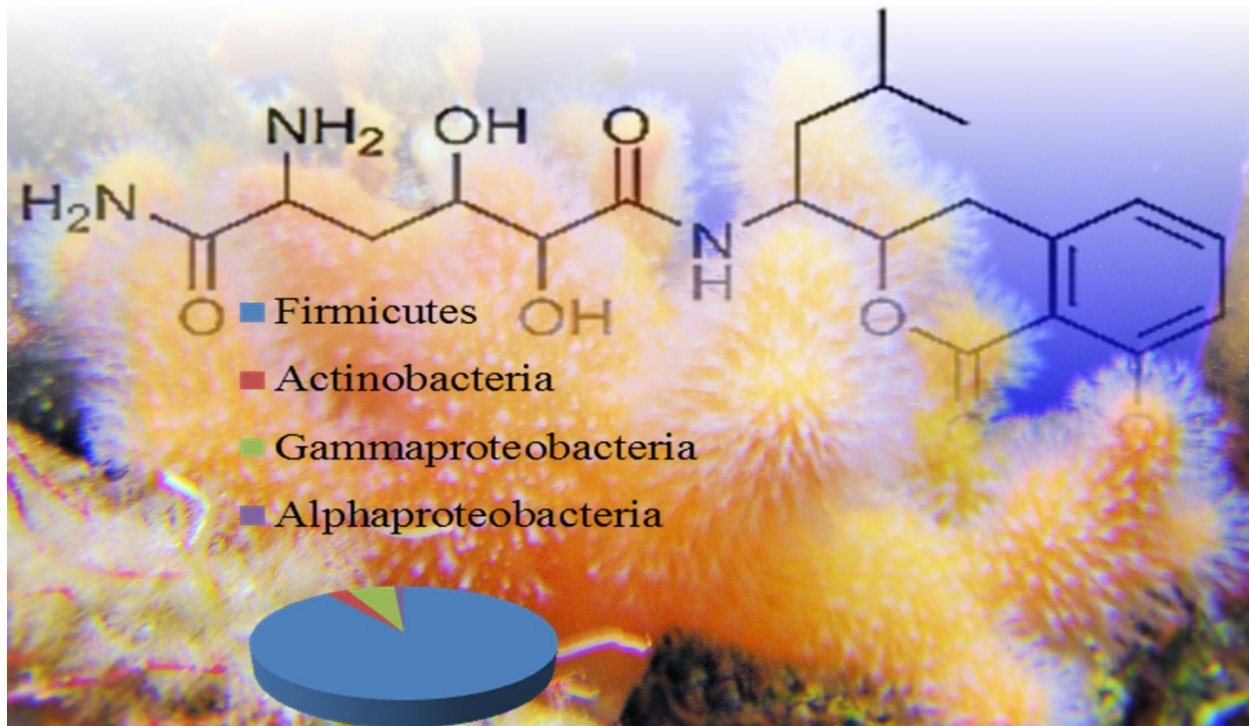


**Community of soft coral *Alcyonium digitatum*
associated bacteria and their antimicrobial activities**



Dissertation

**zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Christian-Albrechts-Universität zu Kiel**

Vorgelegt von

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgesellschaft verfasst habe, und dass sie nach Form und Inhalt meine eigene Arbeit ist. Diese Arbeit, auch Teile davon, wurden noch nicht veröffentlicht oder zur Veröffentlichung eingereicht. Sie wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Dies ist mein erstes und einziges Promotionsverfahren.

Kiel, den 01.10.2014

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Scientific contribution

Sampling of soft corals in the Baltic Sea was done by Gregor Steffen (GEOMAR). Sampling of soft corals in the North Sea was done by SCUBA diving team of AWI staff at Helgoland. All bioassays of crude extracts and pure compounds were done by Arlette Wenzel-Storjohann (Marine Microbiology, GEOMAR). NMR measurement was performed at the Otto-Diels Institute of Organic Chemistry (Christian-Albrechts University of Kiel) and NMR data analysis was done by Bin Wu (GEOMAR).

All other experiments were done by Pham Thi Mien under supervision of Johannes F. Imhoff.

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SUMMARY

Discovery of novel natural products from soft corals were reported with many achievement so far. Soft coral associated bacteria have gained interest for several reasons such as for marine environmental protection and bio-mining of natural products from microorganism as compared to the animals. This work focused on communities of soft coral associated bacteria and on the search for new antimicrobial substances and new natural products as potential candidates for new drugs. Thus, soft coral associated bacteria were quite abundant and good sources of antimicrobial producers.

The results from cultivation-based studies and sequence analysis of 16S rRNA genes of bacterial communities associated with the soft coral *A. digitatum* are reported in this study. A total of 251 isolates were identified as belonging to 4 phyla *Firmicutes*, *Actinobacteria*, *Gammaproteobacteria*, and *Alphaproteobacteria*. Four of these isolates may be new species, 2 isolates of *Firmicutes*, 1 *Gammaproteobacterium* and 1 *Alphaproteobacterium*. The high diversity of the bacterial communities and their antimicrobial activities suggest the contribution of the associated bacteria to the coral health.

The results from antimicrobial tests demonstrated that *A. digitatum* was the source of various bacteria producing antimicrobial substances. Antimicrobial activity of the isolates were found only in BM medium (3%), only in GYM medium (2%) or in both of these media (44%). Quite significant, all isolates belonging to the two species *B. amyloliquefaciens* and *B. methylotrophicus* showed antimicrobial activities. Some strains of the two species inhibited all four test microorganisms. Chemical analysis revealed that the two *Bacillus* sp. strains produced various known cyclic peptides as well as presumably new metabolites. They represented highly potent producers of antimicrobials. All isolates of *Actinobacteria* produced antimicrobial substances, that inhibited particulary Gram-positive bacteria.

In addition to a marine *Micrococcus* isolate, three isolates of *Firmicutes* were thoroughly studied in regard to analysis of production of new compounds. In addition a number of known compounds were identified, including several peptides. The metabolites of the four strains were examined and purified by using several HPLC systems. The

biological activities of pure compounds were studied using antibacterial, antifungal, cytotoxic, and enzyme assays. Eleven compounds were purified and applied to bioassays. One of the proposed known cyclic peptides showed antimicrobial activity against both *B. subtilis* and *E. coli*, whereas the others showed inhibition of methicillin-resistant *Staphylococcus aureus* (MRSA) and phosphodiesterase 4 β 2. One of compounds, which were considered as new compounds, inhibited fungus *Trichophyton rubrum* and enzyme phosphodiesterase 4 β 2. The structure elucidations of all pure compounds are in progress.

ZUSAMMENFASSUNG

Die Entdeckung von biologisch aktiven Substanzen aus Weichkorallen führte bereits zu großen Errungenschaften. Bakterien, die mit Weichkorallen assoziiert sind, stehen aus verschiedenen Gründen im Mittelpunkt des Interesses. Beispielsweise können sie eine Schutzfunktion für die Koralle vor schädlichen Umwelteinflüssen ausüben, zum anderen dienen sie der Gewinnung von Wirkstoffen. Die vorliegende Arbeit beschäftigt sich mit den Gemeinschaften von Korallen-assoziierten Bakterien sowie mit der Suche nach neuen antimikrobiellen Substanzen und nach neuen Wirkstoffen, die als Kandidaten für potentielle Medikamente in Frage kommen können. Hilfreich ist hierbei, dass Bakterien, die mit Weichkorallen vergesellschaftet sind, sehr häufig auftreten und eine vielversprechende Quelle für antimikrobielle Substanzen sind.

Die Ergebnisse der kultivierungsabhängigen Untersuchungen und die Analyse der 16S rRNA Gene von Bakterien, die mit der Weichkoralle *A. digitatum* assoziiert sind, werden dargestellt. Insgesamt wurden 251 Isolate identifiziert und den 4 Phyla *Firmicutes*, *Actinobacteria*, *Gammaproteobacteria* und *Alphaproteobacteria* zugeordnet. Vier dieser Isolate sind vermutlich Repräsentanten neuer Arten, von denen zwei zu den *Firmicutes*, eine zu den *Gammaproteobacteria* und eine zu den *Alphaproteobacteria* zählen. Die große Diversität der bakteriellen Gemeinschaften und die antimikrobiellen Aktivitäten der einzelnen Mitglieder weist auf eine positive Auswirkung der assoziierten Bakterien auf die Gesundheit der Koralle hin.

Die Ergebnisse der antimikrobiellen Testungen zeigten, dass *A. digitatum* eine gute Quelle für verschiedene Bakterien darstellt, die antimikrobielle Substanzen produzieren. Nahezu 50% der Isolate war antimikrobiell aktiv. 44% wiesen Aktivitäten sowohl nach Anzucht in BM Medium als auch nach Kultivierung in GYM Medium auf. Bei 3% der Stämme konnten Aktivitäten nur nach Verwendung des BM Mediums nachgewiesen werden, bei 2% nur bei Anzucht in GYM Medium. Auffällig war, dass alle Isolate, die mit den Arten *Bacillus amyloliquefaciens* and *Bacillus methylotrophicus* verwandt sind, antimikrobielle Aktivitäten zeigten. Einige dieser Stämme hemmten sogar das Wachstum aller vier Teststämme. Die chemische Analyse zeigte, dass zwei *Bacillus* sp. Isolate verschiedene bekannte zyklische Peptide aber auch vermutlich neue Substanzen

produzieren. Alle Isolate der *Actinobacteria* produzierten Substanzen, die vor allen Gram-positive Bakterien hemmten. Diese Stämme haben eine hohes Potential zur Produktion neuer antimikrobieller Wirkstoffe.

Sowohl ein *Micrococcus* sp. Isolat als auch drei *Firmicutes* sp. wurden ausführlich in Bezug auf die Bildung neuer Substanzen hin untersucht. Eine Anzahl bekannter Strukturen wurde identifiziert, einschließlich verschiedener Peptide. Die Metabolite der vier Stämme wurden untersucht und mittels unterschiedlicher HPLC- Systeme aufgereinigt. Die biologischen Aktivitäten von insgesamt 11 Reinsubstanzen gegen Bakterien, Pilze, Tumorzelllinien und Enzyme wurden ermittelt. Eines der bekannten zyklischen Peptide hemmte das Wachstum von *B. subtilis* and *E. coli*. Andere Peptide hemmten den Methicillin resistenten *Staphylococcus aureus* (MRSA) Stamm und das Enzym Phosphodiesterase 4 β 2. Eine Substanz, die vermutlich neu ist, wirkte gegen den Pilz *Trichophyton rubrum* and Phosphodiesterase 4 β 2. Die Strukturaufklärung aller Reinsubstanzen läuft derzeit noch.

INTRODUCTION

1. Soft coral *Alcyonium digitatum* (Linnaeus, 1758)

Like sponges, soft corals are sessile filter feeders. The coral polyps are colonial form with 8 tentacles which capture prey from water column. Like hard corals, soft corals exude mucus by their polyps. Coral mucus plays a very important role as energy carrier and particle trap in the reef ecosystem (Wild et al. 2004; Bythell and Wild, 2011). The polyp conveys oxygen and brings a supply of food as mucus via coral mouth through hollow polyp body that has cylindrical shape. Unlike hard corals, soft corals have numerous tiny, needle-like spicules in their tissues. The spicules give these corals some physical support and give spiky and grainy texture of the polyps and colonies that deters predators (van Alstyne et al. 1992).

The soft coral *Alcyonium digitatum* is a species of the Alcyoniidae family, order Alcyonacea, class Anthozoa, phylum Cnidaria. It is a common boreal species. Its habitats are found around the Atlantic coast of North West Europe from Portugal to Norway, with further reports from Britain, Ireland and found in North America. In the Irish Sea, this species is abundant and is commonly dominant in strong current movement where the otherwise algae are limited due to lack of light (Robin, 1968; Hartnoll, 1975). In the absence of current flow, the pattern of expansion and contraction of polyps and tentacle of *A. digitatum* was not different during light (day time) and dark (night time) periods. However, under high flow conditions, coral polyps and tentacles expanded greatly (Bell et al. 2006).

There are two distinct colours with orange morphs and white morphs. The sex ratio is nearly equal to each colour form. The majority of colonies are either male or female. Hermaphrodites were not found amongst several hundred colonies (Hickson, 1895). However, later hermaphrodites were observed in this soft coral at the same study area (Hartnoll, 1975; Matthews, 1917). The largest specimen has a height of 10 cm, a width of 20 cm and a weight of 1 kg between 5 and 10 years old. The life span certainly exceeds 20 years (Hartnoll, 1975).

This soft coral expands and feeds regularly from February to July. The colonies of *A. digitatum* remain contracted for a long period from late July to December, during which time they do not feed and assume a shrunken appearance with a reddish or brownish colour (Figure 1.1). Due to long quiescent period inactivity, the surface of colonies of *Alcyonium* is attractive for foreign settlement. It has been noted, “the ectoderm is almost invariably covered by a coat of transparent mucus, in which grains of sand, minute algae and other foreign bodies occur” (Hickson, 1895). Diatoms, prostrate thalloid, filamentous algae, hydroid, and amphipod are contributed into film of epibiota on the surface of coral colonies (Hartnoll, 1975).

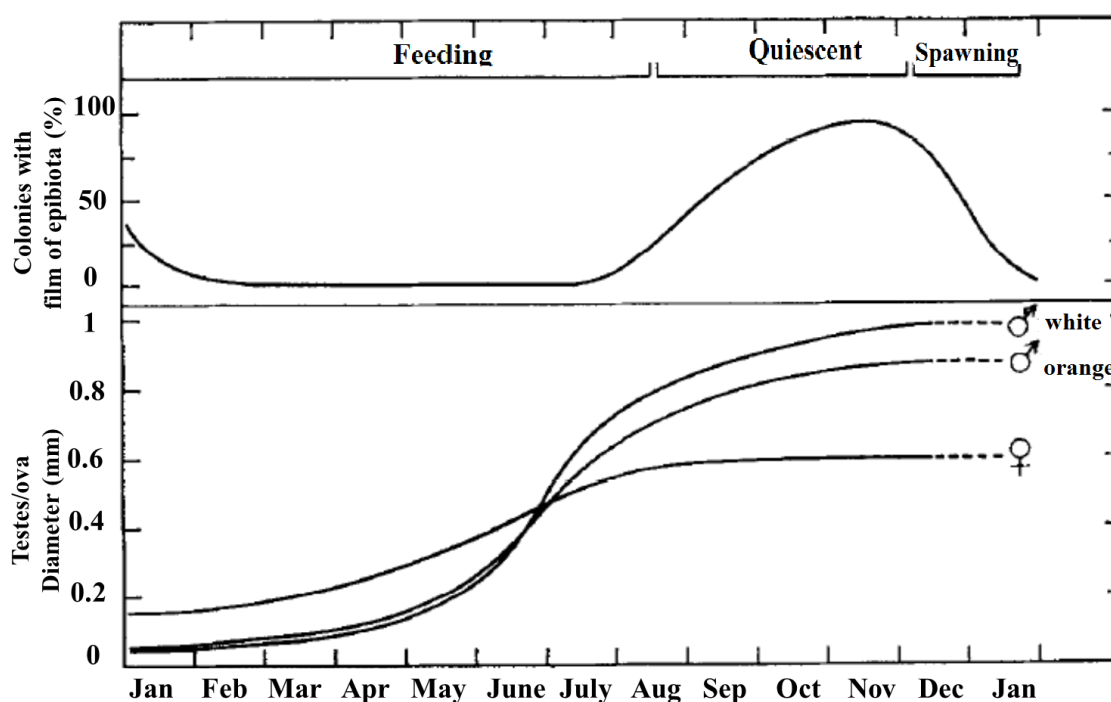


Figure 1.1 The annual cycle of *A. digitatum*. The upper part shows the variation in the proportion of colonies with a prominent film of epibiota on the surface. The lower part shows the maturation of gonads (Hartnoll, 1975).

Small gonads already exist in December and January at that time when the ripe gametes matured over the previous year are being spawned. The prolonged inactivity coincides with the final months of gonad maturation and the film of epibiota slough off immediately precedes the spawning of the gametes. Gametes are expelled from the mouths of the partly expended polyps into the ambient water and fertilization occurs externally (Hartnoll, 1975). The spawning of a colony takes several consecutive days. The zygotes

develop to planulae (free-swimming larva of a cnidarian) after 5 to 7 days and most planulae settle and metamorphose to polyps within one or two following days (Matthews, 1917).

The soft coral *A. digitatum* from the Atlantic coast of Scotland were examined for chemical defences. Methanol extracts of both corals contained substances acting as feeding deterrents for the test fish Dover sole *Solea solea* (Mackie, 1987).

2. Bacteria and coral hosts

2.1 Where we found the bacteria in coral hosts?

For now, there is no article that deals with the bacteria localized in soft coral. Microorganisms found in hard coral host are shown in Figure 1.2. In this model, hard coral *Oculina patagonica* was examined, microorganism were found from mostly whole of coral colony (Rosenberg et al. 2007).

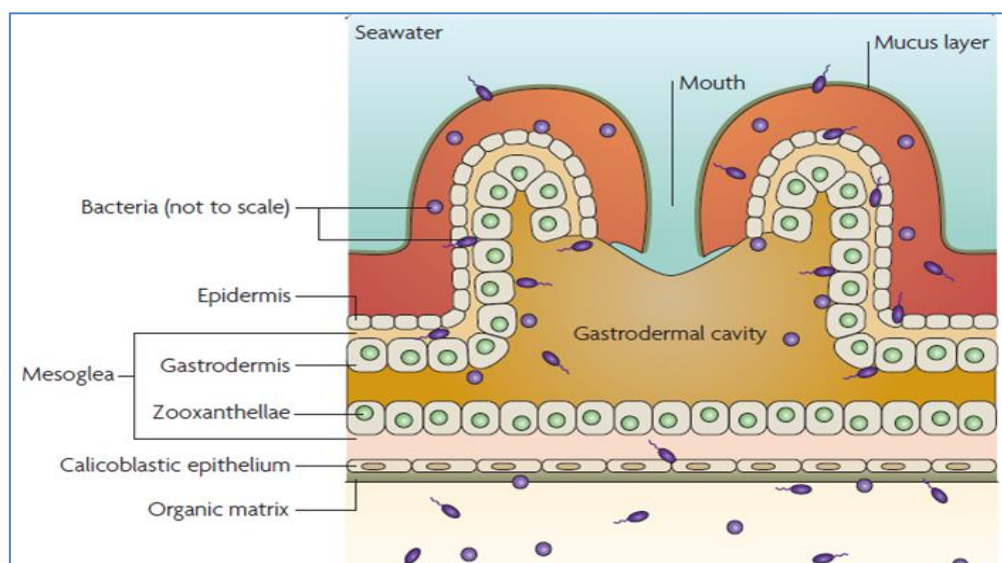


Figure 1.2 Microorganisms in coral host (Rosenberg et al. 2007).

Studies of the diversity of microorganisms associated with soft coral *A. antarcticum* by transmission electron microscopy (TEM) reported that bacteria were detected in the surrounding of the epidermis but not in the subcuticular layers and the mesoglea. However, except epidermis and tentacle regions, bacteria were found within most areas of the coral tissue. Particular high densities of bacteria were detected in the gastro vascular and

mesentery regions by fluorescence in situ hybridization (FISH) (Webster and Bourne, 2007).

In soft corals, bacteria can be found in mucus layer, coral tissues and also in spiny skeletal elements called sclerites but not in the skeleton of hard coral. Because of the most untapped sources, soft coral associated bacteria are likely to lack of information in comparison with sponge-associated microorganisms. The contribution of microorganism to contribute to sponge biomass was found in some sponges to amount to 40% of the sponge biomass (Imhoff and Stöhr, 2003). However, concerning occupation, location, and quantity of bacteria in soft corals no specific information is available.

2.2 Coral associated bacteria

Corals are of particular interest for scientific research for several reasons. On the one hand, effects of climate change are proposed to have a major impact on coastal zone ecosystems in general and on coral reefs in particular. It has been predicted that over 50% of the coral reefs in the world may be destroyed by the year 2030 by global climate change mediated temperature increase (Lough and Barnes, 2000). On the other hand, microorganisms associated with marine invertebrates such as sponges, corals and algae are potent producers of biological active substances with prominent activities and a rich source of novel compounds (Imhoff et al. 2011).

Because of human diseases such as cancer, microbial infections and inflammatory processes as well as quickly increasing antibiotic resistance, the search for antimicrobials becomes more and more urgent. Research on bacterial communities and their antimicrobial compounds from marine microorganisms has focused on associations with sponges (Muscholl-Silberhorn et al. 2008; Thiel and Imhoff, 2003; Thiel et al. 2007), algae (Wiese et al. 2009), ascidians (Tait et al. 2007), holothurian (Ward-Rainey et al. 1996), protozoa (Heindl et al. 2010) and mollusks (Romanenko et al. 2008). Bacteria, fungi, and algae were isolated from soft corals as well and some displayed antifouling activity (Slattery et al. 1995).

Research on bacteria associated with corals has been focussed on hard corals. Studies on the genetic diversity of bacteria associated with corals have revealed extreme species richness and abundances. A vast number of distinct bacterial groups were found

from both coral tissues and coral mucus. *Proteobacteria* (*Gamma*-, *Alpha*-, and *Betaproteobacteria*) were revealed as most abundant coral associated bacteria (Rohwer et al. 2001; Rohwer et al. 2002; Frias-Lopez et al. 2002; Lampert et al. 2008; Bourne and Munn, 2005). Analyses of the diversity of marine microorganisms via molecular techniques have made many advances within the last 15 years. However, many bacteria that can be detected by DNA sequences in the environmental sample cannot be grown on agar plates and in culture media under standard conditions (Pedros-Alio, 2006). Nevertheless, traditional methods and cultivation in pure culture are still needed in order to determine the physiological properties and e.g. the ability to produce antimicrobial compounds and determine their biological activities.

Using culture-base methods, 16S rRNA gene sequence analysis, biochemical testing and antimicrobial susceptibilities have been employed to analyse the diversity of marine microorganisms in mucus of the hard coral *Fungia scutaria* in the Red Sea (Lampert et al. 2006), and a cold water coral *Lophelia pertusa* in the deep sea (Galkiewicz et al. 2011). It was shown that the main bacterial groups associated with the mucus of *F. scutaria* were identified as *Gammaproteobacteria*, *Alphaproteobacteria* and *Actinobacteria* and that more than 30.0% of the 22 different isolates belonged to novel species and a new genus (Lampert et al. 2006). *Gammaproteobacteria* were also the most abundant bacteria associated with the cold water coral *L. pertusa*, but *Betaproteobacteria* and *Actinobacteria* were found in addition (Galkiewicz et al. 2011). A study on cultured bacteria associated with the gorgonian corals *Paragorgia arborea*, *Plumarella superba*, and *Cryogorgia koolsae* from the Aleutian Islands reported that *Gammaproteobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, and *Actinobacteria* were most abundant (Gray et al. 2011). By a culture dependent approach, a total of 36 bacterial strains were recovered and identified from the black coral. They belonged to three bacterial phyla i. e. *Firmicutes*, *Actinobacteria* and *Alphaproteobacteria*. In addition 24 fungal isolates affiliated to four fungal orders *Eurotiales*, *Hypocreales*, *Pleosporales* and *Botryosphaeriales*. A high proportion (51.6%) of microbial isolates displayed distinct antibacterial and antifungal activities (Zhang et al. 2012). Isolates (a total of 76) from four species of South China Sea gorgonians were affiliated to 21 species in 7 genera and most abundant were representatives of the genus *Bacillus* which exhibited a high antimicrobial potential (Peng et al. 2013). *Pseudoalteromonas* spp. and *Vibrio* spp. were most dominant among isolates from the coral *Acropora millepora* in the Great Barrier Reef and displayed good antimicrobial activity against a range of bacteria (Kvennefors et al. 2012). Bacteria associated with the hard coral

Acropora digitifera from Hare Island, the Gulf of Mannar were assigned to *Firmicutes*, *Gammaproteobacteria* and *Actinobacteria*. Both isolates from coral mucus and from coral tissue showed antimicrobial activity. The presence of *Actinobacteria* was established in both the coral mucus and the coral tissue. They produced substances with high activity against pathogens *Staphylococcus aureus* (ATCC11632), *Pseudomonas aeruginosa* (ATCC10145), *Aeromonas hydrophila* (ATCC7966), *Vibrio parahaemolyticus* (ATCC27519), and *Vibrio vulnificus* (ATCC29307) (Nithyanand and Pandian, 2009). *Roseobacter* sp. isolated from mucus of the Mediterranean coral *Oculina patagonica* displayed a broad spectrum of antimicrobial activities against the coral pathogens *Vibrio coralliilyticus* and *Thalassomonas loyana*. This finding suggested the possibility of probiotic effects on microbial communities to the coral holobiont (Nissimov et al. 2009).

Quite a few studies have so far been reported on bacteria associated with soft corals and their antibiotic activities. The study of bacterial communities of soft corals *Dendronephthya* sp. in Port Shelter, Hong Kong reported a distinctive epibiotic community, of which 55.0% belonged to the *Gammaproteobacteria*, 27.0% to the *Alphaproteobacteria*, and 18.0% to the *Bacteroidetes*, respectively (Harder et al. 2003). The bacteria associated with *Dendronephthya* sp. were thought to be contributing to antifouling mechanisms, because they could inhibit larval settlement of the tubeworms *Hydroides elegans* (Dobretsov and Qian, 2004). The diversity of bacteria associated with the soft coral *Alcyonium antarcticum* from Ross Sea, Antarctica was investigated across three different contaminated sites by culture-based and molecular techniques (denaturing gradient gel electrophoresis-DGGE, 16S rRNA gene clone library, and FISH). Phylogenetic analysis of these bacteria showed a close affiliation with psychrophiles from the Antarctic region and high abundance of *Gammaproteobacteria* clades. This study supported the view that specific coral-microbial interactions exist (Webster and Bourne, 2007).

In a comparative study between stony corals and soft corals, bacteria associated with six stony corals (*Platygyra* sp., *Porites* sp., *Fungia granulosa*, *Favia* sp., *Stylophora* sp. and *Pocillopora* sp.) had a higher percentage of antibiotic activity as compared to bacteria associated with two soft corals *Rhytisma fulvum* and *Xenia* sp. (Shnit-Orland and Kushmaro, 2009).

3. Biotechnological potential of soft corals and coral associated bacteria

Marine invertebrates are considered to be a promising source of bioactive compounds to investigate drugs from the sea. Beside foremost good candidate sponges, cnidarians now attract many scientists to exploit marine natural products (MNPs). The soft corals (order Alcyonacea) have contributed with 98.1% of new natural products from sub-class Octocorallia that belongs to the class Anthozoa. The class Anthozoa consisted 99.0% of MNP reported from Cnidaria (Leal et al. 2012). They were most interesting compounds such as antitumor, anti-human immunodeficiency virus, anti-inflammatory, antifouling, antiulcer, and antimicrobial compounds. Antitumor drugs are the main area of interest in the screening of MNPs from cnidarians (41.0%, Figure 1.3).

