Novel anti-infective secondary metabolites and biosynthetic gene clusters from actinomycetes associated with marine sponges

Neue anti-infektive Sekundärmetabolite und biosynthetische Gencluster aus mit marinen Schwämmen assoziierten Actinomyceten

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submitted by

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ERKLÄRUNG

gemäß § 4 Abs. 3 Ziff. 3, 5 und 8
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Hiermit erkläre ich ehrenwörtlich, die vorliegende Arbeit in allen Teilen selbständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt zu haben. Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen. Des Weiteren erkläre ich, dass ich früher weder akademische Grade erworben habe, noch zu erwerben versucht habe.

Würzburg, November 2008 (Sheila Marie Pimentel Elardo)

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FOR MY FAMILY

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Summary

Marine sponges (Porifera) harbor diverse microbial communities within their mesohyl, among them representatives of the phylum *Actinobacteria*, commonly known as actinomycetes. Actinomycetes are prolific producers of pharmacologically important compounds and are responsible for producing the majority of antibiotics. The main aim of this Ph.D. study was to investigate the metabolic potential of the sponge-associated actinomycetes to produce novel anti-infective agents.

The first aim was to cultivate actinomycetes derived from different marine sponges. 16S rDNA sequencing revealed that the strains belonged to diverse actinomycete genera such as *Gordonia, Isoptericola, Micromonospora, Nocardiopsis, Saccharopolyspora* and *Streptomyces.* Phylogenetic analyses and polyphasic characterization further revealed that two of these strains represent new species, namely *Saccharopolyspora cebuensis* strain SPE 10-1^T (Pimentel-Elardo et al. 2008a) and *Streptomyces axinellae* strain Pol001^T (Pimentel-Elardo et al. 2008b).

Furthermore, secondary metabolite production of the actinomycete strains was investigated. The metabolites were isolated using a bioassay-guided purification scheme followed by structure elucidation using spectroscopic methods and subjected to an elaborate anti-infective screening panel. Several interesting compounds were isolated namely, the novel polyketides cebulactam A1 and A2 (Pimentel-Elardo et al. 2008c), a family of tetromycin compounds including novel derivatives, cyclodepsipeptide valinomycin, indolocarbazole staurosporine, diketopiperazine cycloisoleucylprolyl and butenolide. These compounds exhibited significant anti-parasitic as well as protease inhibitory activities.

The third aim of this Ph.D. study was to identify biosynthetic gene clusters encoding for nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) present in the actinomycete strains. Genomic library construction and sequencing revealed insights into the metabolic potential and biosynthetic pathways of selected strains. An interesting NRPS system detected in *Streptomyces* sp. strain Aer003 was found to be widely distributed in several sponge species, in an ascidian and in seawater and is postulated to encode for a large peptide molecule. Sequencing of the PKS gene cluster of *Saccharopolyspora cebuensis* strain SPE 10-1^T allowed the prediction of the cebulactam biosynthetic pathway which utilizes 3-amino-5-hydroxybenzoic acid as the starter unit

followed by successive condensation steps involving methylmalonyl extender units and auxiliary domains responsible for the polyketide assembly.

In conclusion, this Ph.D. study has shown that diverse actinomycete genera are associated with marine sponges. The strains, two of them novel species, produced diverse chemical structures with interesting anti-infective properties. Lastly, the presence of biosynthetic gene clusters identified in this study substantiates the biosynthetic potential of actinomycetes to produce exploitable natural products and hopefully provides a sustainable supply of anti-infective compounds.

Zusammenfassung

Zahlreiche marine Schwämme (Phylum: Porifera) beherbergen eine phylogenetisch diverse mikrobielle Gemeinschaft in der Mesohyl-Matrix, darunter auch viele Vertreter des bakteriellen Phylums *Actinobacteria,* die umgangssprachlich als Actinomyceten bekannt sind. Actinomyceten sind wichtige Produzenten vieler Antibiotika und von weiteren pharmazeutisch relevanten Substanzen. Das Hauptziel dieser Promotionsarbeit war die Untersuchung des Potentials Schwamm-assoziierter Actinomyceten zur Produktion neuer Infektions-hemmender Substanzen.