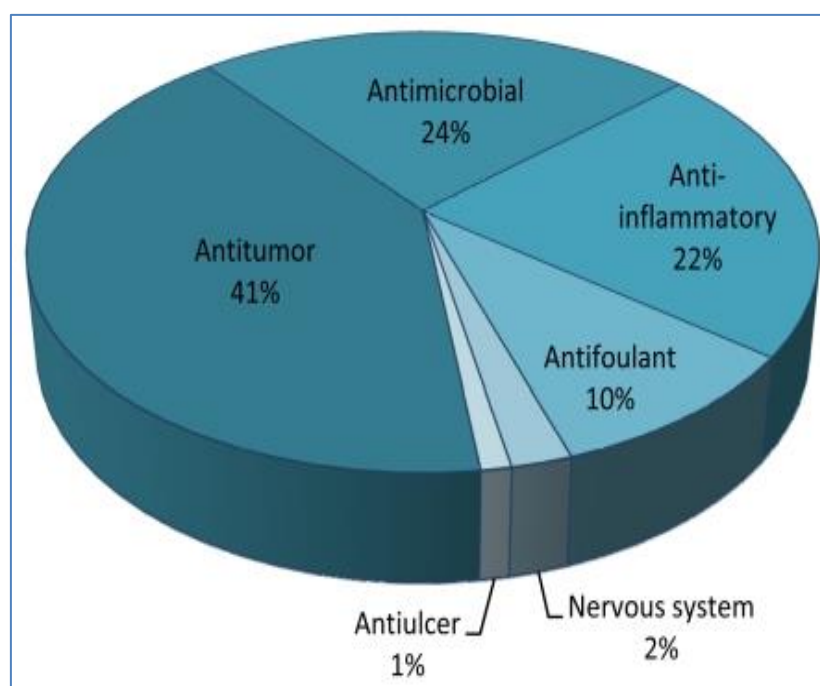


Figure 1.3 Distribution in drug classes of marine bioactive compounds with high biotechnological potential studied from cnidarian species (Rocha et al. 2011).

A total of 5,286 natural products were reported in the last decade (2000 to 2009), most of them were terpenoids (40.5%). Cnidaria were the sources of terpenoids with 54.5% of the total of 2,256 natural products (NPs) (Leal et al. 2012). Rocha et al. (2011) reported that terpenoids (terpenoid, diterpenoid, sesquiterpenoid, sesterterpenoid, cembranoid) are the main chemistry group within the MNPs analysis (61.0%).

It is clear that Cnidaria (especially in order Alcyonacea and order Gorganacea) are a rich source of bioactive compounds. Today over 3000 MNPs have been described from this phylum, most in the last decade (Rocha et al. 2011). Although significant chemical diversity and promising candidates for drug development were revealed from Cnidaria, to the best of our knowledge, no metabolite from them has yet reached the further investigation such as pharmaceutical clinical pipeline or entered clinical trials.

Over 200 species of hard corals (2/3) and soft corals (1/3) were harvested every year from coral reefs to supply the marine aquarium trades. However, NP discovery has been focused on soft corals much more than on hard corals because of biased bioprospecting efforts. Screening efforts have been made with around 30 stony coral species in comparison with approximately 300 soft coral species (Blunt and Munro, 2012). Coral mariculture (*in situ*) and captive breeding (*ex situ*) are offered to provide material for drug discovery. In view of the fact, that coral aquaculture is still in its infancy and has a number of unresolved problems that need to be considered, a number of questions need to be answered, e.g. who is producer of the compounds, the host or the associated microorganisms and what drives metabolite production? How can metabolite production be optimized? (Leal et al. 2013).

Although marine invertebrates show a huge diversity of metabolites, increasing evidence suggested that their symbiotic microorganisms are the true sources of these bioactive compounds in many instances, most obviously in the case of sponges (Piel, 2004). Corals were no exception. The pseudopterosin, a potent anti-inflammatory inhibitor of diterpene cyclase, was obtained from the marine octocoral *Pseudopteroergorgia elisabethae* (Roussis et al. 1990; Correa et al. 2011) and was used in commercially available skin care products. However, the site of biosynthesis of pseudopterosin was found in a macroalgal preparation by using differential centrifugation and it was concluded that the coral symbiont dinoflagellate *Symbiodinium* sp was the original pseudopterosin producer (Mydlarz et al. 2003).

Additionally, the significant increase of the number of NPs isolated from marine microorganisms demonstrated their attraction for scientists in efforts to exploit new natural compounds.

A summary of the number of NPs isolated from sponges, cnidarians and marine microorganisms from 2008 to 2012 is shown in Table 1.1. Data sources are found in the reviews of Blunt et al. (2010-2014).

Table 1.1 Number of NPs reported from sponges, cnidarians and marine microorganisms.

Sources	Years				
	2008	2009	2010	2011	2012
Sponges	278	287	283	296	355
Cnidarians	264	201	188	290	213
Marine microorganisms	208	273	312	405	> 450

In contrast to soft corals harvested for NPs research (normally in large amounts of kg), only tiny amount of the original samples are needed to isolate microorganism. Cultivation, change in cultivation conditions and other factors to optimize natural product production is easily implemented.

Biological activity of coral-associated bacteria is not only restricted to activity against bacteria, fungi but has also been found against tumour cell lines, for example the cyclic depsipeptide thiocoraline which showed antitumor activities (breast, colon, renal, and melanoma) and was obtained from a *Micromonospora* isolate derived from an unidentified soft coral collected in the Indian Ocean near the coast of Mozambique (Romero et al. 1997). Therefore, studies of microorganisms associated with soft corals can lead to answer the question about the host and pathogen and can open interesting research in term of exploiting new drug from nature.

The present study for the first time analyses the diversity of bacteria associated with the soft coral *Alcyonium digitatum* and their antimicrobial activities using culture-based approaches and 16S rRNA gene analysis to identify the isolates.

4. Thesis outline

The diversity of hard coral associated bacteria has been reported so far, however, there were only few reports on soft coral associated bacteria. Searching of new metabolites from marine bacteria associated with corals has been increased recently. It is clear that research on soft coral associated bacteria now is at its beginning. The aim of this study is the investigation of the diversity and the biotechnological potential of bacteria associated with the soft coral *Alcyonium digitatum*. In particular, three objectives were addressed:

Diversity of bacteria associated with the soft coral by cultivation-dependent approach.

Soft corals were sampled in the Baltic Sea for the investigation of bacterial communities and the antimicrobial activities of the isolates. Soft coral associated bacteria were isolated in four different media and at two different temperatures. The diversity of isolates was conducted by using of cultivation based methods and 16S rRNA gene sequence analysis. The sequences were compared with BLAST/NCBI. By the other hand, cultivation dependent work also pays the way for searching of bioactive compound producer bacteria.

Antimicrobial activities of the isolates. All bacterial isolates obtained in the cultivation studie were analysed in regard to the production of new metabolites. The antimicrobial activities of the isolates were screened in two different media. Broth cultures were extracted by organic solvent and tested for antimicrobial activity against a standard set of Gram-negative (*Escherichia coli*) and Gram-positive bacteria (*Staphylococcus epidermidis* and *Bacillus subtilis*) and a human pathogenic yeast (*Candida albicans*). The crude extracts also were applied for chemical screening with high-performance liquid chromatography diode array detection mass spectrometry (HPLC-DAD-MS), and dictionary of natural products (DNPs).

Discovery of natural products from coral associated bacteria. Based on data of metabolites and antimicrobial activites, the following strains were chosen for scale up big volume cultivation: Inhibition of all four test organisms or specific inhibition (one test organism), possible new metabolite producers, easily to growth in screening media. The natural substance were purified with several HPLC. The pure compounds were conducted both chemical and biological analyses in order to identify the biological activity and identify the antimicrobial substances.

MATERIAL AND METHODS

1. Sampling and cultivation processes

Nine living soft coral colonies *Alcyonium digitatum* with white or orange colour were randomly collected at three different sampling sites (Table 3.1). All the sampling sites were located in the Baltic Sea.

Three soft coral colonies were collected at each station. To remove loosely attached microbes from their surfaces, the soft corals were washed three times with 0.2- μm -filtered sterile seawater for 5 minutes. Each colony was cut into 4 branches with a sterile scalpel, and 3 out of 4 branches were stored in 50 ml tubes and kept in -10°C for molecular analysis. The last branch was kept in 16 ml of filtered-sterile Baltic Sea water in a 50 ml tube and stored at 4°C until cultivation.

The soft coral samples from 16 ml of filtered-sterile Baltic seawater were homogenised with Ultraturrax (IKA T25 basic; 30 s, 17.500 speeds; steel tip sterilized with 70% ethanol and washed with filtered seawater immediately before use). Ten-fold serial dilutions of the homogenised samples were prepared to a dilution of 10^{-5} and 100 μL of each dilution were spread into triplicate on 4 different media.

- Tropic Marine Medium (TM)

5.0	g	Peptone from soya bean	Merck
1.0	g	Yeast extract	Bacto
15.0	g	Agar	Difco
30.0	g	Tropic marine salt	Wartenberg/Angersbach
1000	ml	Distil water	

- Baltic Seawater Medium (BS)

15.0	g	Tropic Marine Salt	Wartenberg/Angersbach)
15.0	g	Agar	Difco
1000	ml	Baltic Sea water (filtrated, 15‰)	Whatman (0.2 μm)

- R2A Medium (R2A)

0.5	g	Yeast extract	Bacto
0.5	g	Proteose peptone	Bacto
0.5	g	Casamino acids	Bacto
0.5	g	Glucose x H ₂ O	Merck
0.5	g	Soluble starch	Merck
0.3	g	Sodium pyruvate	Roth
0.3	g	K ₂ HPO ₄	Merck
0.05	g	MgSO ₄ x 7H ₂ O	Merck
15.0	g	Agar	Difco
30.0	g	Tropic marine salt	Wartenberg/Angersbach
1000	ml	Distil water	

- Seawater Medium (SW)

5.0	g	Proteose peptone	Bacto
2.5	g	Yeast extract	Bacto
1.0	g	Glucose x H ₂ O	Merck
0.2	g	K ₂ HPO ₄	Merck
0.05	g	MgSO ₄ x 7H ₂ O	Merck
15.0	g	Agar	Difco
15.0	g	Tropic Marine Salt	Wartenberg/Angersbach
1000	ml	Baltic Sea water (filtrated, 15°/oo) Whatman (0.2 µm)	

Plates were incubated at 28°C. For isolation of slow growing bacteria plates were incubated at 10°C for several months. Representatives of each colony morphotype were serially streak-plated onto fresh media to obtain pure isolates. The pure isolates were conserved in 1000 µL Cryobank System tubes (Mast Diagnostica GmbH Reinfeld, Germany) following manufacturer and stored at -80°C.

2. DNA extraction and phylogenetic classification

Freshly and pure colony from a culture of each morphotype was suspended in 100 µl of DNA-free water for molecular biology (Sigma-Aldrich, USA). The bacterial suspension was centrifuged at 10,000 rpm/min for 15 min at 4°C, after taking off the supernatant, the

cells were re-suspended into 200 µl DNA-free water, added silica beads 0.1 mm diameter (BioSpec Products-Roth) and shaken with bead mill (type MM200, Retsch, Germany) for 6 minutes at 30 Hz/s frequency. The lysed bacterial suspensions were centrifuged at 10,000 rpm for 5 min. The suspension contained of extracted DNA was directly used for polymerase chain reaction (PCR) or stored at -20°C for subsequent analyses.

Amplification of the 16S rRNA fragment was performed using the universal primers, forward primer 27f (5'-GAGTTTGATCCTGGCTCAG-3') and reverse primer 1492r (5'-GGTTACCTTGTTACGACTT-3') or 1525r (5'-CGGGCGGTGTGTACAAGG-3') (Lane, 1991) in puReTaq™Ready-To-Go™ PCR Beads (Heathcare) with final volume of 25 µl including of 5 µl of DNA template, 1 µl of each primer at final concentration of 10 µM and H₂O (BioChemika, Sigma-Aldrich). PCR was performed with following conditions initial denaturation (2 min at 94°C) followed by 30 cycles of primer annealing (40s at 50°C), primer extension (90s at 72°C), and denaturation (1 min at 42°C), a final primer annealing (1 min at 42°C), and a final extension (5 min at 72°C).

To check the available of 16S rRNA gene in PCR products before applying to sequence, the PCR products were run in the gel electrophoresis. The gel was made with 2% agarose (Biozym, Germany) in 1 x TBE (1 mol/L Tris, 0.83 mol/L Boric acid, 10 mmol/L Na-EDTA) and dyed with SYBR Gold stain (Invitrogen). PCR products were mixed with gel loading blue (BioLabs, Germany) and loaded into the gel. Then, gel was run for 25 minutes at 150 volt (Power Pack P25, Biometra, Germany) and visualized under UV light (UV systeme, Fisherbrand). The presence of 16S rRNA in PCR products were compared to the DNA marker (Marker X, Roche, Germany) on the same gel.

Sequencing was performed with two forwards primers 342f (5'-TACGGGAGGCAGCAG-3'), 790f (5'-GATACCCTGGTAGTCC-3'), and reverse primer 543r (5'-ATTACCGCGGCTGCTGG-3'). All sequences were done at the Institute for Clinical Molecular Biology (University Hospitals Kiel, Germany). Sequence data were edited with SeqMan™II (DNAStar) and next relatives were determined by comparison to 16S rRNA genes from the National Centre for Biotechnology Information (NCBI) GenBank using database basis local alignment search tool (BLAST) searches (Altschul et al. 1990).

Type strain relatives of all isolates were determined by comparison of 16S rRNA genes using and the Ribosomal Database Project II (RDP-II) Sequence Match Program (Cole et al. 2014) and the tool "Align two or more sequences"/Nucleotide blast/NCBI

(<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Isolates with the same type strain were grouped into arbitrary taxonomic units (ATUs).

3. Extraction procedure for all isolates

The screening bacteria were re-cultivated in the fresh tropic marine (TM) agar medium for 3-5 days before inoculating into 300 ml Erlenmeyer flasks containing of 100 ml medium GYM (glucose 4 g/l, yeast extract 4 g/l, malt extract 4 g/l, tropical sea salt 15 g/l) and Bannett medium-BM (yeast extract 1 g/l, beef extract 1 g/l, tryptone Bacto 2 g/l, glucose 10 g/l, tropic marine salt 20 g/l) for 3 day cultivations with shaking at 120 rpm and 28°C.

The metabolites from both supernatant and bacterial cell were homogenised by using of UltraTurrax T25 basic (IKA-Werke GmbH & Co., Staufen, Germany) for breaking the cell at 17.500 U/min for 30s. The homogenised broth was extracted with ethyl acetate with ratio 1/1 (volume/volume). Then crude extracts were dried by vacuum rotation (Buchi, Switzerland). Dried extract were re-suspended in 1 ml of methanol (Merck). The extracts were applied to High-performance liquid chromatography diode array detection mass spectrometry (HPLC-DAD-MS) for chemical screening as well as used for antimicrobial tests with four organisms for antimicrobial screening.

4. Chemical and antimicrobial screening

Deionized water was purified by Milli-Q system (Millipore, Bedford USA). MiQ water, methanol (grade for HPLC-Merk) and acetonitrile (Sigma Aldrich) were used as solvents for HPLC. Solvent A: MiQ + 0.1% formic acid (FA), solvent B: Acetonitrile (ACN) + 0.1% FA.

All samples were dissolved in methanol and filtered through 0.2 µm PTFE syringe filter (Grahpic Controls-Germany) before applying in HPLC.

4.1 Chemical screening

The crude extracts were subjected to HPLC-DAD-MS using reversed phase C18 column (C18 column, size 100 x 3.00 mm-Phenomenex Onyx Monolithic) with solvent A and solvent B, application with gradient: 0 min 5% B, 4 min 60% B, 6 min 100% B flow 2

ml/min on the Hitachi Elite LaChrom system. By using of ion trap detector (Esquire 400, Bruker Daltonics) along with LaChrom system to detect the mass. The samples were measured at positive mode.

Hitachi Elite LaChrom system (standard HPLC) contained organizer, diode array detector L 2450 (DAD), pump L 2130, column oven L 2300, and autosampler L 2200.

The mass spectra and UV chromatography of the metabolites were analysed by using of Bruker Daltonics-CompassAnalysis 4.0 and Compasshystar Post Processing software respectively. Molecular weight, UV maxima and biological sources were used for identification of the compounds by using of dictionary of natural products DNPs (database DNPs, 2012).

Compounds were marked as unknown or possibly new compound when there was no hit from data of literature by searching of molecular weight, UV maxima and biological sources. The structures of possible known compounds were modified from the structure from database by searching of PubChem Compound (<http://www.ncbi.nlm.nih.gov>).

4.2 Antimicrobial activities screening

The determination of the antimicrobial activity was carried out on extracts from all isolates. The following test organisms were used: *B. subtilis* DSM 347 and *S. epidermidis* DSM 20044 as representatives of Gram-positive bacteria, *E. coli* DSM 498 as a Gram-negative strain, and the yeast *C. albicans* DSM 1386, all obtained from the German Culture Collection (DSMZ, Braunschweig, Germany).

TSB medium (1.2% tryptic soy broth, 0.5% NaCl) and M186/3 medium (0.1% yeast extract, 0.1% malt extract, 0.17% peptone from soybeans, 0.33% glucose) were used for bacteria and yeast cultivation, respectively. The indicator organisms were prepared overnight cultivation, diluted to an optical density (600 nm) of 0.01-0.05. 200µl of the cell suspension indicator organisms were added to each 96-well microtiter plate containing of 10µl of the crude extracts. 10µl of a resazurin solution (0.2 mg ml⁻¹ phosphate-buffered saline) was added to each well after 5 h incubation of microtiter plates at 37°C. Then the microtiter plates were continuous incubated at 37°C for 5-30 min.

To evaluate cell viability, the reduction of resazurin to resorufin was assessed by measuring the absorbance 600 nm (reference 690 nm). The resulting values were compared with a positive (10 μ M chloramphenicol for bacteria; 10 μ M nystatin for the yeast) and a negative control (no compound) on the same plate.

5. Cultivation and extraction procedures for four selected strains

Selected strains were re-cultivated in the media used for their isolation (TM for C-2-29, C-1-10, R2A for H-6, and SW for A-2-28) for 2 days at 28°C. Then the isolates were inoculated in 2 litre Erlenmeyer flasks containing of 1 litre of GYM (for C-2-29, A-2-28, and C-1-10) or BM (for H-6) media. Cultures were shaken at 120 rpm, 28°C for 4 days. The broth cultures were homogenised by breaking of the bacterial cell with UltraTurrax T25 and extracted two times with ethyl acetate 1:1 (v/v). After evaporation 0.05 mg of crude extract/ml methanol was applied to standard HPLC for analysis of interested compounds before applying to preparation.

6. Compound purification of four selected strains

As mentioned above, MiQ water, methanol and acetonitrile as well as solvents A and B were used as solvents for preparative HPLC. All samples were dissolved in methanol and filtered before applying in preparative HPLC.

6.1 Preparative HPLC with the VWR International LaPrep system

VWR International LaPrep system consisted of column C18 110A AXIA, 100 x 50.00 mm (Phenomenex Gemini), pump P110, detector P311, autosampler smartline 3900 (Knauer), dynamic mixing chamber (Knauer), fraction collector: LABOCOL Vario 2000.

Specific gradient of solvent A and solvent B were applied in this HPLC system for selected samples. VWR International LaPrep system separates the compounds or fractions by monitoring absorption at wavelength. Therefore, many of compounds were collected with this system. All products were contained in fractions and collected automatically into test tubes 180x18 mm (Reagenzgläser, Assistant, Germany). The solvents were removed by rotation vacuum. Then, fractions were re-suspended in methanol and transferred in storage vials (11.6x32 mm, Macherey-Nagel, Germany). Methanol was evaporated by gas N₂ at

40°C and stored at 4°C. The purification of separated compounds was checked by standard HPLC.

6.2 Preparative HPLC with the Merck Hitachi Elite LaChrom system 1

Merck Hitachi Elite LaChrom system 1 included reversed phase C18 110A AXIA column, size 100 x 21.20 mm (Phenomenex Gemini), organizer, autosampler L 2200, and diode array detector DAD L 2450.

Selected extracts and selected fractions were carried out separation with semi preparative HPLC in order to get pure compounds. With this system all products were manually collected with visible UV signals. All further steps to get pure compound were implemented as mentioned above.

6.3 Preparative HPLC with the Merck Hitachi Elite LaChrom system 2

Merck Hitachi Elite LaChrom system 2 consisted of 00G-4336-P0-AX Synergi 4u Polar-RP 80A column, size 250 x 21.20 mm 4 micron (Phenomenex), organizer, autosampler L 2200, and diode array detector DAD L 2450.

The different between LaChrom systems 1 and 2 was column, alternative the column was thought to benefit to purification of different compounds, so that the LaChrom system 2 was used for separation of selected fraction and for purify impure compounds.

7. Bioassays of purified compounds

The *B. subtilis* (DSM 347), *S. epidermidis* (DSM 20044), *Staphylococcus aureus* MRSA (DSM 18827), and *Propionibacterium acnes* (DSM 1897) were representatives of Gram-positive bacteria in addition *E. coli* (DSM 498) and *Klebsiella pneumoniae* (DSM 30104) were used for Gram-negative test. *C. albicans* (DSM 1386), *Septoria tritici* and *Trichophyton rubrum* were employed for antifungal tests. G. Rimbach (University of Kiel, Germany) kindly provided the mouse fibroblast cell line (NIH-3T3). The human hepatocellular carcinoma cell line (HepG2) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Those cell lines were implemented for cytotoxic assays. Four enzymes acetylcholinesterase (AChE), glycogen

synthase kinase, phosphodiesterase 4 (PDE-4 β 2) and protein-tyrosine-phosphatase were used for enzyme inhibition assays.

Antimicrobial activity test against *B. subtilis*, *E. coli* and *P. acnes* were performed as described by Schneemann et al. (2010). Antimicrobial testing in the same manner as *S. epidermidis* were applied for *S. aureus* MRSA and *K. pneumoniae* according to Silber et al (2013). A phytopathogenic *S. tritici* and a dermatophyte *T. rubrum* were tested according to Jansen et al. (2013). The cytotoxic activity against NIH-3T3, HepG2, and inhibitory against phosphodiesterase (PDE-4 β 2) were determined according to Schulz et al (2011). Inhibitory activity against acetylcholinesterase (AChE) and the human pathogenic yeast *C. albicans* were performed according to Ohlendorf et al (2012). The enzyme inhibition test for glycogen synthase kinase-3 β was performed according to Baki et al. (2007) and the test for protein tyrosine phosphatase was implemented following to Helaly et al. (2009).

The final concentration of the pure compounds used in the assays was 100 μ M for antibacterial and antifungal activity, 50 μ M for cytotoxic activity, and 10 μ M for enzymatic inhibition.

8. NMR analyses

NMR spectra were analysed on Bruker DRX 500 (500 and 125 MHz) for ^1H and ^{13}C NMR, respectively, using the residual solvent signals δH 3.31 ppm and δC 49.0 for MeOD_4 as internal references. Measurements of high-resolution mass spectra were performed on a benchtop time-of-flight spectrometer (mirOTOF-II, Bruker) with positive ESI. NMR measurement was performed at the Otto-Diels Institute of Organic Chemistry (Christian-Albrechts University of Kiel).

RESULTS

1. Phylogenetic analysis and identification of the isolates

The bacterial community of soft coral *Alcyonium digitatum* as represented by 251 pure culture isolates is composed of *Firmicutes* (4 genera, 23 species), *Actinobacteria* (2 genera, 4 species), *Gammaproteobacteria* (5 genera, 11 species), and *Alphaproteobacteria* (3 genera, 3 species). Information on sampling sites as well as numbers of total and of antimicrobial active isolates and ATUs, is given in Tables 3.1 and Table 3.2.

Table 3.1 The numbers of total isolates and active isolates, as well as the number of total ATU and active ATU are showing for each sampling sites. The samples were collected in 23-30 m depth by dredging (140 cm x 30 cm) in Kattegatt, Baltic Sea on 4th May 2011.

Sampling sites	Sample styles	Number of bacteria		ATU	
		Isolates	Active	Total	Active
1. Baltic 1 (57°00.773 N-11°34.835 E)	A-1	30	8	13	5
	B-1	35	13	14	6
	C-1	27	16	12	5
2. Baltic 2 (56°58.805 N-11°35.166 E)	A-2	29	12	12	5
	B-2	17	9	10	5
	C-2	31	13	14	7
3. Baltic 3 (56°42.041 N-11°44.185 E)	A-3	19	13	7	2
	B-3	32	20	13	6
	C-3	31	18	9	3
Total		251	122		

In the following the term arbitrary taxonomic unit (ATU) is used as equivalent for species, if the sequence similarity with recognized type strains is >99.0%. The phylogenetic next neighbour and the corresponding type strains are indicated for each ATU. Out of the 41 identified species, only 5 were found in all soft coral specimens or in all but 1. These are *B. amyloliquefaciens*, *B. methylotrophicus*, *B. pumilus*, *B. thuringiensis* and *Lysinibacillus fusiformis*. All other species were less frequent or occurred only occasionally (Table 3.2).

Most abundant isolates were *Firmicutes*, including representatives of the genera *Bacillus* (192 isolates, 17 ATUs-BA1 to BA17), *Exiguobacterium* (1 isolate, 1 ATU-EX), *Paenibacillus* (4 isolates, 1 ATU-PA) and *Lysinibacillus* (29 isolates, 4 ATUs-LY1 to LY4). Among 17 distinct *Bacillus* species isolated, most abundant species were *B. methylotrophicus* (BA8, 52 isolates) *B. thuringiensis* (BA16, 35 isolates) and *B. amyloliquefaciens* (BA2, 33 isolates) (Table 3.2).

The *Actinobacteria* isolated in the present study belong to *Blastococcus* and *Micrococcus*. The genus *Blastococcus* is represented by a single strain (ATU-BL), which is closely related to the type strain of *Blastococcus saxobsidens*. Two isolates each of *Micrococcus* were found of the three species *Micrococcus flavus* (ATU-MIF), *Micrococcus luteus* (ATU-MIL), and *Micrococcus yunnanensis* (ATU-MIY).

In total 18 *Proteobacteria* isolates were affiliated to 11 ATUs of *Gammaproteobacteria* and 3 ATUs of *Alphaproteobacteria*. The *Gammaproteobacteria* were identified as *Pseudoalteromonas aliena* (PSA1, 3 isolates), *Pseudomonas peli* (PSM2, 2 isolates), *Psychrobacter nivimaris* (PSY2, 2 isolates), and one isolate each of *Pseudoalteromonas prydzensis* (PSA2), *Pseudomonas guineae* (PSM1), *Psychrobacter alimentarius* (PSY1), *Psychrobacter piscatorii* (PSY3), *Shewanella baltica* (SH1), *Shewanella colwelliana* (SH2), *Shewanella putrefaciens* (SH3), and *Vibrio lentus* (VI).

The *Alphaproteobacteria* were identified as *Rhizobium vignae* (RH, 1 isolate), *Labrenzia alba* (LA, 1 isolate) and *Sulfitobacter marinus* (SU, 1 isolate). Unless otherwise stated, all isolates had a sequence similarity of >99.0% with the most closely related type strain as indicated in Tables 3.2 and Table 3.3.

Isolates with sequence similarity less than 98.0% to the valid described species are assumed to be new species (Stackebrand and Ebers, 2006). A relative of *Psychrobacter nivimaris* 88/2-7^T (GenBank accession number AJ313425), the isolate A-1-28, had a similarity of 97.5% to this type strain. In addition, the isolate C-1-32 was found to be related to *Rhizobium vignae* CCBAU 05176^T (GenBank accession number GU128881) with 97.6% sequence similarity). Therefore, these two isolates and also strain A-1-2B (97.8% sequence similarity to *Lysinibacillus fusiformis* DSM2898^T) and isolate B-2-18 (97.5% sequence similarity to *Paenibacillus lautus* JCM 9073^T) may turn out to be representatives of new species.

Table 3.2 Number of isolates affiliating to the same type strains.

ATUs	Next related type strains	Baltic coral samples 1			Baltic coral samples 2			Baltic coral samples 3			Total number
		Number of isolates			Number of isolates			Number of isolates			
		A-1	B-1	C-1	A-2	B-2	C-2	A-3	B-3	C-3	
BA1	<i>Bacillus aerophilus</i> 28K ^T	0	0	0	0	0	0	0	1	0	1
BA2	<i>Bacillus amyloliquefaciens</i> NBRC15535 ^T	4	3	3	4	2	1	3	7	6	33
BA3	<i>Bacillus cereus</i> ATCC14579 ^T	1	1	0	1	3	2	0	2	0	10
BA4	<i>Bacillus fusiformis</i> NRS-350 ^T	0	0	1	0	0	2	0	2	0	5
BA5	<i>Bacillus hwajinpoensis</i> SW-72 ^T	0	0	0	0	0	0	0	1	0	1
BA6	<i>Bacillus licheniformis</i> DSM13 ^T	0	5	3	4	0	0	2	4	3	21
BA7	<i>Bacillus megaterium</i> IAM13418 ^T	0	0	0	0	0	0	1	0	0	1
BA8	<i>Bacillus methylotrophicus</i> CBMB205 ^T	1	6	9	3	0	5	10	9	9	52
BA9	<i>Bacillus niabensis</i> 4T19 ^T	0	1	1	4	0	1	1	0	1	9
BA10	<i>Bacillus pumilus</i> ATCC7061 ^T	1	1	2	1	4	1	0	1	3	14
BA11	<i>Bacillus safensis</i> FO-036b ^T	0	0	1	0	0	1	0	1	0	3
BA12	<i>Bacillus simplex</i> DSM1321 ^T	0	0	0	0	0	1	0	0	0	1
BA13	<i>Bacillus stratosphericus</i> 41KF2a ^T	0	1	1	0	0	0	0	1	0	3
BA14	<i>Bacillus subtilis</i> DSM10 ^T	0	0	0	0	1	0	0	0	0	1
BA15	<i>Bacillus subtilis</i> NRRLB-23049 ^T	0	0	0	0	0	0	0	1	0	1
BA16	<i>Bacillus thuringiensis</i> IAM12077 ^T	8	9	1	4	1	5	0	1	6	35
BA17	<i>Bacillus vallismortis</i> DSM11031 ^T	0	0	0	0	1	0	0	0	0	1
EX	<i>Exiguobacterium oxidotolerans</i> T-2-2 ^T	1	0	0	0	0	0	0	0	0	1
PA	<i>Paenibacillus lautu</i> JCM9073 ^T	0	1	0	0	2	0	0	0	1	4
LY1	<i>Lysinibacillus boronitolerans</i> 10a ^T	4	0	0	2	1	0	0	0	0	7
LY2	<i>Lysinibacillus fusiformis</i> DSM2898 ^T	4	0	3	1	1	2	1	1	1	14
LY3	<i>Lysinibacillus sphaericus</i> DSM28 ^T	0	0	0	1	0	0	0	0	0	1

ATUs	Next related type strains	Baltic coral samples 1			Baltic coral samples 2			Baltic coral samples 3			Total number
		Number of isolates			Number of isolates			Number of isolates			
		A-1	B-1	C-1	A-2	B-2	C-2	A-3	B-3	C-3	
LY4	<i>Lysinibacillus xylanilyticus</i> XDB9 ^T	0	2	0	0	0	4	0	0	1	7
BL	<i>Blastococcus saxobidens</i> DSM44509 ^T	0	0	1	0	0	0	0	0	0	1
MIF	<i>Micrococcus flavus</i> LW4 ^T	0	0	0	2	0	0	0	0	0	2
MIL	<i>Micrococcus luteus</i> DSM20030 ^T	0	0	0	2	0	0	0	0	0	2
MIY	<i>Micrococcus yunnanensis</i> YIM65004 ^T	0	0	0	0	0	2	0	0	0	2
PSA1	<i>Pseudoalteromonas aliena</i> KMM3562 ^T	0	0	0	0	0	3	0	0	0	3
PSA2	<i>Pseudoalteromonas prydzensis</i> MB8-11 ^T	1	0	0	0	0	0	0	0	0	1
PSM1	<i>Pseudomonas guineae</i> LMG24016 ^T	1	0	0	0	0	0	0	0	0	1
PSM2	<i>Pseudomonas peli</i> R-20805 ^T	0	2	0	0	0	0	0	0	0	2
PSY1	<i>Psychrobacter alimentarius</i> JG-100 ^T	1	0	0	0	0	0	0	0	0	1
PSY2	<i>Psychrobacter nivimaris</i> 88/2-7 ^T	2	0	0	0	0	0	0	0	0	2
PSY3	<i>Psychrobacter piscatorii</i> T-3-2 ^T	1	0	0	0	0	0	0	0	0	1
SH1	<i>Shewanella baltica</i> NCTC10735 ^T	0	1	0	0	0	0	0	0	0	1
SH2	<i>Shewanella colwelliana</i> ATCC39565 ^T	0	0	0	0	0	1	0	0	0	1
SH3	<i>Shewanella putrefaciens</i> LMG26268 ^T	0	1	0	0	0	0	0	0	0	1
VI	<i>Vibrio lentus</i> DSM13757 ^T	0	0	0	0	1	0	0	0	0	1
LA	<i>Labrenzia alba</i> CECT5094 ^T	0	0	0	0	0	0	1	0	0	1
SU	<i>Sulfitobacter marinus</i> SW-265 ^T	0	1	0	0	0	0	0	0	0	1
RH	<i>Rhizobium vignae</i> CCBAU05176 ^T	0	0	1	0	0	0	0	0	0	1
Total		30	35	27	29	17	31	19	32	31	251

2. Antimicrobial activities of all isolates

From a total of 251 isolates, ca 49% (122 isolates) exhibited antimicrobial activities against at least one of four indicator microorganisms. Active isolates were found in both GYM and BM media (44%) while only 3% active strains were reported in BM only and 2% in GYM only (Figure 3.1).

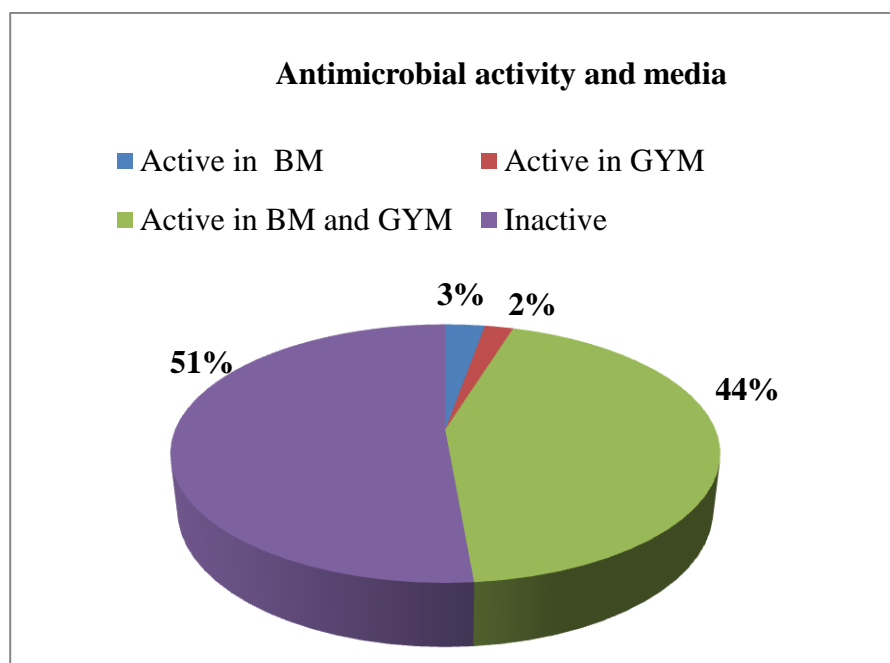


Figure 3.1 Antimicrobial activity of isolates using two different media.

The antimicrobial activity of all antibiotic active isolates is shown in Table 3.3. Antibiotic activities were found in 21 species (122 isolates) including members of the *Firmicutes* (8 ATUs, 105 isolates of *Bacillus*-BAs, 2 ATUs, 2 isolates of *Lysinibacillus* - LY1 and LY4), *Actinobacteria* (1 ATU, 1 isolate of *Blastococcus*-BL, 3 ATUs, 6 isolates of *Micrococcus*- MIF, MIL and MIY), and *Proteobacteria* (7 ATUs, 8 isolates).

Eight out of 17 distinct species of the genus *Bacillus* showed antimicrobial activities, especially all isolates of *B. amyloliquefaciens* (BA2) and *B. methylotrophicus* (BA8) showed antimicrobial activity against at least three of four indicator microorganisms. That is all of them inhibited *B. subtilis*, *S. epidermidis* and *E. coli*, and some of them (five isolates of BA2 and three isolates of BA8) in addition *C. albicans*.

All *Actinobacteria* isolated from the present study exhibited antimicrobial activity. *M. yunnanensis* inhibited only *S. epidermidis* on GYM medium, while *M. flavus* and *M. luteus* showed antimicrobial activities against *B. subtilis* and *S. epidermidis* in both GYM and BM media. *B. saxobidens* possessed antimicrobial activity against *B. subtilis*, *S. epidermidis* and *E. coli* only in BM medium (Table 3.3)

Seven isolates out 15 *Gammaproteobacteria* displayed antimicrobial activities. They were assigned to *P. aliena*, *P. nivimaris*, 3 *Shewanella* species, and *V. lentus*. Bacteria *P. aliena* and *V. lentus* possessed antimicrobial activities against the three bacterial test organisms but not against the yeast *C. albicans*, while *S. baltica* had antimicrobial activities against the three indicator organisms but not against *E. coli*.

Isolates of the 3 *Shewanella* species showed different activity profiles but all were active against *B. subtilis* and *S. epidermidis*. Notable *S. baltica* (SH1) inhibited in addition *C. albicans* (but only after growth in GYM medium), while *S. colwelliana* (SH2) (grown in both BM and GYM media) in addition inhibited *E. coli*. Inhibition of *B. subtilis* and *S. epidermidis* by *S. putrefaciens* (SH3) was found only if grown in BM medium. Among two isolates of *P. nivimaris*, one individual showed inhibition to *B. subtilis* only after growth in BM medium. Among the *Alphaproteobacteria* only crude extract of *S. marinus* (SU) inhibited *S. epidermidis* only after growth in GYM medium (Table 3.3).

Among 122 active isolates, 119 isolates (97.5%) showed inhibition of *S. epidermidis*. Evidently, all 14 isolates of *B. pumilus* exhibited antimicrobial activities to *S. epidermidis*. Impressing, *B. pumilus* possessed antimicrobial activities favour in BM medium. All crude extracts of *B. pumilus* grown in BM medium showed inhibition to *S. epidermidis* (Table 3.3)

A number of *Bacillus* strains, including the ATUs BA3, BA4, BA5, BA6, BA7, BA9, BA12, BA13, and BA16 displayed no antimicrobial activity. Also other *Firmicutes* including of *L. fusiformis* (LY2, 14 isolates) *L. sphaericus* (LY3, 1 isolate) *P. lautus* (PA, 4 isolates) and *Exiguobacterium oxidotolerans* (EX, 1 isolate) were inactive to all four test strains. No antimicrobial activity was found as well in *P. prydzensis* PSA2, *P. guineae* PSM1, *P. peli* PSM2, *P. alimentarius* PSY1, and *P. piscatorii* PSY3.

Table 3.3 Active isolates: affiliation to ATUs, isolation conditions, and antimicrobial profiles.

*The value indicate percent (%) growth inhibition in BM/GYM media

ATU _s	Isolates	Media for Isolation	Next relative type strain (RDP-II)	<i>B. subtilis</i> *	<i>S. epidermidis</i> *	<i>E. coli</i> *	<i>C. albicans</i> *
BA1	B-3-8	TM 28°C	<i>B. aerophilus</i> 28K ^T (AJ831844: 99.2%)	0/0	86/80	0/0	0/0
BA10	A-2-32	SW 28°C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289: 99.4%)	0/0	57/0	0/0	0/0
BA10	A-1-32	TM 10°C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289: 99.7%)	88/0	85/79	0/0	0/0
BA10	B-2-15	SW 10°C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289: 99.7%)	0/0	67/0	0/0	0/0
BA10	B-2-16	TM 10°C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289: 99.7%)	0/0	81/84	0/0	0/0
BA10	B-2-17	TM 10°C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289: 99.7%)	0/0	76/84	0/0	0/0
BA10	C-2-21	TM 10°C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289: 99.7%)	0/0	100/0	0/0	0/0
BA10	C-1-27	SW 28°C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289: 99.8%)	0/25	100/100	0/0	0/0
BA10	C-3-34	R2A 10°C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289: 99.8%)	58/0	90/91	0/0	0/0
BA10	B-1-33	SW 28°C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289: 99.9%)	91/0	86/84	0/0	0/0
BA10	C-1-16	SW 28°C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289: 99.9%)	93/0	96/77	0/0	0/0
BA10	B-2-12	R2A 10°C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289: 99.9%)	0/0	91/55	0/0	0/0
BA10	C-3-36	R2A 10°C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289: 99.9%)	0/0	45/0	0/0	0/0
BA10	C-3-33	R2A 10°C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289: 99.9%)	66/0	85/89	0/0	0/0
BA10	B-3-17	TM 28°C	<i>B. pumilus</i> ATCC 7061 ^T (AY876289: 99.7%)	0/0	97/97	32/42	0/0
BA11	C-1-15	R2A 28°C	<i>B. safensis</i> FO-036b ^T (AF234854: 99.9%)	0/0	82/82	0/0	0/0
BA11	B-3-34A	SW 10°C	<i>B. safensis</i> FO-036b ^T (AF234854: 99.9%)	0/0	92/0	0/0	0/0
BA14	B-2-7	TM 28°C	<i>B. subtilis</i> DSM10 ^T (AJ276351: 99.8%)	0/0	25/86	0/0	0/0
BA15	B-3-37	TM 10°C	<i>B. subtilis</i> NRRL B-23049 ^T (AF074970: 99.9%)	0/30	100/100	0/0	0/0
BA17	B-2-4	SW 28°C	<i>B. vallismortis</i> DSM 11031 ^T (AB021198: 99.6%)	71/43	76/74	86/90	26/31

ATU _s	Isolates	Media for Isolation	Next relative type strain (RDP-II)	<i>B. subtilis</i> *	<i>S. epidermidis</i> *	<i>E. coli</i> *	<i>C. albicans</i> *
BA2	A-2-4	SW 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 100%)	73/84	79/78	0/78	0/0
BA2	A-2-23A	TM 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 100%)	44/42	85/97	66/98	0/0
BA2	C-3-39	R2A 10°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 100%)	79/87	83/79	87/92	0/0
BA2	A-1-13	R2A 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.4%)	41/63	66/68	34/73	0/0
BA2	B-2-5	TM 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.4%)	73/82	72/74	87/0	0/20
BA2	A-3-18	TM 10°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.4%)	36/84	70/80	89/100	0/0
BA2	C-2-14	R2A 10°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.5%)	86/94	85/90	80/90	0/0
BA2	C-3-21	R2A 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.5%)	78/90	77/85	67/85	0/0
BA2	C-3-20	R2A 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.5%)	92/96	95/87	88/100	0/0
BA2	A-1-36	SW 10°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.6%)	44/72	58/71	42/80	0/0
BA2	A-1-6A	TM 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.6%)	0/79	0/76	0/86	0/0
BA2	B-1-8	R2A 10°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.6%)	90/87	87/85	87/91	0/0
BA2	B-1-11	TM 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.6%)	70/82	70/78	91/100	0/0
BA2	B-3-1	TM 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.6%)	91/82	81/76	0/51	0/0
BA2	C-1-11	TM 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.7%)	90/95	78/80	87/92	0/0
BA2	A-3-17	SW 10°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.7%)	0/84	79/76	0/77	0/0
BA2	B-3-6	SW 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.7%)	90/100	87/89	30/89	0/24
BA2	B-3-33	SW 10°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.7%)	46/89	97/99	99/100	0/45
BA2	C-3-23	TM 10°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.7%)	91/63	89/87	0/93	0/0
BA2	C-3-30	R2A 10°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.7%)	74/0	82/89	86/0	0/0
BA2	A-1-18	SW 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8%)	84/89	80/81	73/93	0/0
BA2	B-1-28B	SW 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8%)	84/87	68/76	98/100	0/0
BA2	C-1-10	TM 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8%)	88/80	94/99	96/100	0/33

ATU _s	Isolates	Media for Isolation	Next relative type strain (RDP-II)	<i>B. subtilis</i> *	<i>S. epidermidis</i> *	<i>E. coli</i> *	<i>C. albicans</i> *
BA2	A-2-5	SW 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8%)	59/88	85/89	95/100	0/0
BA2	A-2-14	R2A 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8%)	39/68	89/99	88/99	0/0
BA2	B-2-2	R2A 10°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8%)	84/0	88/83	89/91	0/0
BA2	A-3-13	SW 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8%)	87/88	81/83	0/85	0/0
BA2	B-3-12	R2A 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8%)	91/92	84/87	94/93	0/0
BA2	B-3-21	R2A 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8%)	53/90	96/99	97/100	0/0
BA2	B-3-16	SW 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8%)	89/91	83/85	83/93	0/0
BA2	B-3-32	R2A 10°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8%)	84/91	94/100	36/100	0/0
BA2	C-3-35	R2A 10°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.8%)	69/86	82/88	77/81	0/0
BA2	C-1-9	R2A 28°C	<i>B. amyloliquefaciens</i> NBRC 15535 ^T (AB255669: 99.9%)	43/28	66/69	80/76	0/0
BA8	B-3-26	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 100%)	89/88	83/77	91/91	0/0
BA8	C-1-17	SW 10°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.5%)	87/93	79/83	89/89	0/0
BA8	C-2-10	R2A 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.5%)	70/82	71/80	91/100	0/0
BA8	B-3-36	TM 10°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.5%)	92/97	88/100	84/100	0/0
BA8	C-2-5A	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.6%)	87/86	81/83	82/80	0/0
BA8	C-2-9	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.6%)	87/85	80/80	93/91	0/0
BA8	B-3-18	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.6%)	67/93	57/88	97/100	0/0
BA8	C-2-32	R2A 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.6%)	80/84	89/95	97/99	0/0
BA8	A-1-34	SW 10°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	64/85	78/84	87/92	0/0
BA8	B-1-13	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	59/51	85/80	0/87	0/0
BA8	B-1-27	R2A 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	79/80	66/77	35/100	0/0
BA8	B-1-31	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	86/88	77/82	0/46	0/0

ATU _s	Isolates	Media for Isolation	Next relative type strain (RDP-II)	<i>B. subtilis</i> *	<i>S. epidermidis</i> *	<i>E. coli</i> *	<i>C. albicans</i> *
BA8	C-1-7	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	32/91	90/93	95/100	0/0
BA8	C-1-21	R2A 10°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	36/33	74/71	93/82	0/0
BA8	C-1-22	R2A 10°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	34/89	76/80	88/90	0/0
BA8	C-1-25	TM 10°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	73/85	62/71	92/91	0/0
BA8	C-1-5	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	90/0	81/81	0/61	0/0
BA8	A-2-2	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	77/87	82/90	99/100	0/0
BA8	C-2-5B	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	88/95	89/91	83/78	0/0
BA8	A-3-3	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	0/91	75/82	80/95	0/0
BA8	A-3-6C	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	72/90	95/96	99/99	0/0
BA8	A-3-22	TM 10°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	62/78	68/77	70/85	0/0
BA8	A-3-23A	R2A 10°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	82/86	83/85	87/90	0/0
BA8	A-3-23B	R2A 10°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	86/77	74/75	89/80	36/0
BA8	A-3-6A	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	45/72	82/85	98/99	0/0
BA8	A-3-6B	SW 28°C	<i>B. methylotrophicus</i> CBMB205 ^T (EU194897: 99.7%)	66/84	68/73	92/100	0/0
BA8	A-3-8	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	51/96	89/99	70/100	0/0
BA8	B-3-24	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	48/80	71/74	96/100	0/49
BA8	B-3-13	R2A 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	37/50	81/83	99/99	0/0
BA8	B-3-23	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	66/90	81/81	0/87	0/0
BA8	C-3-14	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.7%)	79/74	83/82	0/91	0/0
BA8	B-1-14	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	60/88	78/85	51/100	0/0
BA8	C-1-12	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	72/0	75/73	45/64	0/0
BA8	C-1-13	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	0/94	76/85	0/93	0/0
BA8	C-1-24	R2A 10°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	88/83	100/82	97/92	0/0

ATU _s	Isolates	Media for Isolation	Next relative type strain (RDP-II)	<i>B. subtilis</i> *	<i>S. epidermidis</i> *	<i>E. coli</i> *	<i>C. albicans</i> *
BA8	A-2-23B	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	0/47	71/80	58/99	0/28
BA8	A-3-2	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	57/87	81/82	0/83	0/0
BA8	A-3-7B	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	22/0	88/88	100/100	0/0
BA8	B-3-2	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	76/90	91/99	95/100	0/0
BA8	B-3-28	R2A 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	73/79	73/86	75/99	0/0
BA8	B-3-20	R2A 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	37/86	63/89	95/99	0/0
BA8	C-3-2	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	24/42	84/81	0/92	0/0
BA8	C-3-4	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	88/82	82/85	89/93	0/0
BA8	C-3-6	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	42/79	83/91	81/93	0/0
BA8	C-3-10	R2A 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	86/88	80/82	89/91	0/0
BA8	C-3-19	R2A 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	72/61	80/77	0/91	0/0
BA8	C-3-7	TM 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	74/68	81/87	64/94	0/0
BA8	C-3-9	R2A 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	66/0	80/87	91/82	0/0
BA8	C-3-17	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.8%)	98/91	93/86	91/91	0/0
BA8	B-1-28A	R2A 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.9%)	80/79	85/83	0/88	0/0
BA8	B-1-30	SW 28°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.9%)	82/89	79/84	0/82	0/0
BA8	A-2-17	R2A 10°C	<i>B. methylotrophicus</i> CBMB 205 ^T (EU194897: 99.9%)	75/71	86/88	94/100	0/0
LY1	A-1-40B	TM 28°C	<i>L. boronitolerans</i> 10a ^T (AB199591 : 98.6%)	0/0	0/0	0/0	34/42
LY4	C-2-29	TM 10°C	<i>L. xylanilyticus</i> XDB9 ^T (FJ477040: 99.0%)	0/0	0/0	0/0	57/68
BL	C-1-31	BS 28°C	<i>B. saxobsidens</i> DSM 44509 ^T (FN600641: 98.8%)	50/0	94/0	86/0	0/0
MIF	A-2-19A	SW 28°C	<i>M. flavus</i> LW4 ^T (DQ491453: 99.2%)	0/0	35/60	0/0	0/0
MIF	A-2-28	SW 10°C	<i>M. flavus</i> LW4 ^T (DQ491453: 99.3%)	0/0	30/68	0/0	0/0
MIL	A-2-7	TM 28°C	<i>M. luteus</i> DSM 20030 ^T (AJ536198: 99.7%)	0/0	60/74	0/0	0/0

ATU _s	Isolates	Media for Isolation	Next relative type strain (RDP-II)	<i>B. subtilis</i> *	<i>S. epidermidis</i> *	<i>E. coli</i> *	<i>C. albicans</i> *
MIL	A-2-19B	SW 28°C	<i>M. luteus</i> DSM 20030 ^T (AJ536198: 99.8%)	0/0	60/62	0/0	0/0
MIY	C-2-28	TM 10°C	<i>M. yunnanensis</i> YIM 65004 ^T (FJ214355: 99.4%)	0/0	0/97	0/35	0/0
MIY	C-2-27	TM 10°C	<i>M. yunnanensis</i> YIM 65004 ^T (FJ214355: 99.9%)	0/0	0/83	0/0	0/0
PSA1	C-2-1	R2A 10°C	<i>P. aliena</i> KMM 3562 ^T (AY387858: 99.2%)	72/69	78/61	0/22	0/0
PSA1	C-2-19	SW 10°C	<i>P. aliena</i> KMM 3562 ^T (AY387858: 99.3%)	76/76	56/29	0/0	0/0
PSY2	A-1-30	SW 10°C	<i>P. nivimaris</i> 88/2-7 ^T (AJ313425: 99.0%)	31/0	0/0	0/0	0/0
SH1	B-1-21	R2A 10°C	<i>S. baltica</i> NCTC 10735 ^T (AJ000214: 98.5%)	0/67	0/41	0/0	0/32
SH2	C-2-3	R2A 10°C	<i>S. colwelliana</i> ATCC 39565 ^T (AY653177: 99.9%)	74/62	84/88	89/89	0/0
SH3	B-1-22	R2A 10°C	<i>S. putrefaciens</i> LMG 26268 ^T (X81623: 99.0%)	91/0	92/0	0/0	0/0
SU	B-1-18	BS 10°C	<i>S. marinus</i> SW-265 ^T (DQ683726: 99.7%)	0/0	0/88	0/0	0/0
VI	B-2-1	R2A 10°C	<i>V. lentus</i> DSM 13757 ^T (AJ278881: 98.8%)	79/00	85/87	90/84	0/0

3. Chemical substances produced by soft coral-associated bacteria

Based on the results of antimicrobial tests and chemical analysis in the screening experiments, four soft coral associated isolates were selected for scale-up of the production of metabolites. These are *Lysinibacillus* sp. strain C-2-29, *Bacillus* sp. strain H-6, *Micrococcus* sp. strain A-2-28, and *Bacillus* sp. strain C-1-10.

The chemical analysis from HPLC-DAD-MS of crude extract of strain C-2-29 (next relative *Lysinibacillus xylanilyticus* XDB9^T) showed that this strain produced at least 8 compounds present in cells grown in both of the screening media. Three out of these 8 were present in large amounts and showed good UV signals. The crude extract also showed inhibition only against *C. albicans* in both GYM and BM media. Species of the new genus *Lysinibacillus* were transferred from *Bacillus* (Ahmed et al. 2007). This species was not reported for bioactive compound producer so far. The strain *L. xylanilyticus* XDB9^T was reported to be a xylan-degrading bacterium isolated from forest humus (Lee et al. 2010).