Ein erstes Ziel dieser Doktorarbeit war die Kultivierung von Actinomyceten aus verschiedenen marinen Schwammarten. Die Sequenzierung der respektiven 16S rRNA Gene zeigte eine phylogenetische Zugehörigkeit der Isolate zu verschiedenen Actinomyceten-Familien, wie *Gordonia, Isoptericola, Micromonospora, Nocardiopsis, Saccharopolyspora* und *Streptomyces*. Durch phylogenetische Analysen und umfangreiche taxonomische Charakterisierungen konnten zwei neue Actinomyceten-Arten, *Saccharopolyspora cebuensis* strain SPE 10-1^T (Pimentel-Elardo et al. 2008a) und *Streptomyces axinellae* strain Pol001^T (Pimentel-Elardo et al. 2008b) beschrieben werden.

Des Weiteren sollten die Actinomyceten-Isolate auf die Produktion von Sekundär-Metaboliten hin untersucht werden. Die Substanzen wurden „bioassay-guided“ aufgereinigt und isoliert sowie deren Struktur mittels spektroskopischer Methoden aufgeklärt. Anschließend wurden die Substanzen ausführlichen Screening-Methoden unterzogen, um sie auf anti-infektive Wirkungen hin zu untersuchen. Zahlreiche interessante Verbindungen konnten so isoliert werden, u. a. die neuen Polyketide Cebulactam A1 und A2 (Pimentel-Elardo et al. 2008c); eine Familie von Tetromycin-Substanzen inklusive neuartiger Derivative; das Cyclodepsipeptid Valinomycin, Indolocarbazole Staurosporine, Diketopiperazine Cycloisoleucylprolyl und Butenolide. Die Verbindungen zeigten signifikante anti-parasitische und Protease-hemmende Aktivitäten.

Das dritte Ziel dieser Arbeit war es, die für nicht-ribosomale Peptidsynthetasen (NRPS) und Polyketidsynthasen (PKS) kodierenden, biosynthetischen Gen-Cluster in den Actinomyceten-Isolaten zu identifizieren. Die Konstruktion von Genbanken sowie die Sequenzierung ausgewählter Cosmidklone lieferte erste Einblicke in das Stoffwechsel- und Biosynthesepotential ausgewählter Isolate. Beispielsweise konnte ein interessantes NRPS-System in *Streptomyces* sp. Stamm Aer003 identifiziert werden, welches in

verschiedenen Schwammarten, einer Ascidienart sowie im Meerwasser gefunden wurde. Die Sequenzierung eines PKS-Genclusters aus *Saccharopolyspora cebuensis* strain SPE 10-1^T ermöglicht die Voraussage des Cebulactam-Biosynthesewegs in dem 3-Amino-5-Hydroxybenzoesäure als Ausgangsprodukt dient, welches durch sukzessive Kondensationsschritte sowie Verlängerungen durch Methylmalonyl- und Zusatzdomänen zum endgültigen Polyketid führen.

Zusammenfassend konnte in dieser Promotionsarbeit gezeigt werden, dass marine Schwämme mit diversen Vertretern aus verschiedenen Familien der Actinomyceten assoziiert sind. Die Bakterienisolate, von denen zwei neue Arten repräsentieren, produzierten mehrere chemische Substanzen mit interessanten anti-infektiven Eigenschaften. Des Weiteren konnte mit dieser Arbeit durch die Identifizierung von Biosynthese-Genclustern das Potential von Actinomyceten zur Produktion verwertbarer bioaktiver Substanzen bekräftigt und somit ein Beitrag zur Entdeckung neuer anti-infektiver Substanzen erbracht werden.