Most abundant isolates from this study were members of the genus *Bacillus*. Marine *Bacillus* sp. strains also were common producers of bioactive compounds. The strain H-6 (next relative *Bacillus pumilus* ATCC7061^T) was isolated from soft coral *A. digitatum* from Helgoland in the North Sea and showed strong antimicrobial activity against *S. epidermidis* in both media used for the screening experiments. The strain H-6 produced different compounds in two different screening media. Two of these compounds might be unknown. Most isolates closely related to *B. pumilus* ATCC7061^T as next relative showed rather weak UV signals. The best UV signals were shown by strain H-6 and therefore this strain was chosen for further studies.

Micrococcus sp. was the most common bacterium among the isolates from the soft corals. Only few investigations of natural compounds from *Micrococcus* sp. were reported previously (Palomo et al. 2013). In this study, *Micrococcus* sp. strain A-2-28 (next relative *Micrococcus flavus* LW4^T) was further considered for large scale cultivation, chemical analyses, and biological activities. Crude extract of the strain A-2-28 inhibited only *S. epidermidis*. The metabolites of the strain A-2-28 were completely different to those of the selected strains of *Bacillus* spp. strain C-1-10 and strain H-6 which also inhibited only *S. epidermidis*. The strain A-2-28 produced a number of compounds with three main compounds and good UV signals.

Unlike strain H-6, crude extracts of the strain C-1-10 (next relative *Bacillus amyloliquefaciens* NBRC15535^T) showed inhibition of all four test organisms. In addition, the chemical analysis revealed that this strain produced a greater number of metabolites, some of which are known according to the DNPs and others could so far not be identified.

3.1 *Lysinibacillus* sp. C-2-29

3.1.1 *Origin, cultivation and identification of the strain C-2-29*

The strain C-2-29 was isolated from soft coral *A. digitatum* Baltic 2 at 10°C in TM medium, and was identified as *Lysinibacillus* sp., because the sequence of the 16S rRNA gene showed 99.0% similarity with *Lysinibacillus xylanilyticus* XDB9^T (GenBank accession number FJ477040), 98.9% similarity with *Lysinibacillus boronitolerans* 10a^T (GenBank accession number AB199591), and 98.3% sequence similarity with *Lysinibacillus fusiformis* DSM 2898T^T (GenBank accession number AJ310083), which are derived from the RDP-II (Cole et al. 2014).

Based on a taxonomic study, especially in cell wall peptidoglycan structures, the genus *Lysinibacillus* was proposed by Ahmed et al. (2007) and several species were transferred from the genus *Bacillus* to this new genus. Today it consists of 5 species with validly published names *Lysinibacillus boronitolerans*, *Lysinibacillus fusiformis*, *Lysinibacillus sphaericus* (Ahmed et al. 2007), *Lysinibacillus parxiboronicapiens* (Miwa et al. 2009), and *Lysinibacillus xylanilyticus* (Lee et al. 2010).

3.1.2 *Cultivation and extraction of the strain C-2-29*

The screening approach using 100 ml GYM or BM medium, respectively, revealed the detection of 8 compounds in both screening media, with enhanced production in GYM medium. In a second step the production was tested using a cultivation volume of 1 L.

After 4 days cultivation, 100 ml broth culture was harvested from 1 L flask culture, and centrifuged (8.000 g, 4°C). Then the bacterial cells were extracted with 100 ml of methanol, and the supernatant was extracted with 100 ml of ethyl acetate. The extracts were concentrated and injected in HPLC-DAD-MS for analysis of the metabolite profiles in supernatant or bacterial cells, respectively. Three out of 8 compounds produced by this strain were found both in supernatant (extracted with ethyl acetate) and bacterial cells (extracted with methanol). Because the production of those three compounds was confirmed, a cultivation volume of 40 L in GYM medium was used for extraction and purification of large amounts. Culture broth (total 40 L) was extracted with ethyl acetate. The crude extract was tested against *C. albicans* and purified using HPLC because of its inhibitory activity.

3.1.3 Compound purification

Four grams of crude extract were separated with VWR International LaPrep system monitoring absorption at a wavelength of 210 nm with a gradient 0 min 15% B, 26 min 86% B, 27 min 100% B, flow 100 ml/min. 10 mg of fraction 1, 30 mg of fraction 2, and 5 mg of fraction 3 were harvested.

To purify compound **1**, semi preparation of 10 mg of fraction 1 was carried out on Merck Hitachi Elite LaChrom system 1 with a gradient from 20% B at 0 min increasing to 55% B at 15 min, to 100% B from 27 min to 28 min, flow 15 ml/min. The compound **1** (MW 223) was eluted at 10 min, and yielded 2 mg.

30 mg of pure compound **3** was harvested from the fraction 2 by preparation of 4 grams of crude extract using VWR International LaPrep system.

For purification of compound **5**, 5 mg of fraction 3 was applied on Merck Hitachi Elite LaChrom system 2 with a gradient from 30% B at 0 min increasing to 100% B at 25 min, flow 14 ml/min and yielded 0.8 mg of purified compound **5** (MW 467) at 19 min.

3.1.4 Metabolic profile

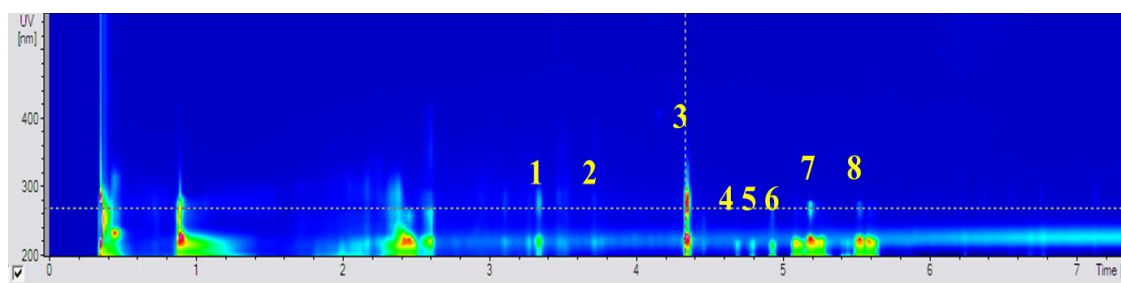
The strain C-2-29 produced at least 8 compounds which could be identified by the mass and UV maxima (Table 3.4, Figure 3.2, Figure 3.3). The same compounds were produced in both 100 ml volume cultures in GYM and BM media.

The compounds **1-8** were found in both 100 ml cultures in GYM and BM media (Figure 3.2, Figure 3.3). The compounds **1** (MW 223) and **3** (MW 338) were found as main peaks in 100 ml cultures in GYM and BM media and also in 1 L cultures in GYM medium. The compound **2** (MW 299), compound **4** (MW 283), and compound **6** (MW 253) were present in very low amounts in both 100 ml and 1 L cultures with weak UV signals. The compound **5** (MW 467) was found in low amounts but demonstrated rather good UV signals in the 100 ml volume cultures and the 1 L cultures. Peak 7 (MW 456, MW 148) and compound **8** (MW 643) were found only in the 100 ml cultures. Therefore, compounds **2**, **4**, **6**, peak 7, and compound **8** could not be purified and further analysed.

Table 3.4 Chemical profiles and purified compounds of the strain C-2-29.

CNo/ Peak	Media	MW	Rt [min]	UV [nm] maxima	CP/yield	CN/comment
1	GYM/100 ml BM/100 ml GYM/1 L	223	3.2	224, 275	Purification; 2 mg	Maybe a deacetylanisomycin
2	GYM/100 ml BM/100 ml GYM/1 L	299	3.9	219, 281	No purification	Low amounts
3	GYM/100 ml BM/100 ml GYM/1 L	338	4.2	224, 276, 325	Purification; 30 mg	2,5-bis (3- indolylmethyl) pyrazine
4	GYM/100 ml BM/100 ml GYM/1 L	283	4.5	223, 276	No purification	Not present after preparation
5	GYM/100 ml BM/100 ml GYM/1 L	467	4.7	227, 279	Purification; 0.8 mg	Unknown Maybe new
6	GYM/100 ml BM/100 ml GYM/1 L	253	4.9	216, 266, 273	No purification	Not present after preparation
7*	GYM/100 ml BM/100 ml	456 148	5.1-5.3	223, 266, 273	No purification	Not present in 1 L cultures
8	GYM/100 ml BM/100 ml	643	5.4	224, 267, 277	No purification	Not present in 1 L cultures

* Peak 7 consists of 2 compounds (MW 456 and MW 148), CNo: compound number; MW: molecular weight; Rt: retention time; CP: compound purification; CN: compound name.

**Figure 3.2** UV spectrums of C-2-29 in GYM medium (100 ml volume).

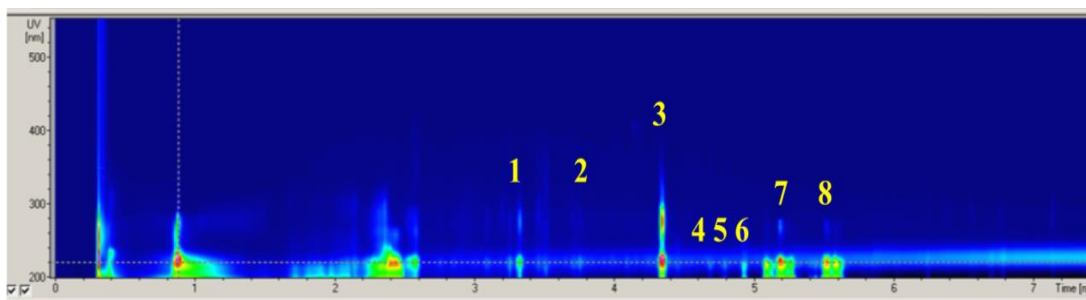


Figure 3.3 UV spectrums of C-2-29 in BM medium (100 ml volume).

Compounds **1**, **3** and **5** were isolated from 1 L cultures from strain C-2-29 and purified (Table 3.4, Figure 3.4). By comparing the data from literature it was noted that the compound **1** (MW 223) may be deacetylanisomycin. The compound **3** was identified as 2,5-bis (3-indolylmethyl) pyrazine and the compound **5** was an unidentified or new compound.

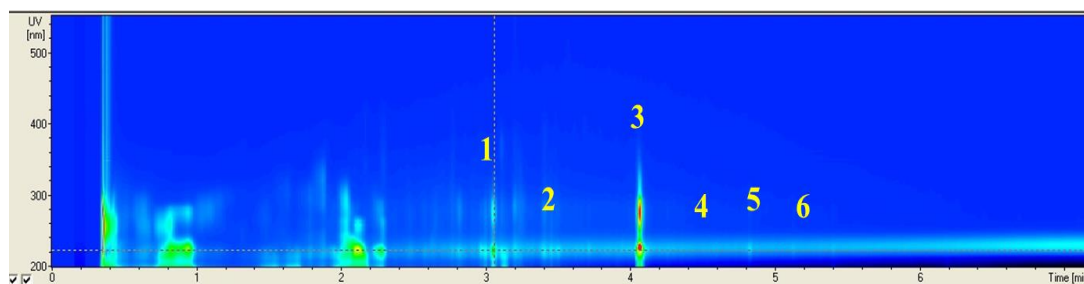


Figure 3.4 UV spectrums of C-2-29 in 1 L cultures in GYM medium.

Searching of data from DNPs it was found that compound **1** (MW 223, UV max 224, 275) might be a deacetylanisomycin (MW 223, UV max 223, 276) that was found from *Streptomyces* sp. SA (Sobin and Tanner, 1954) because of matching of UV maxima and molecular weight as well. The structure elucidation of compound **1** is in progress.

The known compound **3** (MW 338) was identified as 2,5-bis (3-indolylmethyl) pyrazine (Figure 3.5), which was first isolated from *Cytophaga* sp. strain AM 13.1 from the North Sea (Shaaban et al. 2002).

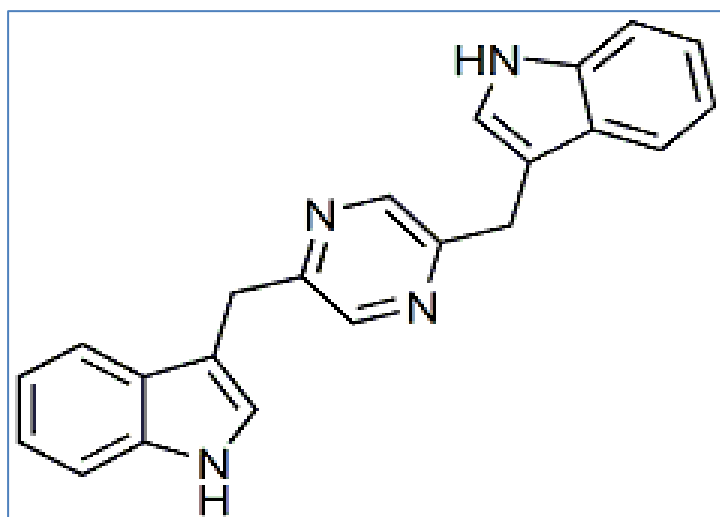


Figure 3.5 Structure of the compound **3** which is 2,5-bis (3-indolylmethyl) pyrazine.

Also by searching data from DNPs, there were 4 hits for compound **5** (MW 467, UV 227, 279) which were not matching in biological sources. It inferred that there are different compounds which had the same MW but were not produced by *Lysinibacillus* sp. strains. For example, marinamycin was isolated from *Streptomyces mariensis* (Soeda, 1959; Soeda and Mitoni, 1962), and considered similar to compound **5** because of sharing of molecular weight (467) and UV maxima (220, 278). Marinamycin was an antitumor substance (Soeda, 1959; Soeda and Mitoni, 1962).

The other three hits for the compound **5** were the antibiotic K252a (MW 467, UV max 227, 249, 264, 280, 290, 320 334, 335), cyclopiamine A (MW 467, UV max 230, 261, 344), cyclopiamine B (MW 467, UV max 232, 260, 345), and laxiracemosin D (MW 467, UV max 208, 220, 262, 306). Antibiotic K252a was isolated from *Nocardiosis* sp. and *Streptomyces longisporoflavus* R19, and showed protein kinase C and phosphorylase kinase inhibition, calmodulin inhibitor, antiallergic agent, and vasorelaxant (Kase et al. 1986). Cyclopiamine A and B were isolated from *Penicillium cyclopium*. This compound was toxic (Bond et al. 1979). Laxiracemosin D was isolated from plant *Dysoxylum laxiracemosum*, it possessed cytotoxic activity against human myeloid leukemia HL-60 at $IC_{50}=6.8\mu M$ (Zhang et al. 2010). The structure elucidation of compound **5** is in progress.

3.1.5 Bioactivities of the isolated compounds

The biological activities of three purified compounds are shown in Table 3.5. The compound **1** and **5** were inactive in all tests, whilst the compound **3** showed weak inhibition of *S. aureus* MRSA with 29.0%.

Table 3.5 Bioactivities assays of pure compounds isolated from C-2-29.

Compounds	Antibacterial						Antifungal			Cytotoxic		Enzyme inhibition			
	<i>Bacillus subtilis</i>	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus aureus</i> MRSA	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Propionibacterium acnes</i>	<i>Candida albicans</i>	<i>Septoria tritici</i>	<i>Trichophyton rubrum</i>	NIH-3T3 (mouse fibroblasts)	Hep G2 (human liver cancer)	Acetylcholinesterase	Glycogen synthase kinase	Phosphodiesterase 4 B2	Protein-tyrosine-phosphatase
1 (MW 223)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3 (MW 338)	-	-	29	-	-	-	-	-	-	-	-	-	-	-	-
5 (MW 467)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The numbers indicate the percentage of inhibition. (-) indicate negative results.

As mentioned above, the known compound **3** (MW 338) was identified as 2,5-bis (3-indolylmethyl) pyrazine. This compound has been reported to be an inactive compound to three microalgae, the fungus *Mucor miehei*, the yeast *C. albicans* and the bacteria *S. aureus*, *B. subtilis*, *E. coli* and *Streptomyces viriochromogenes* (Shaaban et al. 2002). The compound **3** isolated from *Lysinibacillus* sp. strain C-2-29 in this study was inactive to applied biological tests beside a very weak activity against *S. aureus* MRSA.

3.1.6 Discussion

Two main compounds were produced from the strain *Lysinibacillus* sp. C-2-29, compound **1** (MW 223) and compound **3** (MW 338) in both 100 ml volume cultures in GYM and BM media. They were found in both supernatant and bacterial cell extracts. The crude extract from 1 L cultures in GYM medium had 2 main compounds (**1**, **3**) (Figure 3.4)

and showed inhibition of *C. albicans* in addition. This inferred that the compound responded for *C. albicans* inhibition existed in crude extract of 1 L cultures. It could be none of the three purified compounds, because these were inactive to *C. albicans* (Table 3.5). However, it is proposed that compound **1** is deacetylanisomycin, a hydrolysed product of anisomycin. Anisomycin (Figure 3.6-I), which inhibited *C. albicans* (Lynch et al. 1954) was hydrolysed to deacetylanisomycin during cultivation or extraction procedure (Figure 3.6-II). Success of isolation of deacetylanisomycin (MW 223, $C_{12}H_{17}NO_3$) instead of anisomycin (MW 265, $C_{14}H_{19}NO_4$) after partition chromatography was more usually than isolation of anisomycin (Butler, 1966). Anisomycin or flagecidin antibiotic was first isolated from a mixed fermentation of two species of *Streptomyces* sp. as an antiprotozoan agent (Sobin and Tanner, 1954). Anisomycin was replaced by nystatin as an antifungal agent in Thayer-Martin medium (Martin and Lewis, 1977). This medium was used specifically to isolate the Gram-negative human pathogenic bacteria *Neisseria gonorrhoeae* from specimens containing a mixture of bacteria and fungi (Granato et al. 1980). Now anisomycin is a well-known compound to be added to Martin-Lewis medium to inhibit *C. albicans*.

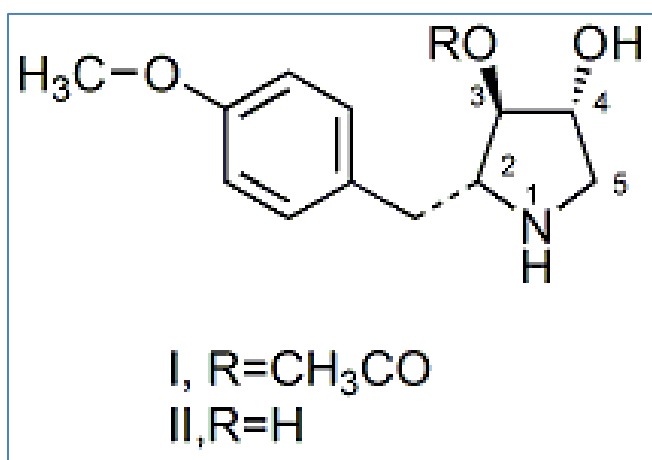


Figure 3.6 Structure of anisomycin (I) and deacetylanisomycin (II).

3.2 *Bacillus* sp. H-6

3.2.1 Origin, cultivation and identification of the strain H-6

The strain H-6 was isolated from the soft coral *A. digitatum* in Helgoland (North Sea, GPS 54°10, 405'N, 7°52, 955'E). All sampling procedures were displayed in Material and Methods part 1. In summary, soft corals were collected by SCUBA diving at ca. 10 m depth, sub-sampled in laboratory at Alfred Wegener Institute (AWI) at Helgoland and bacterial cultivation was carried out at GEOMAR. The strain H-6 was isolated from coral tissues in R2A medium.

Analysis of the 16S rRNA gene sequence of the strain H-6 from RDP-II (Cole et al. 2014) showed that this strain was a *Bacillus* sp. and shared 99.8% sequence similarity with *Bacillus pumilus* ATCC7061^T (GenBank accession number AY876289), 99.6% sequence similarity with *Bacillus safensis* FO-036b^T (GenBank accession number AF234854) and 99.3% sequence similarity with both *Bacillus aerophilus* 28K^T (GenBank accession number AJ831844) and *Bacillus stratosphericus* 41KF2a^T (GenBank accession number AJ831841).

3.2.2 Cultivation and extraction of the strain H-6

Chemical analyses of metabolites of strain H-6 produced the same compounds but in different amounts in both screening media. There were three interesting compounds which were found in BM medium in large amounts. Therefore, a total of 20 L of cultures were extracted and used for preparation.

3.2.3 Compound purification

The 2.3 grams of crude extract obtained from 20 L culture were separated with VWR International LaPrep system monitoring absorption at a wavelength of 210 nm with gradient 0 min 15% B, 26 min 86% B, 27 min 100% B, flow 100 ml/min and yielded a total of 60 mg fraction 1 (mix of compounds **1**, **2**, **4** and **5**) and a total of 100 mg fraction 2 (mix of compounds **3**, **4** and **5**), 7.7 mg of fraction 3 (compound **7**), and 30 mg of fraction 4 (compound **8**). Compound **6** (MW 389) was not found in any fraction because this compound was not produced in 1 L cultures.

Compound **5** was present in large amounts in fraction 2 and in low amounts in fraction 1, this compound also was considered as an unknown compound. Compounds **1** and **2** were main components of fraction 1 and both compounds were known compounds. The fraction 1 and fraction 2 were purified in the semi-preparative-Merck Hitachi Elite LaChrom system 1 for isolation of compound **5** (MW 476). However, no purification could be achieved, which might be due to the same UV maxima of different compounds contained in the fractions and instability of the compound **5** in addition (see discussion).

The fraction 4 consisted of compound **8** (MW 1035) and an additional peak of two compounds (MW 835, 1049) which was found only in 1 L cultures, this fraction was separated with the semi preparative-Merck Hitachi Elite LaChrom system 1 to purify compound **8** (MW 1035) which presented as a main compound in fraction but this work was not successful due to overlap of the mass (MW 1035 and MW 1049) and UV maxima at the same retention time.

The fraction 3 yielded 7.7 mg and contained only compound **7** with a rather good UV signal, so that this compound was purified with semi preparation and yielded 2.5 mg. Semi preparation of 7.7 mg of compound **7** (MW 444) was carried out with a semi preparative HPLC on Merck Hitachi Elite LaChrom system 2 with a gradient from 30% B at 0 min increasing to 80% B at 15 min, to 100% B from 27 min to 27.5 min, flow 14 ml/min, yielded 2.5 mg of purified compound **7** at 13 min.

3.2.4 Metabolic profile

The main metabolites of strain H-6 are listed in Table 3.6. Strain H-6 produced at least 8 compounds both in 100 ml volume cultures in GYM and BM media. Compound **1** (MW 423) and compound **3** (MW 438) were main peaks in 100 ml volume cultures in GYM medium. Other compounds (compound **2**, **4**, **5**, **6**, **7** and **8**) were presented in low amounts (Table 3.6, Figure 3.7). The compound **1** (identified as amicoumacin A), the compound **2** (identified as bacilosarcin B), the compound **6** (considered to be AI-77F), and the compound **8** (a cyclic peptide, see part 3.4) possessed the same molecular weight, UV maxima as well as biological sources with data in the literature. Those four compounds might be noted as known compounds. Other compounds from H-6 were not identified.

Table 3.6 Chemical profiles and purified compounds of the strain H-6.

CNo Peak	Media	MW	Rt [min]	UV [nm] maxima	CP/yield	CN/comment
1	GYM/100 ml BM/100 ml BM/1 L	423	2.6	212, 248, 315	No purification	Maybe a amicoumacin A
2	GYM/100 ml BM/100 ml BM/1 L	493	2.7	212, 249, 315	No purification	Maybe a bacilosarcin B
3	GYM/100 ml BM/100 ml	438	2.9	212, 248, 315	No purification	Unknown Maybe new
4	GYM/100 ml BM/100 ml	508	3.1	212, 251, 315	No purification	Unknown Maybe new
5	GYM/100 ml BM/100 ml BM/1 L	476	3.2	212, 248, 315	Not successfully purified	Unknown Maybe new
6	GYM/100 ml BM/100 ml	389	3.5	213, 246, 311	No purification	Maybe a AI- 77F
7	GYM/100 ml BM/100 ml BM/1 L	444	4.5	225	Purification; 2.5 mg	Unknown Maybe new
8*	GYM/100 ml BM/100 ml BM/1 L	1035 835 1049	6.4	224, 278	No purification	Maybe bacillopeptins

* Peak 8 consists of 3 compounds (MW 1035, MW 835, and MW 1049); CNo: compound number; MW: molecular weight; Rt: retention time; CP: compound purification; CN: compound name.

The compounds **1** (MW 423) and **2** (MW 493) were main compounds in 100 ml volume cultures in BM medium. In addition, the compounds **3** (MW 438), **4** (MW 508), and **5** (MW 476) were found. Other compounds (**6**, **7** and **8**) were also found in 100 ml volume cultures in BM medium with low amounts according to mass detection (Figure 3.7).

The strain H-6 was scaled up in 1 L in BM medium for isolating of compounds **3**, **4** and **5**. However, the strain H-6 produced 5 main compounds compound **1**, **2**, **5**, **7**, and **8**, but no compounds **3**, **4**, and **6** in the 1 L cultures. In addition, a group of compounds with MW 835 and MW 1049 were observed in 1 L cultures in BM medium. Both compounds shared the same UV maxima to compound **8** (MW 1035) and showed very close retention time also (Table 3.6, Figure 3.8).

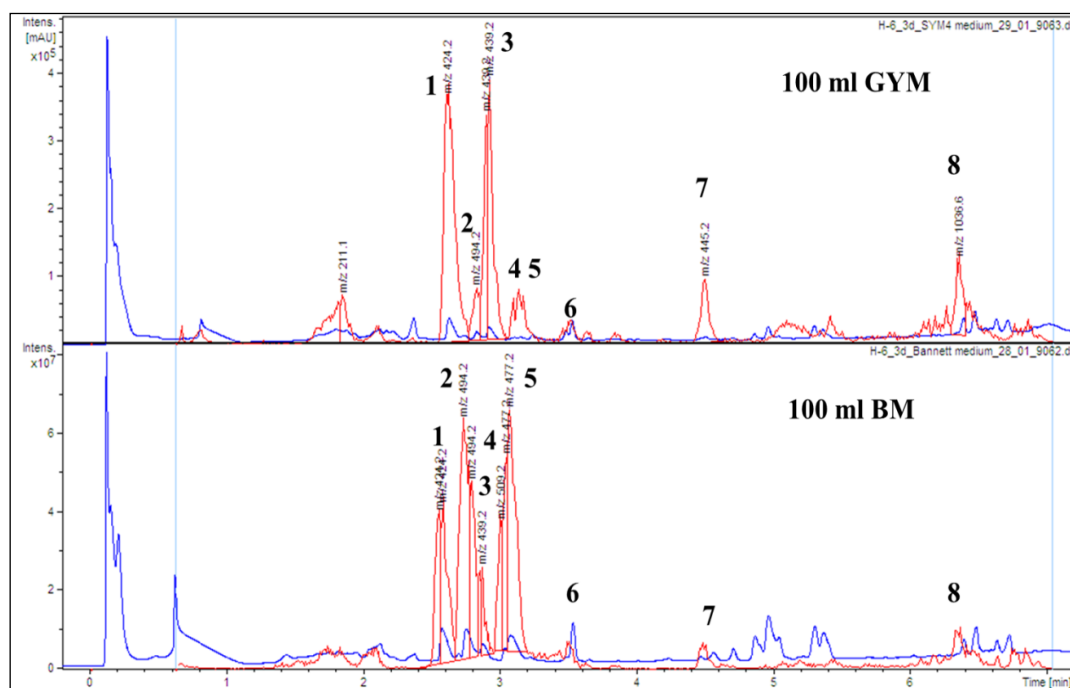


Figure 3.7 Chemical profiles of H-6 in 100 ml cultures. Red-mass, blue-UV.