Chapter 1
Introduction

1.1 Marine sponges (Porifera)

1.1.1 Biology and microbial diversity

Marine sponges (Porifera) are ancient metazoans dating back to the Precambrian era (Li et al. 1998). The phylum Porifera is divided into three classes: *Hexactinellida* (glass sponges), *Calcarea* (calcareous sponges) and *Demospongiae* (demosponges), with the last group containing an estimated 85% of the formally described living species (Hooper and van Soest 2002). These sessile invertebrates inhabit a wide variety of marine and freshwater systems and are found throughout tropical, temperate and polar regions (Hooper and van Soest 2002). The growth habits of sponges encompass various shapes, colors and sizes (Fig.1.1) (Brusca and Brusca 1990). Furthermore, sponges are comprised of a basic body plan (Bergquist 1978; Simpson 1984; Brusca and Brusca 1990). The outer surface, pinacoderm consists of pores called ostia which extend along interior canals that permeate through the sponge. Specialized flagellated cells called choanocytes form a series of choanoderm chambers where the choanocytes beat to pump water in through the ostia as well as through the aquiferous systems within the sponge body. These flagellated choanocytes also filter out food particles from the surrounding water, including bacteria and microalgae which are then transferred to the mesohyl and digested by archaeocytes through phagocytosis. After water is filtered through the choanocytes, it is then expelled from the sponge via the osculum rendering the effluent essentially sterile. Sponges are capable of turning over large volumes of water through its aquiferous canal system equaling to several thousands of liters for a 1-kg sponge per day (Vogel 1977).

Dense communities of microorganisms are present in the mesohyl of most sponges (Vacelet and Donadey 1977; Wilkinson 1978; Friedrich et al. 1999). These types of sponges have been termed ‘bacteriosponges’ or ‘high-microbial-abundance sponges’ (Vacelet and Donadey 1977; Hentschel et al. 2003) where bacterial densities may reach up to 10^8-10^{10} bacteria per gram of sponge wet weight (Hentschel et al. 2006), exceeding that of seawater by several orders of magnitude (Friedrich et al. 2001) and accounting to up to 40% of the sponge biomass (Vacelet and Donadey 1977). On the other hand, the mesohyl of other sponges that co-exist in the same habitat are essentially devoid of microorganisms, hence these are termed as ‘low-microbial-abundance sponges’ with 10^5-

10^6 bacteria per gram of sponge wet weight which are within the range of natural seawater (Hentschel et al. 2006) (Fig. 1.2).