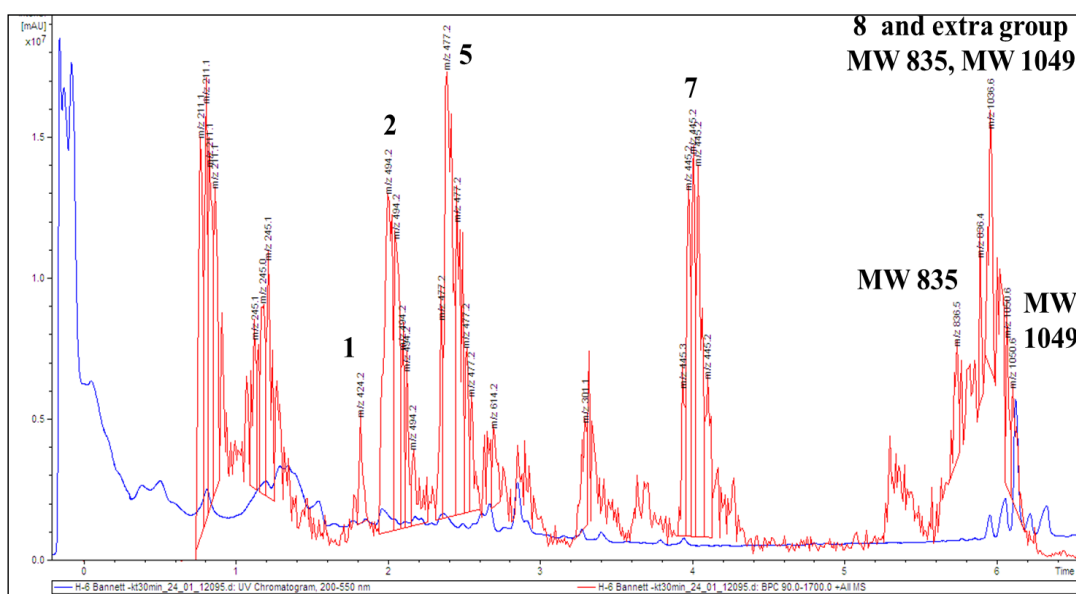


Figure 3.8 Chemical profiles of H-6 in 1 L cultures in BM medium. Red-mass, blue-UV.

The compounds **5** and **7** were regarded as unknown compound found from 1 L cultures of strain H-6 in BM medium. Therefore, these compounds were purified.

3.2.5 Bioactivities of the isolated compounds

The compound **7** (MW 444) was found in 100 ml volume cultures at low amounts but was main mass peak in 1 L cultures with good UV signals (Table 3.6, Figure 3.8). This compound was successfully isolated. The structure elucidation is in progress. This compound did not show any antibacterial activities and did not possess any cytotoxic and enzyme inhibition activities.

3.2.6 Discussion

By comparison of molecular weight, UV maxima and biological sources from DNPs, the compound **1** (MW 423, UV max 212, 248, 315) may be an amicoumacin A (MW 423, UV max 216, 245, 315) which was first isolated from *B. pumilus* (Itoh et al. 1981). Compound **1** was the main compound of strain H-6 in 100 ml volume cultures in GYM and BM media. Crude extract of strain H-6 of 100 ml broth cultures showed 100% inhibition of *S. epidermidis*. The compound **1** might be responsible for *S. epidermidis* inhibition. It is evident that amicoumacin A (Figure 3.9) produced by *B. pumilus* BN-103 inhibited *S. epidermidis* ATCC 14990 at very low concentration (0.2 mcg/ml) (Itoh et al. 1981 and 1982).

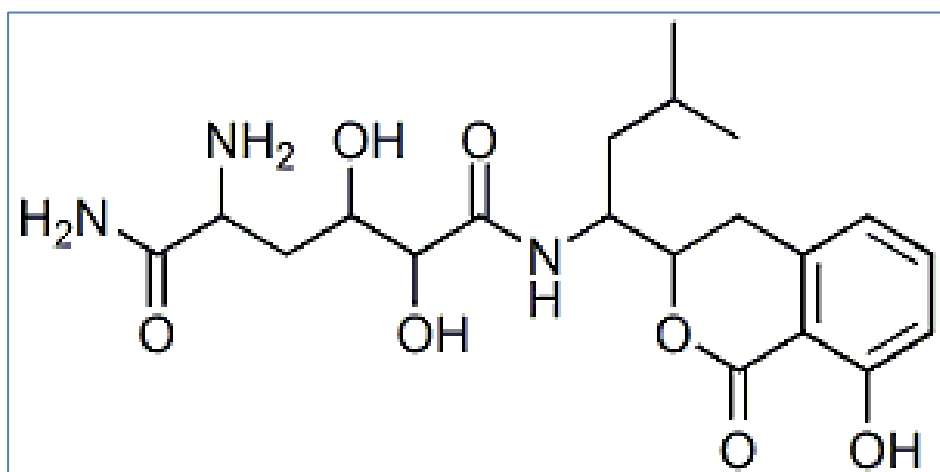


Figure 3. 9 Structure of amicoumacin A from *Bacillus* sp.

Properties of compound **2** (MW 493, UV max 212, 249, 315) are most similar to bacilosarcin B (MW 493, UV max 206, 247, 315) which was produced by *B. subtilis* strain TP-B0611 (Azumi et al. 2008). Bacilosarcin B is a member of the amicoumacin group.

Amicoumacin A (C₂₀H₂₉N₃O₇) and bacilosarcin B (Figure 3.10) have an amide functional group at C-12', exhibited antibacterial activities against *S. aureus*, and possessed cytotoxicity (IC₅₀=33.6 μM) against the human HeLa cells (Li et al. 2012).

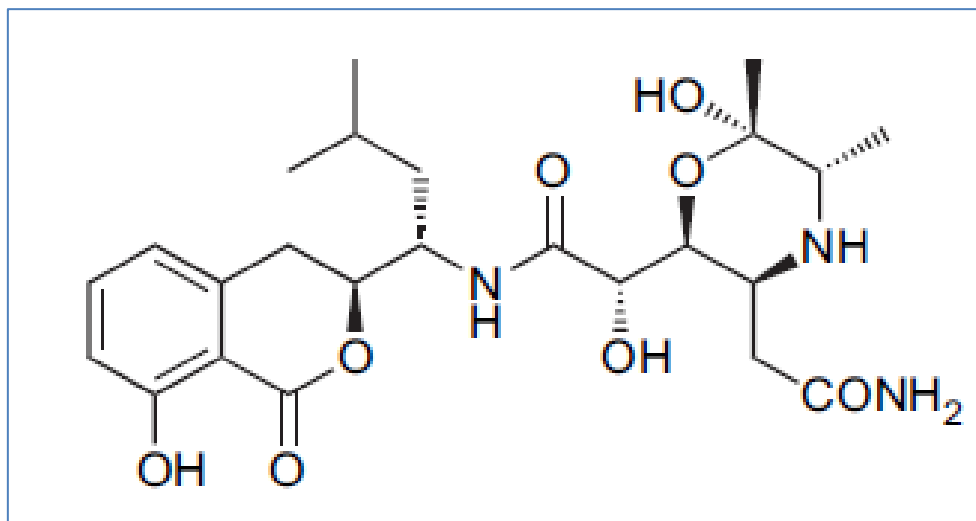


Figure 3.10 Structure of bacilosarcin B from *B. subtilis* TP-B0611 (Azumi et al. 2008).

There were several hits for the compound **3** (MW 438) which was found mainly in GYM medium (in 100 ml volume), and 10 hits for the compound **4** (MW 508). However, they did not match in UV maxima and biological sources. There were two hits for the compound **5** (MW 476) from DNPs by searching of the biological sources and molecular weight. It was tetraprenyl-β-curcumene (MW 476, C₃₅H₃₅) which was produced by marine *B. subtilis* (Böröczky et al. 2006, Kontnik et al. 2008). However, there was no UV information available. Also no UV spectrum was available for the second compound trihexocin (MW 476). This antifungal antibiotic was isolated from a soil *Bacillus* sp. strain SY-414 (Shin and Yoo, 1995). By searching of molecular weight and UV maxima from DNPs there were 11 hits for compound **5** (MW 476), 23 hits for compound **7** (MW 444, UV max 225). But all hits did not perfectly match.

The compound **6** (MW 389, UV max 246, 311) was found only in 100 ml both cultures in GYM and BM media (Figure 3.7). This compound was regarded as AI-77F (MW 389, UV max 245, 312) which was isolated from soil *B. pumilus* AI-77 (Shimojima et al. 1982a). The substance AI-77F was not clearly approved as being of biogenetic origin or produced by degradation (Shimojima et al. 1982b). In addition, this substance was reported as inactive against *B. subtilis* and *S. aureus* (Li et al. 2012).

At first point of view, compounds **3** (MW 438), **4** (MW 508), and **5** (MW 476) might be interesting compounds. However, they shared the same UV maxima and had retention times very similar to the compound **1**, and **2**, (range Rt at 2.9, 3.1, and 3.2 min). That was the big problem for separation by HPLC-UV method. Although the fractions 1, 2 were attempted to be separated with semi-preparative HPLC, the compounds could not be purified.

The compound **5** was identified with base peak chromatogram (BPC) analysis and was clearly visible in individual fractions next after of crude extract preparation (Figure 3.11).

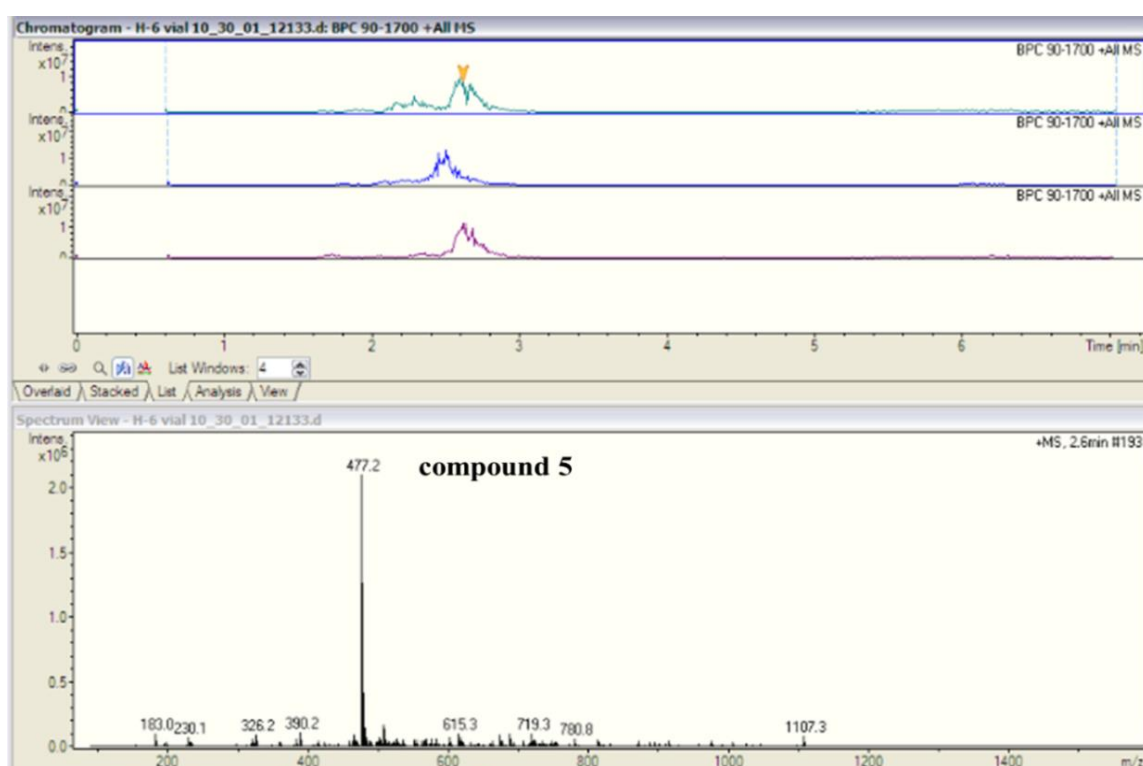


Figure 3.11 The presentation of the compound **5** (MW 476) in fractions after preparation.

The fraction 2 included compound **5** is shown in Figure 3.12. The fraction 2 is illustrated in sky colour and the presence of compound **5** is confirmed by extracted ion chromatogram analysis with red colour. The compound **5** was found as main component of the fraction 2. After 2 and 4 weeks storing, the compound **5** disappeared. Instead compound **3** (MW 438), compound **4** (MW 508), and unidentified mass 566 were observed (Figure 3.12).

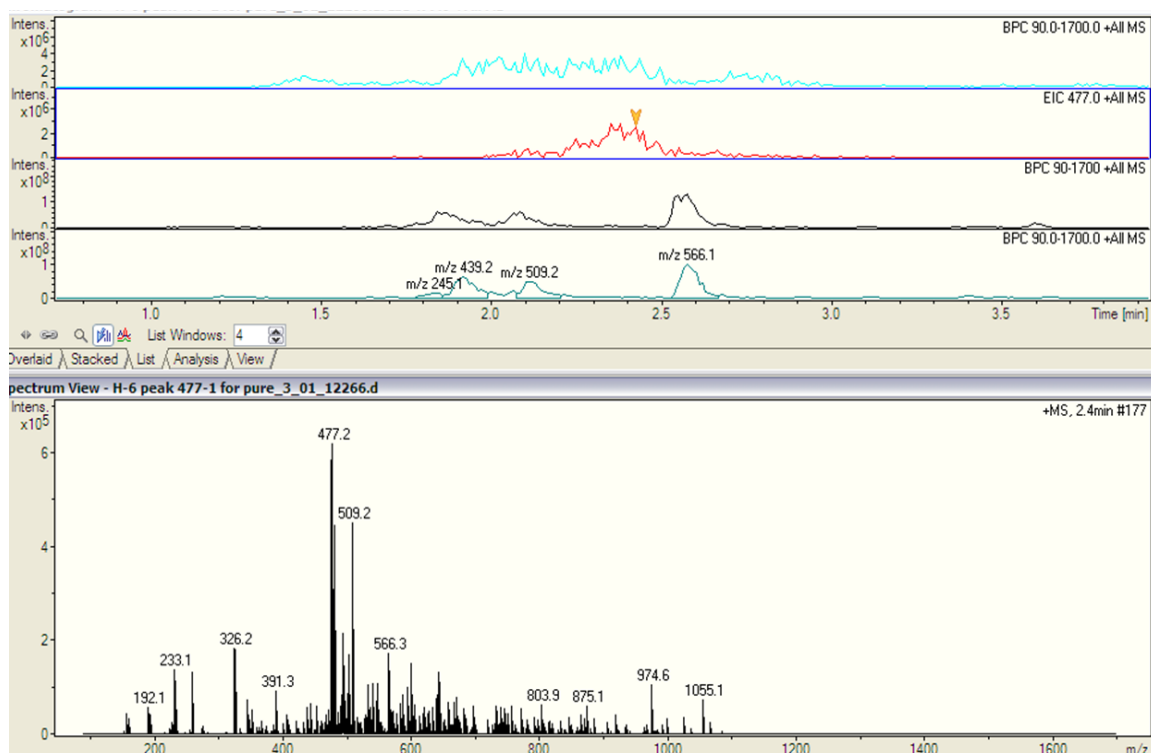


Figure 3.12 The fraction 2 (sky line), compound **5** (MW 476-red line), after preparation 2 weeks and 4 weeks (black and blue) consists of compound **3** (MW 438) and compound **4** (MW 508).

Significantly, the compound **3** (MW 438), and the compound **4** (MW 508) were not found in crude extract of 1 L cultures in BM medium (Figure 3.8) but in the mixture of fraction 2 after preparation and 2 weeks storage (Figure 3.12). Consequently, the compound **5** might be an unstable compound because the mass of this compound changed by time.

Finding of the compound **3** (MW 438) in the fraction 2 after preparation of crude extract of 1 L cultures in BM medium was unexpected, because of its absence in crude extract. It is suggested that this compound was might be a product of degradation of other compounds. It was thought that it was impossible to isolate unstable compounds because of time consuming. One other obstacle was that most of the compounds produced by this strain have very similar UV spectra and retention times. Therefore, this strain H-6 is a potential candidate for more detailed studies.

3.3 *Micrococcus* sp. A-2-28

3.3.1 *Origin, cultivation and identification of the strain A-2-28*

The strain A-2-28 was isolated from soft coral *A. digitatum* Baltic 2 at 10°C in SW medium and identified as *Micrococcus* sp., because analysis of 16S rRNA gene sequence showed 99.3% sequence similarity with *Micrococcus flavus* LW4^T (GenBank accession number DQ491453), 98.6% similarity with *Micrococcus yunnanensis* YIM65004^T (GenBank accession number FJ214355), and 98.5% sequence similarity with *Micrococcus luteus* DSM 20030^T (GenBank accession number AJ310083) which are derived from the “Ribosomal Database Project” (Cole et al. 2014).

3.3.2 *Cultivation and extraction of the strain A-2-28*

Cultivation of strain A-2-28 was scaled up in 1 L in GYM medium. The 100 ml of broth culture was extracted after 2, 3 and 4 days, applied to HPLC-DAD-MS to check the presence of three main compounds which were identified in screening media. The identified metabolites in 1 L cultures disappeared after 4 days cultivation. Therefore, a total of 12 L broth was harvested after 72 h cultivations, extracted with ethyl acetate and separated with semi HPLC.

3.3.3 *Compound purification*

Semi-preparative HPLC was used for separation of the metabolites which were produced by A-2-28. Semi preparation of 1.2 grams crude extract obtained from 12 L cultures was carried out with Merck Hitachi Elite LaChrom system 2 with a gradient from 20% B at 0 min increasing to 80% B at 15 min, to 100% B from 27 min to 27.5 min, flow 14 ml/min.

100 mg fraction 1 consisted of 3 compounds MW 129, MW 158, and MW 175 was eluted at 15 min. 180 mg fraction 2 was eluted at 5 to 7 min and 60 mg pure compound **2** (MW 327/654) was obtained. 28 mg fraction 3 [compound **3** (MW 716), compound **2**, and compound with MW 158] was eluted at 16.5 min, and 7 mg fraction 4 [compound **2** (MW 327/654) and compound **4** (MW 334/668)] was eluted at 18 min.

To purify fraction 1 (MW 129, 158, 175), preparation of 100 mg fraction 1 was carried out by using of Merck Hitachi Elite LaChrom system 2 m with a gradient from 5% B at 0 min increasing to 100% B at 25 min to 27 min, flow 14 ml/min. 1.5 mg of pure compound with MW 129 was eluted at 19 min, and 20 mg of the mixtures of this compound with compound MW 158 were eluted at 15 min. The compound with MW 175 was not found after second preparative HPLC separation.

The same gradient, and column as for purification of compound **1** (MW 129) was applied to purify the compound **3** (MW 716) with Merck Hitachi Elite LaChrom system 2. The compound **3** was eluted at 17 min and 1 mg pure compound was obtained. The mass of this compound was changed during the preparation time along with the emergence of a new mass which was previously not available in the fraction. Consequently, the compound **3** might be an unstable compound and was not further separated. The compound **4** was found in very low amounts in 7 mg of fraction 4, so that this compound was not purified.

3.3.4 Metabolic profile

Chemical analysis of metabolite profiles of the strain A-2-28 are shown in Table 3.7

Table 3.7 Chemical profiles and purified compounds of the strain A-2-28.

CNo Peak	Media	MW	Rt [min]	UV [nm] maxima	CP/yield	CN/comment
1*	GYM/100 ml BM/100 ml GYM/1 L	129 158 175	1.8	229, 273, sh278, 289	Purification; (MW 129) 1.5 mg	Maybe a 3- indolylacetic acid (MW 175)
2	GYM/100 ml BM/100 ml GYM/1 L	327/654	3.5	225, 292 sh360, sh375, 390	Purification; 60 mg	Unknown Maybe new
3	GYM/100 ml BM/100 ml GYM/1 L	716	3.7	226, 326, 405, 536	Purification; 1 mg	Unknown Maybe new
4	GYM/100 ml BM/100 ml GYM/1 L	334/668	3.9	224, 395	No purification	Unknown Maybe new

* Peak 1 consists of 3 compounds (MW 129, MW 158 and MW 175), CNo: compound number; MW: molecular weight; Rt: retention time; CP: compound purification; CN: compound name.

Peak 1 included 3 compounds with MW 129, MW 158 and MW 175. This peak was found in 100 ml culture in GYM and BM at low amounts. Those three compounds possessed similar UV max 229, 273, 278 and 289. Whilst the compound **2** (MW 327, UV max 225, 292 sh 360, sh 375, 390) was found in large amount in both media. The compound **3** (MW 716, UV max 226, 326, 405, 536) was present in very low amounts in BM (100 ml cultures), and the compound **4** (MW 334, UV max 224, 395) was likely found with very low amounts in 100 ml cultures in GYM (Table 3.7, Figure 3.13).

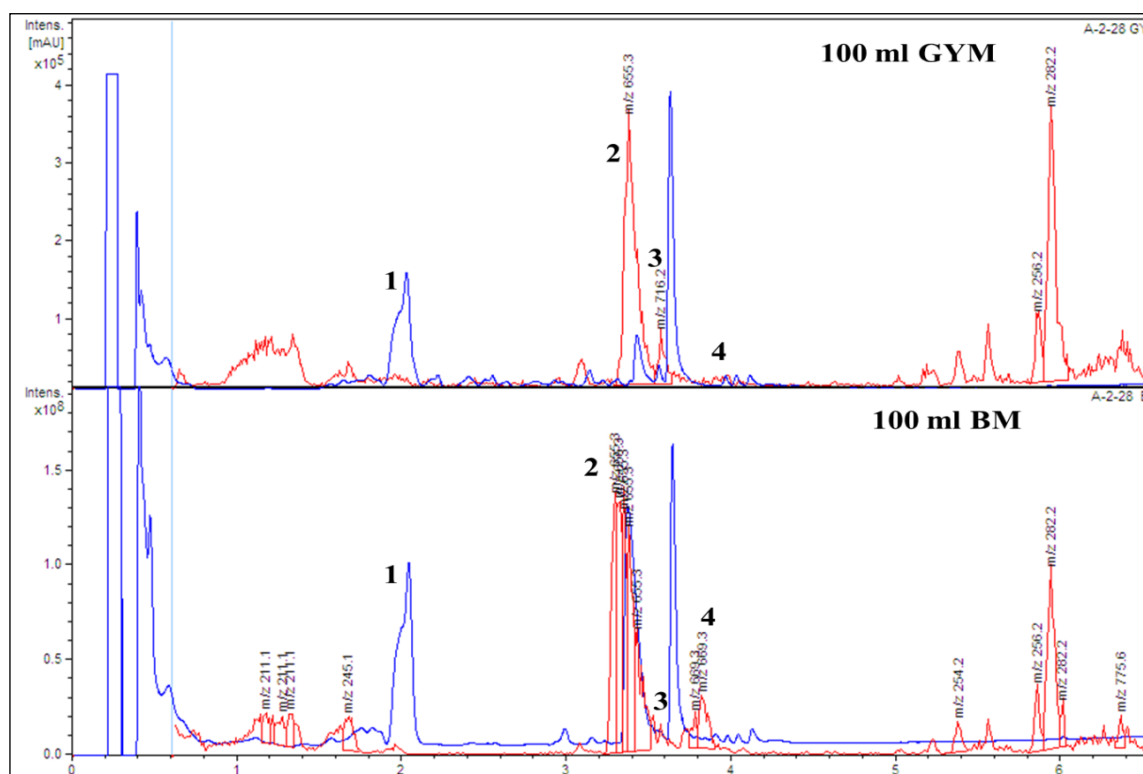


Figure 3.13 Chemical profiles of A-2-28 in 100 ml cultures. Red-mass, blue-UV.

All compounds (compounds in peak 1, compound **2**, **3** and **4**) were produced in 100 ml cultures and in 1 L cultures in GYM, in addition to compound **5** with unidentified mass. Although the compound **5** showed very good UV signals. It could not be isolated due to the very low amounts in the crude extract (Figure 3.14, Figure 3.15). Compounds **2** (MW 327) and **3** (MW 716) were produced in large amounts and had good UV signals, while compounds of peak 1 and the compound **4** (MW 334) were present in very low amounts, the latter compound showed rather weak UV signals as well (Figure 3.14, Figure 3.15).

isolated from this genus so far. Moreover, the compounds with MW 129, 327 and 716 which were produced by this strain showed good enough UV signals for isolation. Therefore, these compounds were purified.

3.3.5 Bioactivities of the isolated compounds

The biological activities of three purified compounds are shown in Table 3.8. The compounds **1** (MW 129) and **2** (MW 327) were inactive in all tests, whilst the compound **3** (MW 716) showed weak inhibition of *T. rubrum* with 24.0% and inhibited the enzyme phosphodiesterase 4 β 2 (PDE4) with 43.0%. The other tests were not done due to lack of material.

Table 3.8 Bioactivities assays of pure compounds isolated from A-2-28.

(The numbers indicate the percentage of inhibition, (-) indicate negative results)

Compounds	Antibacterial						Antifungal			Cytotoxic		Enzyme inhibition			
	<i>Bacillus subtilis</i>	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus aureus</i> MRSA	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Propionibacterium acnes</i>	<i>Candida albicans</i>	<i>Septoria tritici</i>	<i>Trichophyton rubrum</i>	NIH-3T3 (mouse fibroblasts)	Hep G2 (human liver cancer)	Acetylcholinesterase	Glycogen synthase kinase	Phosphodiesterase 4 β 2	Protein-tyrosine-phosphatase
1 (MW 129)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 (MW 327)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3 (MW 716)	-	-	*	-	*	*	-	*	24	*	-	*	-	43	-

* The test was not performed due to limitation of purified compound.

3.3.6 Discussion

Among the compounds of peak 1, there was no hit for compounds with MW 129 (UV max 229, 275) and MW 158 (UV max 230, 281). Whereas, the compound with MW 175 (UV max 229, 273, sh 278, sh 289) might be a 3-indolylacetic acid (IAA) which was isolated from marine alga *Undaria pinnatifida* (Abe et al. 1972). This substance has UV maxima 229, 273, 278, 289 in MeOH which perfectly matched the compound with MW 175

in this study. This compound was isolated from the marine alga and is widely distributed in higher plants, bacteria, algae, yeasts and fungi (DNPs).

The compound with MW 129 that might be considered as unknown compound was purified. The compounds with MW 158 and MW 175 were not purified due to limited amount in the crude extract. The structure elucidation of the compound with MW 129 is in progress.

There was only one close hit for compound **2** (MW 327, UV max 225, 292, sh 360, sh 375, 390). It was proviolacein (MW 327, UV max 271, 290, 385, 412, 492) produced by *Chromobacterium violaceum* (Hoshino et al. 1995). However, proviolacein has been reported as a red pigment in MeOH (DNPs) while the purified compound **2** was not. In addition, the UV maxima of compound **2** did not match the UV spectra of proviolacein. Consequently, the compound **2** was an interesting compound. Structure elucidation is in progress.

Similar, the compound **3** was an unknown compound. Rubiflavin D (MW 716, UV max 204, 230, 245, 280, 313, 408, 429) isolated from *Streptomyces griseus* (Nadig and Séquin, 1987) was found most similar to the compound **3** (MW 716, UV max 226, 326, 405, 536). Moreover, rubiflavin D (DNPs) has been reported as orange substance whereas the purified compound **2** was a pink pigment. As a result, the compound **2** was regarded as unknown compound and attempted to be purified. However, 1 mg of compound **3** was purified and severed for several bioassays. The mass of this compound changed during the preparation along with the emergence of a new mass which was previously not seen in the fraction. Consequently, the compound **3** might be an unstable compound and was not further separated. So the structure elucidation of this compound was not performed due to limited amount of purified compound obtained.

There were 2 hits for compound **4** (MW 334/668). The first hit was 6-methyldihydroindolindione (MW 334, UV max 269, 324, 389) isolated from myxomycete *Lindbladia tubulina*. This compound was red and was inactive against P388/S (murine leukemia sensitive P388 cell line) and P388/VCR (vincristine resistant P338 cell line cell) in the absence of VCR, but it was active against P388/VCR cell in the presence of VCR (Misono et al. 2003). The second hit was palmarumycin CP3 (MW 334, UV 290, 299, 307, 313, 327, 381) isolated from the fungi *Coniothyrium palmarum*. Palmarumycin was colourless and showed equal activity against Gram-positive (*B. megaterium*) and Gram-

negative bacteria (*E. coli*). It was particularly active against fungi *Ustilago violacea*, *Mycotapha microspore*, *Fusarium oxysporum*, *Eurotium repens*, and in addition against the alga *Chlorella fusca* (Krohm et al. 1994).

In contrast to the crude extract of this strain, all three purified compounds (MW 129, MW 327, and MW 716) were inactive against *S. epidermidis*. It was inferred that the corresponding compound for inhibition of test organism was not isolated.

The compound MW 175 (included in peak 1) was regarded as 3-indolylacetic acid or IAA (Figure 3.16). As my knowledge this phytohormone was not reported to be an antibacterial agent so far. The last two metabolites, the compounds with MW 158 (member of peak 1) and the compound **4** (MW 334) were produced in very low amounts in GYM and BM, and might cause the rather weak inhibition of the crude extract toward *S. epidermidis*.

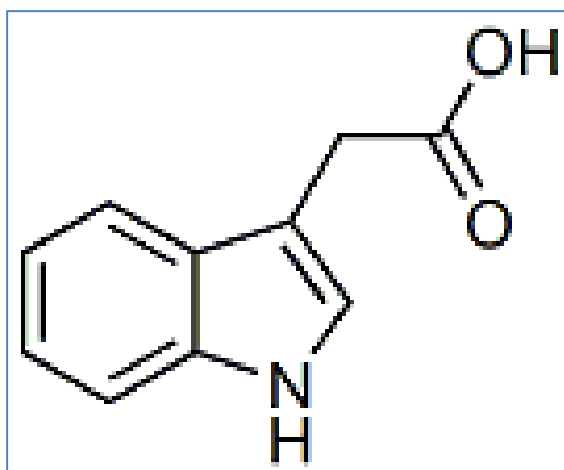


Figure 3.16 Structure of 3-indolylacetic acid (IAA).

3.4 *Bacillus* sp. C-1-10

3.4.1 *Origin, cultivation and identification of the strain C-1-10*

The strain C-1-10 was originally isolated from *A. digitatum* in the Baltic Sea in TM medium and was identified as *Bacillus* sp., based on 16S rRNA sequence analysis. Strain C-1-10 showed 99.8% sequence similarity with *Bacillus amyloliquefaciens* NBRC 15535^T (GenBank accession number AB255669), and shared 99.7% sequence similarity with *Bacillus methylotrophicus* CBMB 205^T (GenBank accession number EU194897) and 99.5% sequence similarity with *Bacillus vallismortis* DSM11031^T (GenBank accession number AB021198) respectively.

3.4.2 *Cultivation and extraction of the strain C-1-10*

Chemical analyses of metabolites after growth in GYM and BM (100 ml cultures) showed that the strain C-1-10 produced 7 compounds in both media. Beside those present in BM medium, additional metabolites were produced in GYM. There were 8 interesting compounds found in 100 ml cultures in GYM medium with rather large amounts and also good UV signals. Therefore, the strain C-1-10 was scaled up to 1 L in GYM medium. Broth of 10 L cultures in total was extracted with ethyl acetate and applied to preparation.

3.4.3 *Compound purification*

Semi-preparative HPLC was used for separation of the metabolites which were produced by C-1-10. Preparation of 2.2 grams crude extract obtained from 10 L cultures was carried out with Merck Hitachi Elite LaChrom system 2 with a gradient from 10% B at 0 min increasing to 100% B from 25 min to 27 min (flow 14 ml/min), and yielded 430 mg fraction 1 at 9 to 16 min, 50 mg fraction 2 at 16 to 18 min, 40 mg fraction 3 at 18 to 22 min, 60 mg fraction 4 at 23 min, 160 mg fraction 5 at 24 min, and 120 mg fraction 6 at 24.5 min.

Compound **1** (MW 686) was found in fraction 1 in very low amounts. This fraction was purified with the same gradient and HPLC system as for the preparation of crude extracts. The fraction 1 was separated into two peaks with different retention time (2.9 min

and 3.3 min), but with identical UV maxima and molecular weight 686. This compound needs to be further purified.

Two isomer compounds (the compound **13** and **14** with MW 462) which also were unknown compounds were found in fractions 2 and 3. In contrast to the UV signals in crude extract (with standard HPLC), the UV signals of those compounds were found very weak in Merck Hitachi Elite LaChrom system 2, when they were applied for preparation. In addition those isomers existed in both fractions with very low amounts. Therefore those isomers were not considered to further preparation.

To purify compounds **4** (MW 993) and **5** (MW 1007), preparation of 60 mg fraction 4 was carried out by using Merck Hitachi Elite LaChrom system 2 with a gradient from 70% B at 0 min increasing to 100% B at 13 min to 14.5 min, (flow 14 ml/min). The pure compound **4** was yielded 3.5 mg and the pure compound **5** was yielded 6.5 mg.

To purify compounds **6** and **7**, 160 mg of fraction 5 were applied to preparation with Merck Hitachi Elite LaChrom system 2 using the same gradient as for the purification of fraction 4. The pure compound **6** (MW 1021) was yielded 60 mg and the pure compound **7** (MW 1035) was yielded 4 mg.

Four compounds (4 to 7) which were considered as known cyclic peptides were isolated from the strain C-1-10. The other fractions 1, 2, and 3 including compound **1** (MW 686), compound **18** (MW 366), the known cyclic peptides (MW 1030 and MW 1044), isomer compound (MW 462), and fraction 6 (mix of 3 known cyclic peptides MW 1007, MW 1021, MW 1035) will be further analysed.

3.4.4 Metabolic profile

The substances identified in strain C-1-10 in 100 ml cultures in GYM and BM media are shown in Table 3.9 and Figure 3.17. This strain produced at least 7 compounds (compound **1-7**) in both media. One out of those compounds was unknown (compound **1**, MW 686). The other compounds might be all known. The compound **2** (MW 772, UV max 231, 275, 328) might be an O-demethylpaulomycin A (MW 772, UV max 236, 274, 321) which was isolated from *Streptomyces paulus* (Argoudelis et al. 1988). Compound **3-7** might be known cyclic peptides. The compound **3** (MW 502, UV max 233) might be a cyclic hexapeptide bistratamide C (MW 502, UV max 233) which was isolated previously

from the ascidian *Lissoclinum bistratum* (Foster et al. 1992). The known cyclic peptides [compound **4** (MW 993), compound **5** (MW 1007), compound **6** (MW 1021) and compound **7** (MW 1035)] were major compounds produced by the strain C-1-10. Those main produced cyclic peptides were successful isolated. The structure elucidation now is in progress.

Table 3.9 Chemical profiles and purified compounds of the strain C-1-10.

CNo Peak	Media	MW	Rt [min]	UV [nm] maxima	CP/yield	CN/comment
1	GYM/100 ml BM/100 ml GYM/1L	686	3.7	233, 271, 323	Not successfully purified	Unknown Maybe new
2	GYM/100 ml BM/100 ml GYM/1L	772	5.2	231,275, 328	No purification	Maybe an 0-demethylpaulo macin A
3	GYM/100 ml BM/100 ml GYM/1L	502	6.0	230	No purification	Maybe a bistratamide C
4	GYM/100 ml BM/100 ml GYM/1L	993	6.2	226, 279	Purification; 3.5 mg	Maybe a surfactin
5	GYM/100 ml BM/100 ml GYM/1L	1007	6.3	226, 280	Purification; 6.5 mg	Maybe a surfactin
6	GYM/100 ml BM/100 ml GYM/1L	1021	6.4	225, 278	Purification; 60 mg	Maybe a bacillopeptin A
7	GYM/100 ml BM/100 ml GYM/1L	1035	6.5	226, 278	Purification; 4 mg	Maybe a bacillopeptin B
8	GYM/100 ml	758	3.4	227, 284, 305 320, 337, 355	No purification	Unknown
9	GYM/100 ml	742	3.8	232, 270, 306 322, 338, 357 387	No purification	Unknown
10*	GYM/100 ml	742 744	4.0	226, 286, 324 341, 362, 384	No purification	Unknown
11	GYM/100 ml	726	4.1	235, 273, 322 343, 350, 362 384	No purification	Unknown
12	GYM/100 ml	580	4.5	223, 285, 327 345, 349, 363 383	No purification	Unknown
13*	GYM/100 ml GYM/1L	745 462	4.8	236, 274, 286	No purification	Unknown
14	GYM/100 ml GYM/1L	462	5.1	235, 273, 286	Not successfully purified	Unknown

CNo Peak	Media	MW	Rt [min]	UV [nm] maxima	CP/yield	CN/comment
15	GYM/100 ml	446	5.8	233, 272, 286	No purification	Unknown
16	BM/100 ml	586	3.6	235, 270, 309 324	No purification	Unknown
17	BM/100 ml GYM/1L	1030	3.9	227, 276	No purification	Maybe a bacillomycin D1
18	BM/100 ml GYM/1L	366	4.1	235, 268, 311	No purification	Maybe a paecilomycin E/F
19	BM/100 ml GYM/1L	1044	4.2	227, 275	No purification	Maybe a bacillomycin D2
20	BM/100 ml GYM/1L	1058	4.3	Could not be determined	No purification	Maybe a bacillomycin D4/5
21	BM/100 ml	766	4.7	231, 275, 287	No purification	Unknown
22	BM/100 ml GYM/1L	780	4.9	235, 273, 287	No purification	Unknown
23	BM/100 ml	787	5.1	233, 274, 287	No purification	Unknown
24	BM/100 ml	779	5.2	Could not be determined	No purification	Unknown

* Each of peaks 10 and 12 consist of 2 compounds MW 742, MW 744 and MW 745, MW 462, respectively. CNo: compound number; MW: molecular weight; Rt: retention time; CP: compound purification; CN: compound name.

Searching of molecular weight and biological sources revealed that *Bacillus* spp. were sources of lipopeptides including members of surfactin class and iturin class. Compounds with molecular weight 993, 1007, 1021 and 1035 were surfactins produced by *B. subtilis* OKB 105 (Kowall et al. 1998). But no UV information was available for these surfactins. The compounds **4** (MW 993, UV max 226, 279) and **5** (MW 1007, UV max 226, 280) which were produced by *Bacillus* C-1-10 in this study might be surfactin relative substances. Compound **6** (MW 1021, UV max 225, 278) and compound **7** (MW 1035, UV max 226, 278) might be bacillopeptin A (MW 1021, UV max 228, 278) and B (MW 1035, UV max 228, 278), respectively (Kajimura et al. 1995).

In addition to these compounds produced in both media (black numbers), this strain produced compounds **8-15** (blue numbers) in 100 ml cultures in GMY medium and compounds **16-24** (green numbers) in 100 ml cultures in BM medium (Figure 3.17).

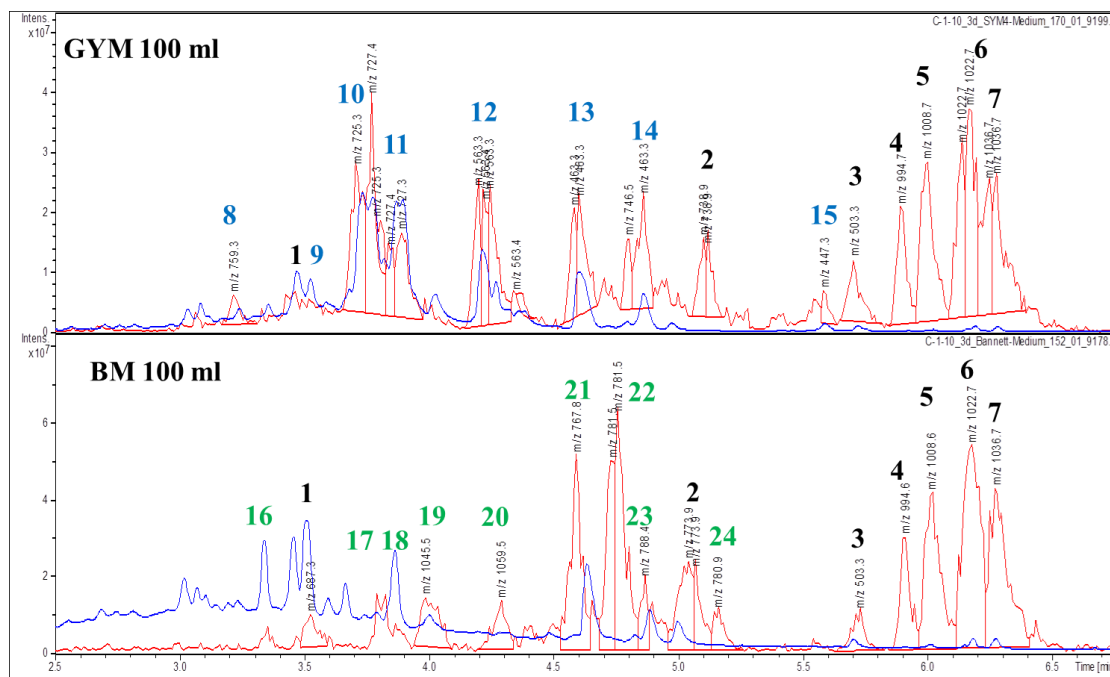


Figure 3.17 Chemical profiles of C-1-10 in 100 ml cultures. Red- mass, blue-UV.

These 8 different compounds which were produced in 100 ml cultures in GYM medium were unknown compounds, while at least 3 out of 9 compounds produced in 100 ml cultures in BM were known cyclic peptides (compounds **17**, **19** and **20**). In addition of the 5 known cyclic peptides mentioned above, this strain might be produce bacillomycin D derivatives, the compound **17** (MW 1030), the compound **19** (MW 1044) and the compound **20** (MW 1058) might be considered as bacillomycin D1, bacillomycin D2, and bacillomycin D4/5, respectively, all known as products of *B. subtilis* (Peypoux et al. 1984).

The mass analysis is shown in Figure 3.17. At 4.0 min in GYM 100 ml was m/z 725 and m/z 727 in positive mode. It were found m/z 725 $[M+H-H_2O]^+$, m/z 743 $[M+H]^+$, and m/z 765 $[M+H+Na]^+$ together with m/z 727 $[M+H-H_2O]^+$, m/z 745 $[M+H]^+$, and m/z 767 $[M+H+Na]^+$. Therefore, the true molecular weights for these compounds were 742 and 744. Because the compounds MW 742 and MW 744 existed in the same retention time and had similar UV maxima so that these compounds were listed in peak 10. Also, the mass of the compound **12** which was given by automatic analysis in Figure 3.17 at 4.5 min was 563.

The true molecular weight of this compound was decided as 580 because of the m/z 563 $[M+H-H_2O]^+$, m/z 581 $[M+H]^+$, and 603 $[M+H+Na]^+$.

Beside the major cyclic peptide products, the compound **10** (MW 742, MW 744), the compound **11** (MW 726), the compound **12** (MW 580), and two interesting isomer compounds (**13**, **14**) with molecular weight 462 were presented in rather high amounts and showed good UV signals (R_t at 4.0 to 5.1 min) in GYM 100 ml cultures (Table 3.9, Figure 3.17, Figure 3.19). At the similar retention time of compounds **10** to **12**, known cyclic peptides were found. These were compound **19** (MW 1044) and **20** (MW 1058) either present at rather low amounts and with weak or no UV signals (Table 3.9, Figure 3.17). At similar retention time and UV maxima, compounds **21** (MW 767), **22** (MW 780), and **23** (MW 787) were found as major compounds in screening BM, while two isomers (compound **13** and **14**) were found in 100 ml cultures in GYM medium (Table 3.9, Figure 3.17, Figure 3.19).

Other compounds were produced in 100 ml cultures in both media either at low amounts or revealed weak UV signals. These were the compounds **8**, **9**, and **15** in GYM, and the compounds **16**, **17**, and **24** in BM.

The metabolites of the strain C-1-10 in 1 L cultures in GYM medium are shown in Table 3.9, Figure 3.18, and Figure 3.19

This strain produced 7 compounds [compound **1** (MW 686), compound **2** (MW 772), and 5 cyclic peptides (compounds **3-7**)]. In addition, compound **13**, **14** which were produced in GYM 100 ml cultures and compound **17**, **18**, **19**, **20** and **22** which were found in BM 100 ml cultures were available in 1 L cultures in GYM medium. The known cyclic peptides (compound **4-7**) which were originally isolated from *Bacillus* species were major products with high amounts in mass and rather good UV signals, whilst compound **3** which is considered as hexapeptide from the ascidian *Lissoclinum bistratum* and others existed in very low amounts. The known cyclic peptides which were produced in 100 ml cultures in BM medium were also found in big scale cultivation in 1 L in GYM medium. These were compounds **17** (MW 1030), **19** (MW 1044), and **20** (MW 1058) with rather low amounts and weak UV signals. The isomer compounds (compounds **13** and **14** with MW 462) were found in rather low amounts in GYM 1 L cultures. The compound **14** with MW 745 (100 ml cultures in GYM) and compound **22** with MW 780 (100 ml cultures in BM) were also found

in GYM 1 L cultures. They shared the same UV maxima and retention time to one out of two isomers (MW 462).

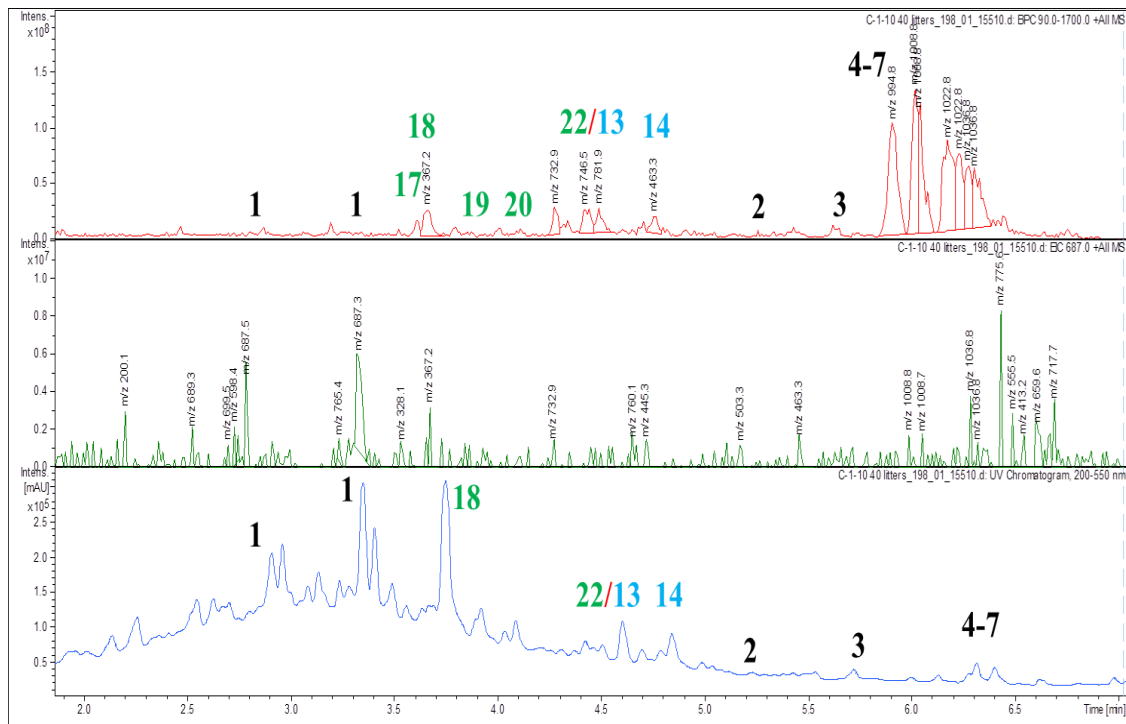


Figure 3.18 Chemical profiles of C-1-10 in 1 L cultures GYM medium. (Red-mass, green-presented of mass 687 in two different times, blue-UV).

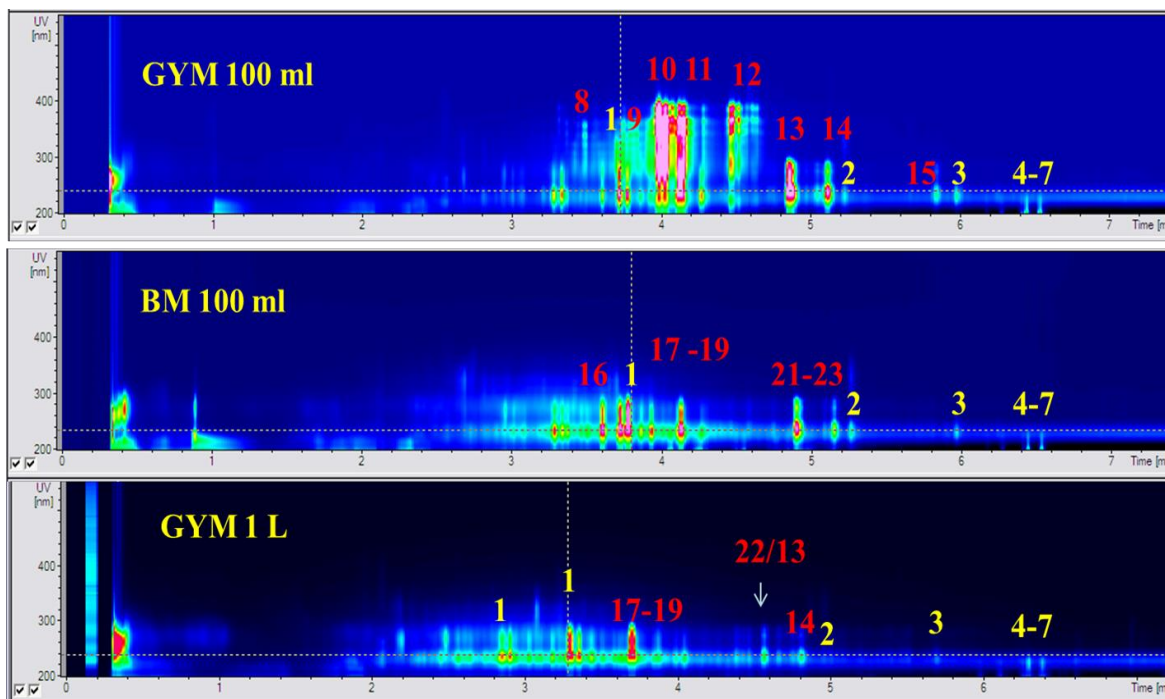


Figure 3.19 UV spectrums of C-1-10 in GYM and BM (100 ml) and 1 L cultures in GYM.

3.4.5 Bioactivities of the isolated compounds

The biological activities of four purified compounds are described in Table 3.10.

Table 3.10 Bioactive activities of pure compounds isolated from C-1-10.

Compounds	Antibacterial						Antifungal			Cytotoxic		Enzyme inhibition			
	<i>Bacillus subtilis</i>	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus aureus</i> MRSA	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Propionibacterium acnes</i>	<i>Candida albicans</i>	<i>Septoria tritici</i>	<i>Trichophyton rubrum</i>	NIH-3T3 (mouse fibroblasts)	Hep G2 (human liver cancer)	Acetylcholinesterase	Glycogen synthase kinase	Phosphodiesterase 4 B2	Protein-tyrosine-phosphatase
4 (MW 993)	-	52	86	-	-	-	-	-	-	-	-	-	-	39	-
5 (MW 1007)	23	96	98	22	-	-	-	-	-	-	-	-	-	-	-
6 (MW 1021)	-	75	74	-	-	-	-	-	-	-	-	-	-	31	-
7 (MW 1035)	23	53	63	-	-	-	-	-	-	-	-	-	-	41	-

The numbers indicate the percentage of inhibition, (-) indicate negative results.

All four compounds showed inhibition of *S. epidermidis* and also MRSA. The compound **5** with molecular weight 1007 showed rather weak inhibition of Gram-negative *E. coli* and was inactive to phosphodiesterase 4, while the other compounds were inactive to *E. coli* and active against phosphodiesterase 4. However, all four pure compounds were inactive to other Gram-negative bacteria and fungi and exhibited no cytotoxic activity, and no inhibition to glycogen synthase kinase, and protein-tyrosine-phosphatase.

3.4.6 Discussion

There were several hits from databases for the compound **1** (MW 686, UV max 233, 271, 323). However, all hits did not perfectly match both the biological sources and the UV maxima. For instance, the compound excelside B (MW 686, UV max 230, 275, 283) was isolated from the seeds of *Fraxinus excelsior* and reported as inhibition of adipocyte differentiation in 3T3-IL1 cell (Bai et al. 2010). The compound mayoside C (MW 686, UV max 226, 276, 350) was a constituent of the bark of *Picramnia teapensis* (Rodríguez-

Gamboa et al. 2000). There was no hit from microbial sources for compound **1**. Therefore, this compound might be interesting for chemical structure analysis. As mentioned above, the fraction 1 was purified in order to isolate the pure compound **1**.

Before preparation of the fraction 1, the UV spectra of metabolites in 1 L cultures in GYM medium showed that there were some compounds with strong UV signals at 2.5 to 3.5 min (Figure 3.18-blue line, Figure 3.19-GYM 1 L). However, the mass of all compounds which were eluted at this retention time could not be identified, due to limitation of amounts of the compounds (Figure 3.18). The compound **1** (MW 686) which was regarded as interesting compound was detected at this retention time. This was confirmed by analysis of extracted ion chromatogram (EIC) of mass 687 (green line in Figure 3.18). The mass 687 (MW 686) was found at 2.9 min and 3.3 min with similar UV maxima (Figure 3.18, Figure 3.19, Figure 3.20). Due to presentation in crude extract with very low amounts, the compound with MW 365/765 was only found in the fraction 1 after preparation of crude extract, this compound was notably remarked because its existence next to the compound **1** in addition with good UV signals (green line in Figure 3.18). Due to limitation amount of crude extract, the compound **1** was conserved for further separation.

After semi-preparation, with the range of retention time 2.5 to 3.5, the compound **1** (MW 686) was found as isomers with UV maxima 230, 273 (MeOH). The mass 365/765 was found in rather low amounts but very good UV signals (UV maxima 237, 268, 323) at 3.2 min (Figure 3.20).

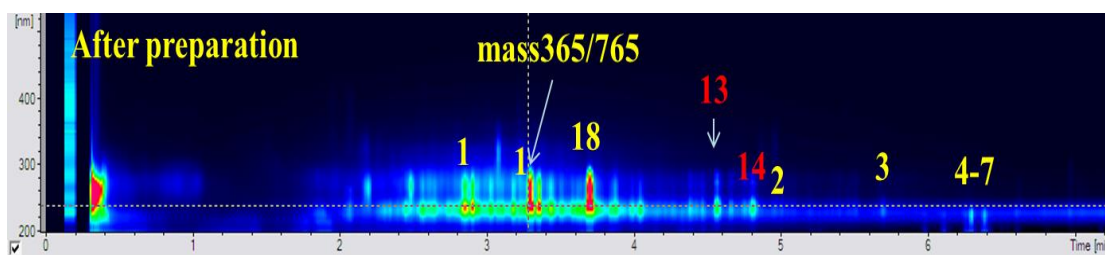


Figure 3.20 UV spectrums of metabolites of the strain C-1-10 after preparation.

The compound with MW 364 and UV max 234, 272, 312 might be an antibiotic LL Z1640-1 (MW 364, UV max 234, 272, 312) isolated from unidentified fungus and possessed antiprotozoal activity (Ellestad et al. 1978). Other hit was a zeaenol or antibiotic LL-Z1640-4 (MW 364, UV max 234, 271, 314) isolated from *Drechslera portulacae*

(Sugawara et al. 1992). Antibiotic LL Z1640-1 and zeaenol isolated from Gorgonian derived fungus *Cochliobolus lunatus* showed antifouling properties (Shao et al. 2011).

The molecular weight and UV maxima of compound **2** (MW 772, UV max 231, 275, 328) matched to those of 0-demethylpaulomycin A (MW 772, UV max 236, 274, 321) which was isolated from *S. paulus*. This compound (Figure 3.21) was reported to be active against Gram-positive bacteria, especially *S. aureus* (Argoudelis et al. 1988a).

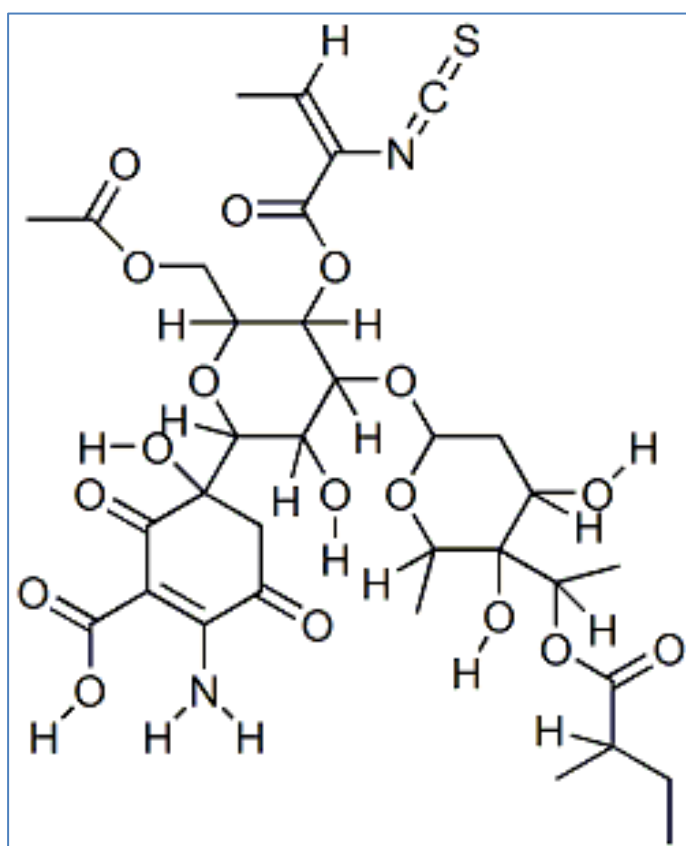


Figure 3.21 Structure of 0-demethylpaulomycin A.

The compound **3** (MW 502, UV max 230) might be the cyclic hexapeptide bistratamide C (MW 502, UV max 234 in CH_2Cl_2), which was isolated from the ascidian *Lissoclinum bistratum* (Foster et al. 1992). The other hit was aleurodiscal (MW 502, UV max 237) isolated from *Aleurodiscus mirabilis*. This compound was reported as antifungal agent inhibition of *Fusarium oxysporum*, *Mucor miehei*, *Absidia glauca*, *Alternaria porri*, *Ascochyta pisi*, *Aspergillus ochraceus*, *Verticillium* sp. 153-83, and *Zygorrhynchus moelleri* with 2 μg /disc by agar diffusion assay method. But no antibacterial activity was observed

with *B. subtilis*, *E. coli*, and *S. aureus* with minimum inhibitory concentration (MIC) >100 µg/ml (Lauer et al. 1989).

As mentioned above for known surfactins which had similar molecular weight to compounds **4** (MW 993) and **5** (MW 1007) there was no UV information (see 3.4.4)

Searching of MW and UV maxima revealed that micropeptin EI 992 (MW 993, UV max 224, 280) and hymenamamide K (MW 1007, UV 221, 280) were similar to compounds **4** (MW 993, UV 226, 279) and **5** (MW 1007, UV max 226, 280) from this present study. Micropeptin EI 992 isolated from cyanobacterium was described as colourless oil, trypsin inhibitor (Ploutno et al. 2002). Hymenamamide K isolated from a marine sponge *Hymeniacion* sp. was a proline containing cyclic octapeptide and showed inhibition of protein tyrosine kinase at concentration 73 µg/ml (Tsuda et al. 1994). However, the compound **4** isolated from C-1-10 was white solid substance and showed antibacterial activity against Gram-positive bacteria and inhibited phosphodiesterase 4, whereas compound **5** inhibited Gram-positive bacteria and also Gram-negative bacteria and showed no inhibition of any tested enzymes.

As mentioned above, compounds **6** (MW 1021) and **7** (MW 1035) were regarded as bacillopeptin A and B. However, these compounds isolated in this present study were antimicrobial substances. They showed antimicrobial activity against Gram-positive bacteria (Table 3.10), while bacillopeptin A and B isolated from *B. subtilis* FR-2 did not show any activity against bacterial and fungal tests including *B. subtilis*, *E. coli*, and *C. albicans* (Kajimura et al. 1995).

The compound **8** with MW 1049 was produced by *Bacillus* sp. strain H-6 (see 3.2.4). It might be a bacillopeptin C (MW 1049, UV max 229, 278), an antifungal cyclic peptide against *C. albicans* (Kajimura et al. 1995). However, this compound was not found in the crude extract of the strain C-1-10 in 100 ml cultures and also in 1 L cultures.

The crude extract of the strain C-1-10 showed inhibition of four test microorganisms. Four purified compounds showed no inhibition of *C. albicans*. Thus, the compound which responded to *C. albicans* inhibition was not identified.

The genus *Bacillus* sp. is a well-known source of cyclic peptides so far. They had similar molecular weight to some compounds isolated in this study. However, UV

information was not available. For examples, surfactins 1 (MW 1035), and 2 (MW 1021) produced by *B. subtilis natto* showed moderate anti HIV activity (Itokawa et al. 1994). Bacillomycin L (MW 1021, normal-C₁₄) isolated from *B. subtilis* showed weak antifungal activity against *Ophiostoma ulmi* and *Cryphonectria parasitica*, while bacillomycin L_{1,2} (MW 1035, anteiso C₁₅ and iso C₁₅) and bacillomycin L_{3,4} (MW 1049, iso C₁₆ and n-C₁₆) showed stronger activity than those of bacillomycin L (Eshita et al. 1995). Halobacillin (Figure 3.22), which was produced by a marine *Bacillus* sp. was an acylpeptide of the iturin class with MW 1035. Halobacillin showed moderate human cancer cell cytotoxicity, no antifungal, and no antibiotic activities (Trischman et al. 1994).

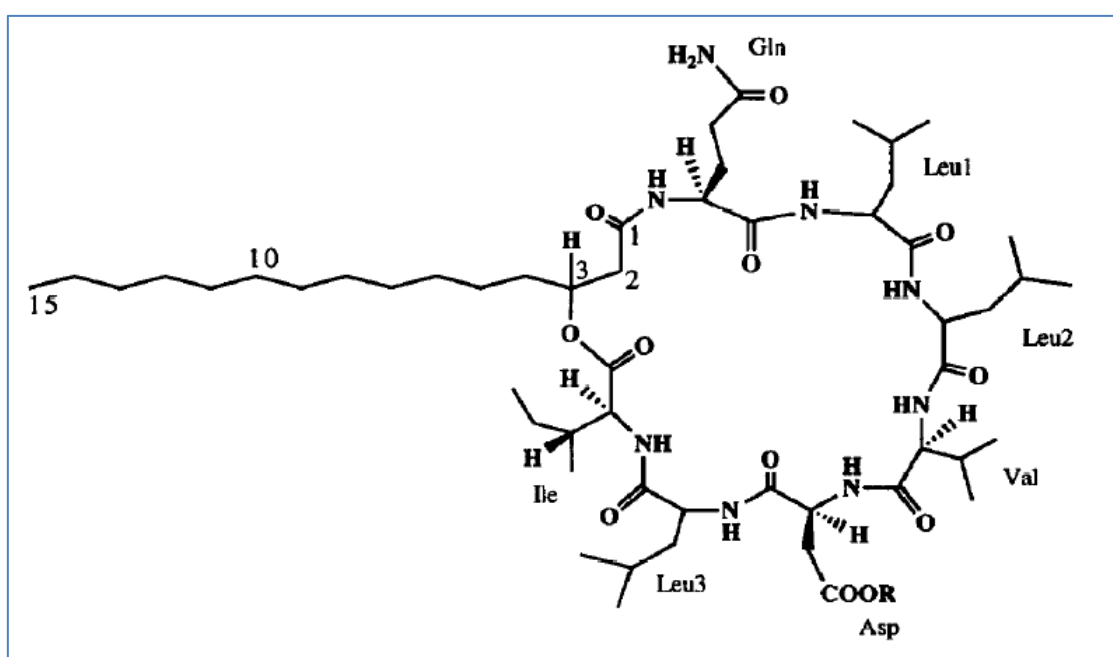


Figure 3.22 Structure of halobacillin (Trischman et al. 1994).

The compounds **4** (MW 993), **5** (MW 1007), **6** (MW 1021), and **7** (MW 1035) were the major compounds produced by strain C-1-10. They were successfully purified by UV-HPLC separation and showed different antimicrobial activities to known cyclic peptides from DNPs. This study suggested that compound **4** (MW 993), **5** (MW 1007), **6** (MW 1021) and **7** (MW 1035) might be relative cyclic peptides. Further detailed NMR analysis is required to confirm the name and structure of each compound.

The search by molecular weight and UV maxima yielded 5 hits for compound **8** (MW 758 UV max 227, 284, 305, 320, 337, 355). Three hits were original sources from microorganisms. These were 0-demethylpaulomycin B (MW 758, UV max 237, 276, 320)

isolated from *S. paulus* (Argoudelis et al. 1988a), antibiotic disorazole A1 (MW 758, UV max 251, 265sh, 272, 298, 306, 321sh) which was produced by a myxobacterium *Sorangium cellulosum* strain So ce 12 (Jansen et al. 1994), and unguisin A (MW 758, UV max 219, 274, 281, 290) isolated from marine fungus *Emericella unguis* (Malmstrom, 1999). Disorazole A1 showed strong inhibition of many fungi but no inhibition of yeasts (*Sacharomyces cerevisiae*, *C. albicans*, *Debaryomyces hansenii*) and bacteria including of *B. subtilis*, *S. aureus*, *Micrococcus luteus*, *Streptococcus faecalis*, *E. coli* and *Salmonella typhimurium* (Irschik et al. 1995; Hopkins and Wipf, 2009). Unguisin A showed moderate inhibition of *S. aureus* and *Vibrio parahaemolyticus* (Malmstrom, 1999). Other hits had the same molecular weight but different in UV maxima and biological sources.

The best hit for compound **9** (MW 742, UV max 232, 270, 306, 322, 338, 357, 387) were bacillaene B (MW 742, UV maxima 292, 328, 343, 361, 382) which was isolated from *B. amyloliquefaciens* strain FZB 42 (Moldenhauer et al. 2010) and licoagrone (MW 742, UV max 230, 278, 324, 396) isolated from hairy root cultures of plant *Glycyrrhiza glabra* (Asada et al. 1999).

The most similar hit to compound **10** (MW 744, UV max 226, 286, 324, 341, 362, 384) was paulomycin D (MW 744, UV max 236, 275, 321) which was isolated from *S. paulus*. This compound showed activity against the Gram-positive bacteria *S. aureus*, *S. epidermidis*, *S. faecalis* and *S. pneumoniae* (Argoudelis et al. 1988b).

Searching by molecular weight and UV maxima revealed that unguisin B (MW 726, UV max 220, 274, 280, 290) which was isolated from the marine fungus *Emericella unguis* (Malmstrom, 1999) and bambusicolaside IV (MW 726, UV max 232, 298, 344, 356, 456) were most similar to compound **11** with MW 726 and UV maxima 235, 273, 322, 343, 350, 362, 384 (Zhang et al. 1997).

The best hit for compound **12** (MW 580, UV max 223, 285, 327, 345, 349, 363, 383) was bacillaene with molecular weight 580 and UV maxima 292, 328, 343, 361, 382. Bacillaene was isolated from *B. subtilis*, and is active against a broad spectrum of bacteria inhibiting prokaryotic protein synthesis and showed strong inhibition of *E. coli* (Patel et al. 1995).

The compounds **13** and **14** with identical MW 462 and UV maxima 235, 272, 286 but different retention time (4. 8 and 5. 1 min) were found in GYM medium (100 ml and 1

L cultures). The two compounds might be considered as isomer compounds. No hits were found for these compounds from DNPs by searching of MW and UV maxima and biological sources as well. The most similar compound was aranotin (MW 462, UV max 222, 270) which was isolated from *Arachniotus aureus*, and showed antiviral property (Nagarajan et al. 1968). Other possible hits to this compound with molecular weight 462 were mostly isolated from plants. For example, bletilol C (MW 462 UV max 234, 274, 285, 302) was found in the tubers of *Bletilla striata* (Yamaki et al. 1993) and phyllodulcon 8-glucoside MW 462, UV max 233, 285, 295 was isolated from leaves of the plant *Hydrangea macrophylla* var. *thunbergii* and supported for the polyphenol biosynthesis in host plant (Suzuki et al. 1977).

Chemical analysis of metabolite of crude extract in 1 L cultures showed that peak 13 including of two compounds with MW 462 and MW 745, compound **14** (MW 462), and compound **22** (MW 780) were found at 4.8 to 5.1 min with similar UV maxima (Table 3.9). Mass spectrum showed that the isomers (MW 462) existed in rather low amounts, and the compound **22** and compound with MW 745 were found in a bit higher amounts. These compounds existed in this retention time showed good UV signals when the crude extract was applied in standard HPLC (Figure 3.18, Figure 3.19). As mentioned above, the isomers were regarded as interesting compounds and purified with semi preparation. The UV spectrum of fractions 2 and 3 after preparation showed that the main compound in those fractions were compound with MW 745 and compound **22**, respectively. The isomers MW 462 which were found in crude extract also presented after preparation of those fractions but in very low amounts, and shared UV maxima with compound MW 745. The compound **22** was found in rather high amounts but in very weak UV signals. As a result, all compounds which were found in fractions 2 and 3 were not purified, because of either their existed in very low amounts or revealed in very weak UV signals.

There was no clear hit for compound **15** (MW 446, UV max 233, 272, 286). The most similar compound was pseudopterosin E (MW 446, UV max 226, 275, 285) isolated from *Pseudopteroorgia elisabethae*. This diterpene glycoside showed very low acute toxicity in mice LC₅₀ >300 mg/kg and was an anti-inflammatory agent (Roussis et al. 1990). Other pseudopterosins possessed identical molecular weight 446 and UV maxima 213, 232, 276, 285 for pseudopterosin P, UV maxima 228, 277, 284 for pseudopterosin T, and UV maxima 277, 284 for pseudopterosin K. Two other hits were derivatives of eruberin A, and pneumatopterin C. A derivate eruberin was abacopterin which was constituent

compound of *Abacopteris penangiana* with MW 446 and UV maxima 232, 276 (Zhao et al. 2007). Pneumatopterin C was isolated from *Pneumatopterus pennigera* with MW 446 and UV maxima 230, 280 (Tanaka et al. 1997).

The compound **16** (MW 586, UV max 235, 270, 309) was found in screening BM medium in very low amounts but very good UV signals. Searching by molecular weight, UV maxima and biological sources revealed that this compound is not known as product of *Bacillus* so far. Among some substances with similar molecular weight but different UV maxima compared to compound 16, the most similar compound was integracin B/C (MW 585, UV max 225, 265, 302) isolated from *Cytonaema* sp. (Singh et al. 2002).

The compound **18** (MW 366, UV max 235, 268, 311) might be a paecilomycin E (MW 366, UV max 233, 271, 311) or paecilomycin F (MW 366, UV max 253, 271, 314). Both were isolated from *Paecilomyces* sp. SC 0924 and a marine-derivate *Cochliobolus lunatus* (Xu et al. 2010).

Compounds **17**, **19** and **20** were considered as bacillomycin D1, bacillomycin D2, and bacillomycin D4/5, respectively (Peypoux et al. 1984). Recently, the compounds a₁, a₂ and a₃ which were bacillomycin D-like lipopeptides of 14, 15 and 16 carbon fatty acid long chains showed strong inhibition of *C. albicans*. Especially, compound a₃ with molecular weight 1058 and very similar bacillomycin D structure had strongest antifungal activity (Tabbene et al. 2011). The compounds **17**, **19**, and **20** were considered as known peptides. All were found in 100 ml cultures in BM medium, and in 1 L cultures in GYM medium but with low amounts and weak UV signals. As a result, those compounds were not able to purify.

The compound **21** (MW 766, UV max 231, 275, 287) had similar molecular weight with the antibiotic SB 22484 (MW 766), but different UV maxima (SB 22484, UV 232, 327). This antibiotic SB 22484 was isolated from *Streptomyces* sp. (Selva et al. 1990).

The most similar hit to compound **22** (MW 780, UV max 235, 273, 287) was kirrothricin isolated from *Streptomyces cinnamomensis* (Thein-Schranner et al. 1982).

The most similar hit to compound **23** (MW 787, UV max 232, 274, 287) was the antibiotic BE 32030 also called BE 32030B (MW 787, UV max 242, 248, sh 258, 305). This substance was isolated from *Nocardia* sp. A 32030 and showed inhibition the growth

of P388 murine leukemia, DLD-1 human colon cancer, PC-13 human lung cancer and MKN-45 human stomach cancer cell lines (Tsukamoto et al. 1997).

The compound **24** (MW 779) was found only in 100 ml cultures in BM medium and its UV maxima could not be determine, therefore this compound was not considered for further analysis.

Analysis data from the literature revealed that compound **2** with MW 772 possessed the UV maxima matched to the UV maxima of the compound 0-demethylpaulomycin with MW 772 which was isolated from *S. paulus*. In addition, *S. paulus* also produced paulomycin D (MW 744), paulomycin D was found as closed hit for the compound **10** from this study.

Unguisin A (MW 758) and unguisin B (MW 726) which were both isolated from marine derived fungus *Emericella unguis*, those compound were the closed hits for the compound **8** (MW 758) and the compound **11** (MW 726) in this study. Unguisins were cyclic heptapeptides contained gamma aminobutyric acid (GABA) in the ring, the synthetic cyclic peptide containing GABA has been tested as antitumor agent (Malmstrom, 1999).

Bacillaene B (MW 742) and bacillaene A (MW 580) isolated from *Bacillus* genus were closed hits for the compound **9** (MW 742) and the compound **12** (MW 580) respectively.

All close hits for compounds which were produced by the strain C-1-10 were bioactive substances. It was interesting to find that best hits for compounds **2** and **10**, compounds **8** and **11**, and compounds **9** and **12** were antibiotic substances which were produced by the same sources (*S. Paulus*: producer of hit compounds for compounds **2** and **10**, *Emericella unguis*: producer of hit compounds for compounds **8** and **11**, *Bacillus* sp.: producer of hit compounds for compounds **9** and **12**).

Actual, further exploitation should be carried out analysis of the metabolites of the strain C-1-10 for identification of all its metabolites and for determination of biological activities as well.

DISCUSSION

1. Phylogenetic classification of coral associated bacteria

The aim of this study was the isolation, phylogenetic identification of bacteria associated with the soft coral *A. digitatum* living in the Baltic Sea, and the assessment of their production of antimicrobial compounds. Colonies derived from the soft coral samples were picked randomly from different media with the purpose to obtain a maximum of distinct characteristic colonies.

From our results it is evident that *Firmicutes*, *Gamma*-, *Alphaproteobacteria*, and *Actinobacteria* contribute to the bacterial diversity of the soft coral *A. digitatum*.

This relates to results from the literature in which another *Alcyonium* species was studied. Bacteria associated with the soft coral *A. antarcticum* yielded a high diversity of bacteria including *Gamma*-, *Alpha*-, *Betaproteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinomycetales*, *Planctomycetes*, sulphate-reducing bacteria, and *Chlorobi* by analysis of 16S rRNA gene sequences, while results from culture dependent analysis revealed *Gammmaproteobacteria* as major group. Psychrophilic bacteria were dominant whereas three out of ten cultures were identified as non-psychrophilic and non-marine bacteria (Webster and Bourne, 2007). Members of *Bacillus* were reported as most abundant bacteria isolated from algae, ascidians, and sponges from the North Coast of Sao Paulo state Brazil (Menezes et al. 2010). The *Bacillus* genus was also considered to be most abundant among the isolates from the black coral *Antipathes dichotoma* from South China Sea (Zhang et al. 2012) and from four sympatric gorgonian corals (*Dichotella gemmacea*, *Melitodes squamata*, *Muricella flexuosa*, and *Subergorgia suberos*) from the South China Sea (Peng et al. 2013). Other studies on the coral *F. scutaria* from the Red Sea did not report any member of the genus *Bacillus* (Lampert et al. 2006).

The genus *Lysinibacillus* was the second dominant group of *Firmicutes* found in *A. digitatum*. To our knowledge, there was no report on *Lysinibacillus* associated with corals so far. It should be noted that representatives of the genus *Paenibacillus* were isolated mainly from terrestrial. Recently *Paenibacillus profundus* sp. was reported as a new species from deep sea sediment (Romanenko et al. 2013).

There were very few reports of *Paenibacillus* members from invertebrates. The genus *Paenibacillus* was isolated from the black coral *A. dichotoma* from the South China Sea (Zhang et al. 2012) and the coral *Mussismilia hispida* from eastern Brazil (de Castro et al. 2010). *Exiguobacterium* sp. had been targeted as a mutualised relationship with coral host in Elkhorn coral *Acropora palmate* from Florida Key (Ritchie, 2006). In this study we reported on the isolation of *Paenibacillus* sp. and *Exiguobacterium* sp. from the soft coral *A. digitatum*.

Several culture-dependent studies showed the presence of actinobacteria associated with the stony coral *F. scutaria* from the Red Sea (Lampert et al. 2006), the reef building coral *A. digitifera* from the Gulf of Mannar (Nithyanand and Pandian, 2009), the reef coral *M. hispida* from eastern Brazil (de Castro et al. 2010). Actinobacteria were also reported on the associated with the cold water coral *L. pertusa* from the northeastern Gulf of Mexico (Galkiewicz et al. 2011), the black coral *A. dichotoma* from the South China Sea (Zhang et al. 2012), and the deep sea Gorgonian coral from the South China Sea (Gray et al. 2011). A few cultivation independent studies have reported that a large number of the *Actinobacteria* were associated with the reef building coral *Pocillopora damicornis* from the Great Barrier Reef (Bourne and Munn, 2005), the hard coral *F. scutaria* from Red Sea (Lampert et al. 2008), the cold water coral *L. pertusa* from the Trondheimsfjord (Norway) (Neulinger et al. 2008), the reef building coral *Acropora palmate*, and with *Porites astreoides* from the Mexican Caribbean (McKew et al. 2012). By cultivation independent methods *Actinobacteria* and *Alphaproteobacteria* recovered from deep sea bamboo corals (in Warwick) from Gulf of Alaska seamounts were classified only as belonging to *Actinomycetales* order, *Rhizobiaceae* family and *Rhodobiaceae* at the family level but not at species levels (Penn et al. 2006). Therefore, it is not possible to correlate these data with the present study at the species level. *Actinobacteria* were isolated from the closely related soft coral *A. antarcticum* (Webster and Bourne, 2007). However, those *Actinobacteria* isolated from previous studies were different to the isolates presented in this study. The actinobacterial species *B. saxobsidens* (C-1-31, ATU BL) and alphaproteobacterium *Rhizobium vignae* (C-1-32, ATU RH) were first reported from soft coral *A. digitatum* by cultivation dependent approach in the present study.

The *Gammaproteobacteria* *Pseudoalteromonas* spp., *Vibrio* spp., and *Shewanella* spp. are commonly found when culturing bacteria from corals (Gray et al. 2011; Lampert et al. 2006). *Pseudoalteromonas*, *Pseudomonas*, *Shewanella*, *Psychrobacter* and *Vibrio* were

reported to be associated with cold water *L. pertusa* from the northeastern Gulf of Mexico (Galkiewicz et al. 2011). *Pseudoalteromonas* and *Vibrio* were found from reef building corals and *Psychrobacter* spp. were first reported as dominant in both corals *Acropora* sp. (Mexican Caribbean and Indonesia) and *Porites* sp. from Indonesia (McKew et al. 2012). *Vibrio* was described as a coral pathogen causing bleaching of the coral *O. patagonica* (Ben-Haim et al. 2003; Kushmaro et al. 1997). *Vibrio* sp. were found associated with the Red Sea coral *F. scutaria*, and Caribbean coral *Montastraea franksi* by cultivation independent methods (Lampert et al. 2008; Rohwer et al. 2001). *Vibrio* sp. were dominant bacteria cultured from both mucus and tissues of *A. digitifera* from the Gulf of Mannar (Nithyanand and Pandian, 2009) and were also recovered in the healthy coral *Pocillopora damicornis* from the Great Barrier Reef (Bourne and Munn, 2005), in *M. hispida* from eastern Brazil (de Castro et al. 2010), in *Acropora hyacinthus*, and in coral *Stylophora pistillata* from Great Barrier Reef corals (Kvennefors et al. 2010). The presence of *Vibrio* in corals was intriguing when they may provide nutrients and second metabolites for the coral host (Ritchie, 2006). These bacteria also have been reported as important nitrogen fixing bacteria of the Brazilian coral *M. hispida* holobiont (Chimetto et al. 2008). Only one isolate of *Vibrio* was isolated from *A. digitatum* in this present study.

The *Alphaproteobacterium Silicibacter lacuscaerulensis* belongs to the *Rhodobacteria* and was reported to be specifically associated with healthy stony coral *M. franksis* from the Caribbean. This particular bacterium was established in the corals at five different reefs with a distance of up to 10 km in Bocas del Toro, (Panama) suggesting that it may play a very important role for growth and health of this coral (Rohwer et al. 2001). *Silicibacter* spp. were also dominant in *Acropora* sp. and in *Porites* sp. living in the Mexican Caribbean. Other *Rhodobacteria-Sulfitobacter* spp. were also reported from both corals from Sampela, Indonesia (McKew et al. 2012). The *Sulfitobacter* strain EE-36 from a salt marsh on the coast of Georgia, USA was demonstrated to convert dimethylsulfoniopropionate (DMSP) to acrylate plus dimethylsulfide (DMS) (Curson et al. 2008). DMSP, DMS, and acrylic acid were suggested as nutrient sources for coral-associated bacteria and those sulphur compounds seemed to be playing an important role in construction of bacterial communities in corals. Four genera, *Roseobacter*, *Spongiobacter*, *Vibrio*, and *Alteromonas* comprised the majority of clones retrieved from clone libraries of the corals *M. aequituberculata* and *A. millepora* from Davies Reef, Great Barrier Reef (Raina et al. 2009). Those bacteria were isolated on media with either DMSP or DMS. The results from this study inferred that coral associated bacteria might be involved in cycling of sulphur in the coral reef as well as in coral health (Raina et al. 2009; 2010). Representatives

of *Rhodobacteraceae* family, which are related to *S. marinus* SW-265^T, *L. alba* CECT 5094^T, and *R. vignae* CCBAU 05176^T were found from *A. digitatum* in this present study.

2. Coral hosts and their associated bacteria

The cultured soft coral associated bacteria in this study were not considered as specifically associated with the coral. In fact, the *Firmicutes* especially *Bacillus* spp. found most abundant in *A. digitatum* (this study) were also reported in the black coral *A. dichotoma* from South China Sea (Zhang et al. 2012) and also from in sympatric gorgonian corals (*D. gemmacea*, *M. squamata*, *M. flexuosa*, and *S. suberos*) from the South China Sea (Peng et al. 2013). The actinobacterium *B. saxobsidens* and alphaproteobacterium *R. vignae* were in the first time reported from soft coral *A. digitatum* by cultivation dependent approach.

By cultivation-independent approaches the relationship of microbial communities and coral hosts were found to present specific coral-microbial interactions (Rohwer et al. 2001; Rohwer et al. 2002; Bourne and Munn, 2005). In addition, other studies on the Great Barrier Reef reviewed that certain bacterial groups were associated specifically with corals (*Acropora millepora*, *A. tenuis*, *A. valida* (Littman et al. 2009). Bacteria associated with *A. hyacinthus* and *S. pistillata* were not only different from the ambient water but were also significantly different from each other (Kvennefors et al. 2010). The microbial consortia were found in species-specific associations with the four coral species *Madracis decactis*, *M. hispida*, *Palythoa caribaeorum* and *Tubastraea coccinea* (Carlos et al. 2013). The microbial communities included *Planctomyces*, *Chlorobi*, and sulphate-reducing bacteria as determined by FISH, whereas those bacteria were not detected by other used methods (clone-library, DGGE). This work also showed evidence to support specific coral-microbial interactions, when the conserved coral associated bacterial groups were observed over wide geographic scale (Webster and Bourne, 2007).

As cultivation base approaches might not completely reflect microbial diversity, they might not be used to point out specific or non-specific host interactions. Nevertheless, the cultivation based method and 16S rRNA gene analysis applied in this study demonstrated a bacterial community to be associated bacteria. The term “associated” is simple meaning a co-existence of these isolates with the coral host. A number of soft coral associated bacteria were isolated with 251 individuals belonging to 4 phyla and 41

genotypes. A large number of bacteria were isolated with oligotrophic media, but they were not easily grown on the original media so that their cultivation was continued on other media (Muscholl-Silberhorn et al. 2008). Actually, the use of different media increased the diversity of the bacteria isolated. Four different media were used for isolation in this present study. The nutrient poor Baltic Sea medium (BS) was the medium for isolating of the actinobacterium *B. saxobsidens*.

The finding of abundant antibiotic producing bacteria *Bacillus* spp., *Shewanella* spp., *Vibrio* spp., *Micrococcus* sp. and *S. marinus* (Figure 3.23) supported the assumption that the associated bacteria play a role in coral health.

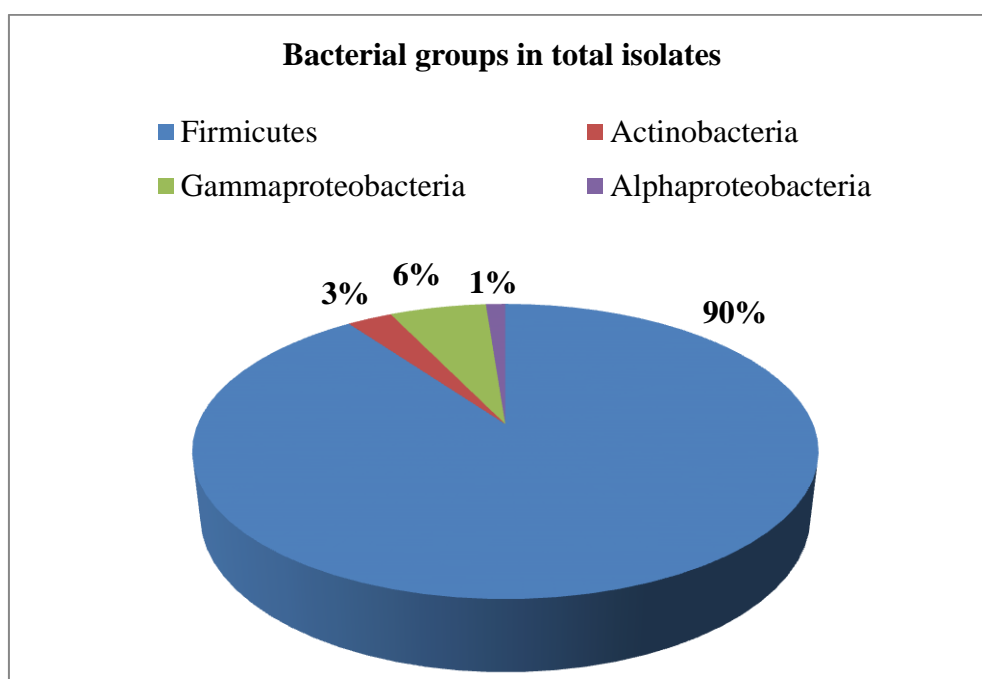


Figure 3.23 Percentage of bacterial groups in total isolates.

A coral colony as a holobiont consisted of the coral animal, zooxanthellae and prokaryotes (bacteria, fungi, protozoa) and unknown components that have yet to be studied (Rohew et al 2002; Rosenberg et al. 2007).

In fact, the microbes found in the coral holobiont certainly play a role in the healthy coral, but also could cause disease of the coral hosts. The coral associated bacteria might be N_2 fixing species (Lema et al. 2012) and could contribute to the coral nutrition. A *Thraustochyrid* sp. was isolated from mucus of healthy coral supported that producer of polyunsaturated fatty acids (PUFAs) might account for rich nutrient sources of the coral

host as well (Harel et al. 2008). In addition, *Shewanella* spp. which were considered as PUFAs sources (Gentile et al. 2003) were commonly associated with corals and other invertebrates. The role of coral associated bacteria was suggested to have “probiotic” function (Reshef et al. 2006). Coral associated microbial involved in cycling of sulphur in the coral reef as well as in coral health (Raina et al. 2009; Raina et al. 2010). They contribute to the coral defense toward pathogenic bacteria by producing of antibacterial compounds (Harder et al. 2003; Ritche et al. 2006; Rosenberg et al. 2007; Shnit-Orland and Kushmaro, 2009; Nissimov et al. 2009; Kvnenefors et al. 2012; Zhang et al. 2012; Peng et al. 2013).

3. Antimicrobial activity of isolates

Our knowledge on antimicrobial compounds from soft coral associated bacteria is poor and their role is not well understood.

All four major groups of bacterial isolates from this present study showed antimicrobial activities. Ten out of 23 distinct ATU of *Firmicutes* isolates possessed antimicrobial activities and represented 47% (107/226) of all bioactive isolates. Most of the active isolates were affiliated to two most abundant strains *B. amyloliquefaciens* (BA2) and *B. methylotrophicus* (BA8).

The antimicrobial pattern of active isolates is shown in Figure 3.24. The antimicrobial activity of isolates from this study targeted both Gram-positive and Gram-negative indicators (abc: 67%). Beside *Bacillus* spp., also *Gammaproteobacteria* (PSA1, SH2, VI) and *Actinobacteria* (BL) inhibited Gram-positive (*B. subtilis*, *S. epidermidis*) and Gram-negative (*E. coli*) indicators.

The antimicrobial pattern of isolates from this study inferred that bacteria associated with the soft coral *A. digitatum* were potential sources of antimicrobial substances, especially BA2 and BA8. It was assumed that these abundant bacteria can play an important role in the community of soft coral associated bacteria and in coral health. In fact, in this study *Bacillus* sp. associated with the soft coral showed antibiotic activity against *B. subtilis*, *S. epidermidis*, *E. coli*, and *C. albicans* with over 7% (9/122).

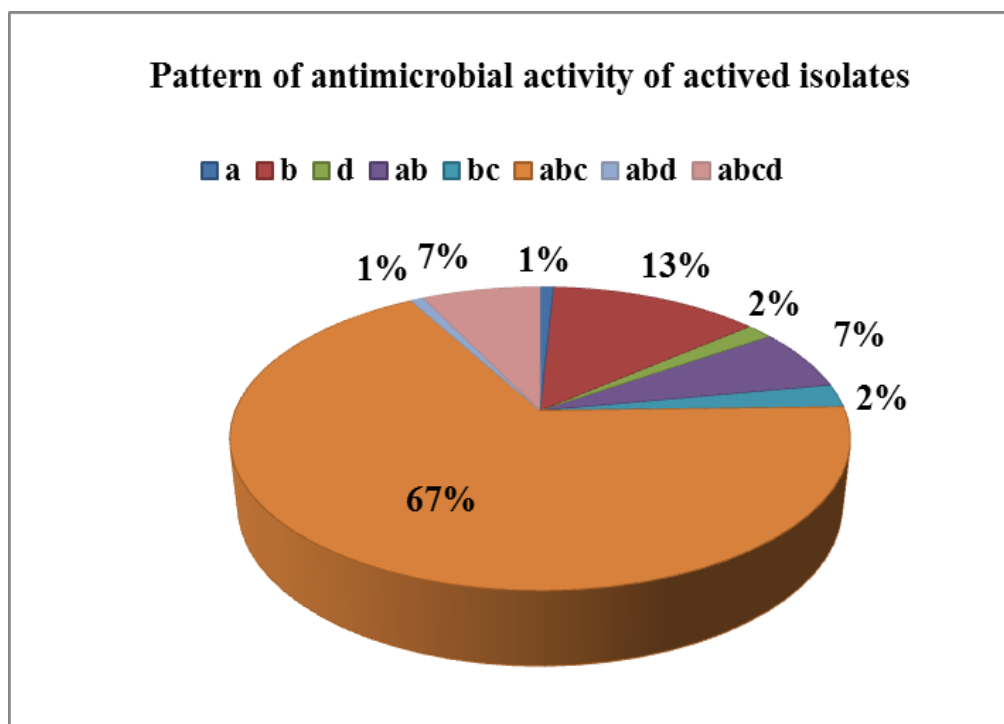


Figure 3.24 The antimicrobial activity pattern of active isolates; a: inhibition of *B. subtilis*, b: inhibition of *S. epidermidis*, c: inhibition of *E. coli*, and d: inhibition of *C. albicans*.

The genus *Bacillus* was considered as an important producer of antimicrobials. Genomic screening demonstrated that 3% of the *Bacillus* genome encoded gene cluster of second metabolites as potential bioactive molecules (Donadio et al. 2007). Also, studies of antimicrobial producer from four sympatric Gorgonian corals in China Sea and the black coral *A. dichotoma* from the South China Sea identified representatives of the genus *Bacillus* as good antimicrobial producers (Peng et al. 2013; Zhang et al. 2012).

All four of *Paenibacillus* isolates, *B. megaterium* and *Exiguobacterium* sp. isolated during this study showed no antimicrobial activities in GYM and BM media. However, *B. megaterium* and *Exiguobacterium* sp., which had mutualistic relationships with the coral host-Elkhorn coral *A. palmate*, produced antibiotic compounds. *B. megaterium* exhibited antimicrobial activity against *B. subtilis*, whilst *Exiguobacterium* sp. showed activity against *Serratia marcescens* PDL100 causing the coral white pox disease (Ritchie, 2006).

Coral associated *Pseudoalteromonas* exhibited antimicrobial activity against both Gram- positive and Gram-negative bacteria but favourably against Gram-positive bacteria (Shnit-Orland et al. 2012; Shnit-Orland and Kushmaro, 2009). *Pseudoalteromonas* sp. isolated from the Mediterranean coral *O. patagonica* inhibited its coral pathogen *Vibrio*

shiloi. This species also has been suggested as a probiotic bacterium for this coral (Nissimov et al. 2009).

In this present study, there were three isolates of *P. aliena* (PSA1), two isolates possessed antimicrobial activities with the same pattern whilst the other showed no bioactivity. All tested *Pseudoalteromonas* sp. from bryozoan in the Baltic Sea showed inhibition of at least 1 of 2 Gram-positive indicator organisms, two out of 37 tested *Pseudoalteromonas* strains showed antimicrobial activity against all three indicator bacteria including the Gram-negative *E. coli* bacterium (Heindl et al. 2010). *Pseudoalteromonas* sp. from brown algae in the Baltic Sea had similar antimicrobial activities as those from bryozoa. Some *Pseudoalteromonas* isolates inhibited *E. coli* and the yeast *Candida glabrata* (Wiese et al. 2009).

Shewanella isolates from the soft coral of this study showed inhibition of both Gram-positive and Gram-negative bacteria as well as *C. albicans*. In addition, all *Shewanella* strain isolated in this study were affiliated to type strains which were different to other *Shewanella* isolates from bryozoa and from brown algae in Baltic Sea habitats (Heindl et al. 2010; Wiese et al. 2009). A study on bryozoan-associated bacteria diversity and antimicrobial activity from different habitats showed that *Bacillus* sp. strains were not found, and *Vibrio* and *Shewanella* were found only from bryozoa from the Baltic Sea and not from the Mediterranean Sea. A number of *Shewanella* isolated from bryozoan in Baltic Sea were reported to inhibit to Gram-positive bacteria (Heindl et al. 2010) while other species of this genus which associated with brown alga from Baltic Sea had no activity to both Gram-positive test strains (Wiese et al. 2009).

The isolate B-2-1 (ATU VI-*Vibrio lentus*) from this study exhibited strong antimicrobial activity against *B. subtilis*, *S. epidermidis*, and *E. coli*. All three isolates of *Vibrio* showed inhibition against *B. subtilis* (Heindl et al. 2010). Among isolates from brown algae or from bryozoans in the Baltic Sea, *Vibrio* sp. strains were reported as promising producers antimicrobials (Heindl et al. 2010; Wiese et al. 2009). Also *Vibrio parahaemolyticus* and *Vibrio natrigens* isolated from *A. digitifera* from the Gulf of Mannar showed strong antimicrobial activity (Nithyanand and Pandian, 2009).

Three representatives of the family *Rhodobacteraceae* of this study were *L. alba*, *S. marinus*, and *R. vignae*. *L. alba* and *R. vignae* showed no antimicrobial activity to any indicator microorganism. *S. marinus* inhibited only *S. epidermidis* if grown on GYM

medium. *Sulfitobacter* sp. DFL-10 isolated from the brown alga *Laminaria saccharina* from the Baltic Sea inhibited *C. glabrata* (Wiese et al. 2009). Another species isolated from the sponge *Haliclona simulans* from Irish water, *Sulfitobacter pontiacus* showed no activity towards any tested microorganisms including Gram-positive bacteria (Kennedy et al. 2009). The *Alphaproteobacteria* isolated from this study may play any other roles (e.g. nitrogen or sulphur cycling) rather than as antimicrobial agent producers.

The finding of *Micrococcus yunanensis* that was isolated from a sponge, supported the statement of Shnit-Orland, who noted that coral-associated bacteria are ubiquitous, as the same species of bacteria could be found in different coral species also in distinct geographical habitats (Shnit-Orland and Kushmaro, 2009). This study found that many of the ATUs showed negative results in antimicrobial tests, while some ATUs with all of their members had antimicrobial activity. Some AUTs (BA10, MIF, MIL) with all members exhibited specific inhibition of indicator microorganisms. In contrast, only individual strains ATU LY1 and LY4 showed activity specific to the human pathogenic yeast *C. albicans*.

4. Biotechnological potential of soft coral associated isolates

An unnamed substance from *Bacillus vallismortis* DSM11031^T isolated from seawater in Korea showed cytotoxic activity on three colon cancer cells, and was suggested as a candidate for antitumor compound development (Jeong et al. 2008). Another study on *B. vallismortis* strain BS07 (99.8% sequence similar to *B. vallismortis* DSM11031^T) which was isolated from soil reported that this strain possessed biocontrol potential and increased fruit yield when it was applied in chili pepper (Park et al. 2013). Comparing of a number of bioactive compounds that were found from *Bacillus* species, very little antitumor compounds have been reported so far.

Interestingly, *Bacillus vallismortis* DSM11031^T is the next relative to isolate B-2-4. The crude extract of this isolate inhibited all four-indicator microorganisms. The metabolite profile of strain B-2-4 showed some different peaks to those of from other *Bacillus* species. It inferred that the strain B-2-4 could produce different metabolites, which were not found in other *Bacillus* isolates of this study. Therefore, the strain B-2-4 might be regarded as a potential candidate for further studies.

Although represented by a few isolates only, all actinobacteria showed antimicrobial activity against Gram-positive *S. epidermidis* either in BM or in GYM medium. Kocurin is a new member of the thiazolyl peptide family of antibiotics, inhibited the growth MRSA but not *B. subtilis*, *E. coli* and *C. albicans*. Kocurin was first produced by a sponge-derived *Kocuria lacustris* (Martín et al. 2013). The first member of the genus *Micrococcus* reported as an kocurin producer, possessed a type II polyketide synthase (PKS-II) gene clusters, and was identified as *Micrococcus yunnanensis* YIM65004^T (FJ214355) (Palomo et al. 2013). Interestingly, strain C-2-28 from this study affiliated to the type strain of *M. yunnanensis* YIM65004^T (FJ214355), and the crude extract of this strain showed antibacterial activity against both Gram-negative (*E. coli*) and Gram-positive (*S. epidermidis*) indicator bacteria in GYM medium only. The metabolite profile from this strain showed that it did not produce kocurin (MW 1514). Its metabolite profiles also were different to those of from *Micrococcus* sp. (strain A-2-28). In addition, the finding of plant hormone IAA in the metabolite profile of strain A-2-28 suggested that this bacterium might be a potential candidate for further research on biocontrol agents. We propose that *Micrococcus* sp. should be exploited in the future for natural compounds because of limited reports of bioactive compounds isolated from this genus so far.

The actinobacterium *B. saxobsidens* (C-1-31, ATU BL) was found for the first time from a soft coral, and possessed antimicrobial activity against three indicator microorganisms, but only after growth on BM medium. This strain should be further examined for production of new natural compounds.

A large number of bioactive compounds were identified from *Vibrionaceae* family (Gram et al. 2010; Mansson et al. 2011). Beside the known antibiotic andrimid, which was found in *Vibrio coralliilyticus* strain S2052 isolated from marine sediment, this strain produced promising metabolites which need to be further examined for new natural bioactive compounds (Wietz et al. 2010).

Vibrio and *Shewanella* genera are prevalent in the marine environment. Recently bacteria associated with the black coral *Anthipathes dichotoma* from the South China Sea were analysed in respect to antimicrobial activity. In addition to antimicrobial producers belonging to *Actinobacteria*, also, members of the genus *Bacillus* were good sources of antimicrobial substances, but none of the *Gammaproteobacteria* was isolated from this black coral. As a result, *Vibrio* and *Shewanella* associated with this black coral and their antimicrobial activity was not reported (Zhang et al. 2012). Other studies on coral

associated microbes and their antimicrobial activities reported that *Vibrio* sp. inhibited both Gram-positive bacteria and Gram-negative bacteria while *Shewanella* sp. inhibited Gram-negative bacteria. Unlike *Vibrio* sp., no bioactive compounds were reported from *Shewanella* sp. by far. The phylogenetic and antimicrobial results showed that *Shewanella* sp. isolated from soft coral *A. digitatum* might be a potential strain for bioactive compound investigations and also for host-interaction studies.

Screening antimicrobial producer in two different media (BM and GYM) were carried out in this study, together with antimicrobial testing (crude extract of cell cultures) and chemical analysis (HPLC/DAD/MS) were applied for searching of diverse metabolite isolates. Thus, the antimicrobial activities of all isolates from this study were observed in two screening media (44.0%). However, BM medium promoted the production of antimicrobial substances of bacteria such as *B. pumilus*, *B. saxobsidens*, *S. putrefaciens*, whereas GYM medium was better for *B. amyloliquafaciens*, *M. yunnanensis*, and *S. baltica*. *B. saxobsidens* was isolated in poor nutrient medium (BS medium). Moreover, the result from antimicrobial tests of crude extract of this strain spurred more investigation of isolation and antimicrobial screening of coral associated bacteria for bioactive compounds. The activity of antimicrobial inhibition of individual bacteria in two tested media (BM and GYM) indicated that the cultivation medium is indeed a factor to be considered for exploring new bioactive substances.

Significantly, purified compound which was isolated from *Bacillus* sp. from this present study showed inhibition two Gram-positive and Gram-negative indicator bacteria. A total of 11 pure compounds which were isolated from this study and several candidate strains suggested that soft coral associated bacteria are promising sources for biotechnological use.

CONCLUSIONS

By using four different media for isolation of bacteria, and in addition two different media for production of metabolite and chemical screening a huge number of isolates were analysed and diverse metabolites were identified. This work demonstrates that soft corals are a good source of bacteria producing antimicrobials. In fact, certain gene clusters which encoded for bioactive substances served as a tool for searching of bioactive metabolite producing bacteria. Molecular screening should be included in further work for exploring the rich sources of biotechnological promising isolates.

The results achieved from this work showed that searching for new bioactive compounds furthermore needs to be focused not only on novel niches or novel microbial species, but should include well known bacteria (*Bacillus* spp.) or common marine bacteria (*Micrococcus* spp.). In fact, some single strains of *Streptomyces* species were deeply examined for drug development, a plenty of metabolites were found and elaborated for biotechnological use.

In this study, many of Gram-positive bacteria were recovered from the soft coral *A. digitatum*. Moreover, antimicrobial substances isolated from Gram-positive bacteria showed inhibition against Gram-positive bacteria and also Gram-negative indicator strains. Data from this study suggest that soft coral associated bacteria may play a role in coral health. Thus, the production of antimicrobial substance by associated bacteria significant can contribute to the chemical defence of the corals. However, the cell-cell interaction among isolates as well as relationship of associated bacteria and coral host need to be investigated more detail in further studies. In addition, the roles of each individual bacteria group in coral host need to be thoroughly examined to highlight the dynamic interaction with each other with the ambient environment. In fact, associated bacteria were very diverse with high potential to produce antimicrobials, but results from cultivation based approach and 16S rRNA gene analysis might not be completely comprehensive for diversity of soft coral associated microbial communities.

It would be interesting to investigate the relationship of coral hosts and their holobiont in detailed studies. Thus, a number of questions remain to be answered, and corals and their associated communities still is an attractive object for future research.

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List of Abbreviations

%	Percent
ATUs	Arbitrary taxonomic units
AWI	Alfred Wegener Institute
BM	Bannett medium
BLAST	Database Basis Local Alignment Search Tool
BPC	Base Peak Chromatogram
BS	Baltic seawater medium
CN	Compound name
CNo	Compound number
CP	Compound purification
DGGE	Denaturing Gradient Gel Electrophoresis
DNPs	Dictionary of Natural Products
DSM	Dimethylsulfide
DSMP	Dimethylsulfoniopropionate
EIC	Extracted Ion Chromatogram
et. al	Et. Alii (Latin) and others
e. g	Exempli gratia (Latin), for example
FISH	Fluorescence in situ hybridization
g/l	Gram/litre
GABA	Gamma Aminobutyric Acid
GYM	GYM medium
h	Hour
HPLC	High Performance Liquid Chromatography

HPLC-DAD-MS	HPLC- diode array detection mass spectrometry
IAA	3-indolylacetic acid
L	Litre
Max	Maxima
MeOD	Methanol
MeOD4	Methanol-d4
mg	Milligram
MIC	Minimum inhibitory concentration
min	Minutes
MiQ	Milli-Q water
ml	Milliliter
MNPs	Marine natural products
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MW	Molecular weight
NCBI	National Centre for Biotechnology Information
NMR	Nuclear Magnetic Resonance
NPKS	Nonribosomal peptide synthetases
NPs	Natural products
°C	Degree celsius
PKS	Polyketide synthase
PUFAs	Polyunsaturated fatty acids
R2A	R2A medium
RDP-II	The Ribosomal Database Project II
rpm	Rotation per minute
Rt	Retention time
SW	Seawater medium

TEM	Transmission electron microscopy
TM	Tropic marine medium
UV	Ultraviolet
v/v	Volume by volume
VCR	Vincristine resistant
w/w	Weight by weight
μl	Microliter
μm	Micrometer

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