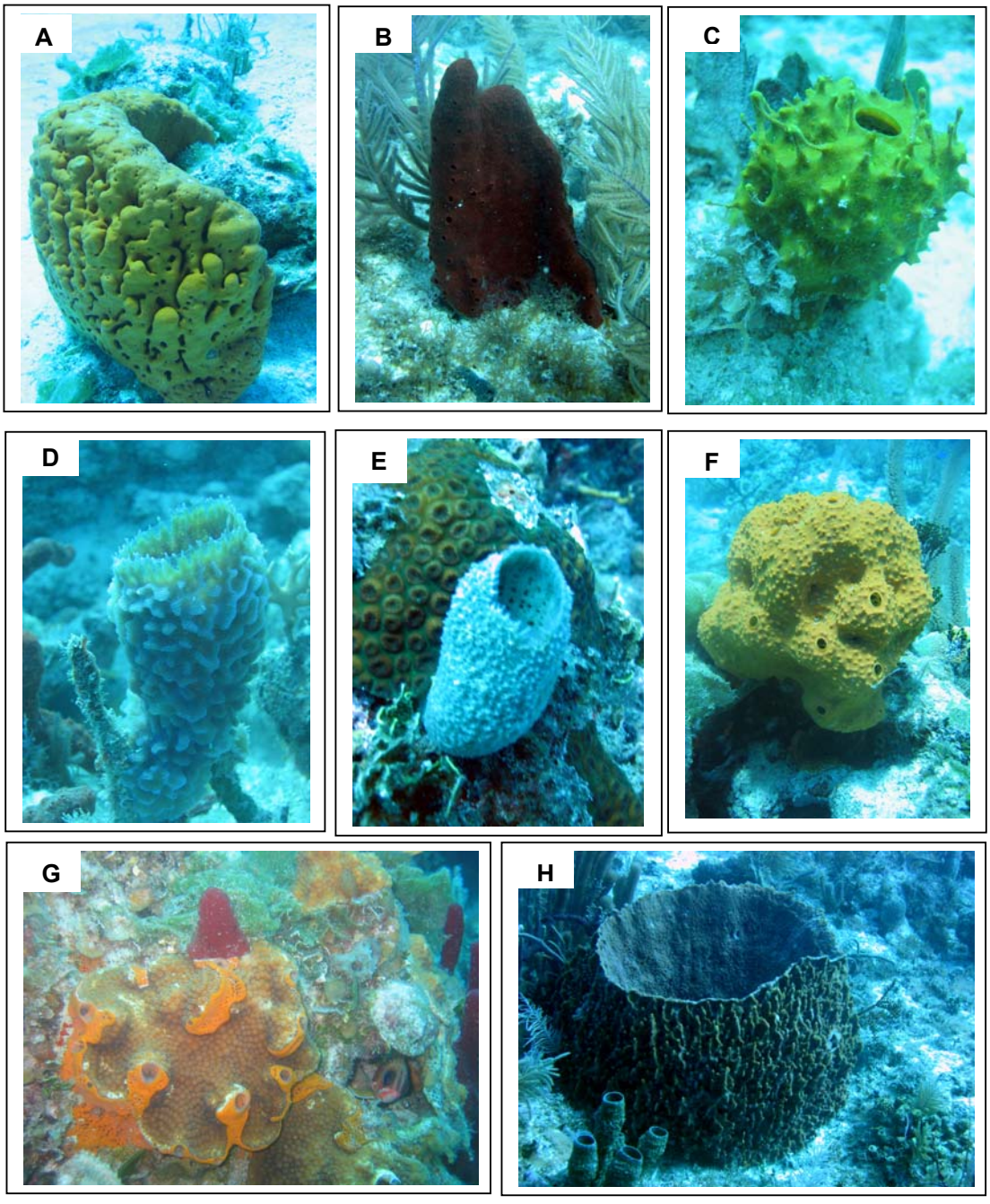


Fig. 1.1 Demosponges: (A): *Agelas clathrodes*; (B): *Amphimedon compressa*; (C): *Aplysina fistularis*; (D): *Callyspongia plicifera*; (E): *Niphates digitalis*; (F): *Pseudoceratina crassa*; (G): *Mycale laxissima*; (H): *Xestospongia muta* (underwater photography by H. Angermeier, University of Würzburg)

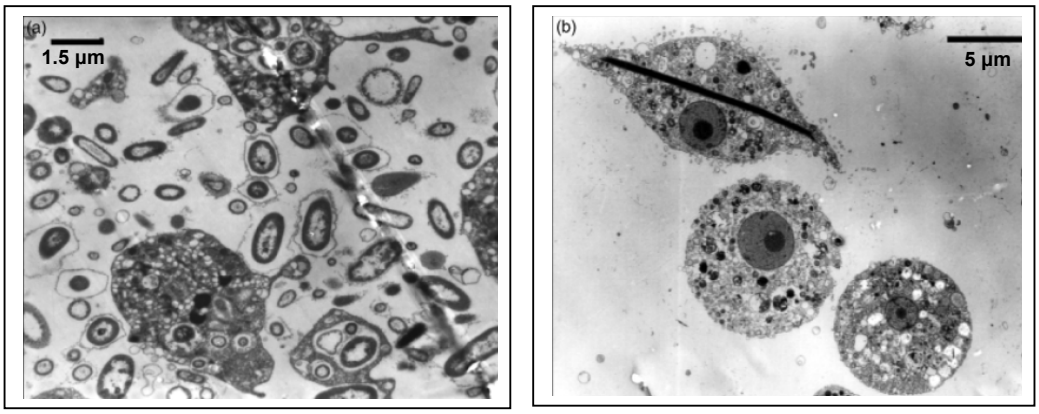


Fig. 1.2 Transmission electron micrographs of (A) *Xestospongia muta* (high-microbial-abundance sponge) and (B) *Callyspongia vaginalis* (low-microbial-abundance sponge) (Hentschel et al. 2006)

The associations between sponges and microorganisms are maintained such that microbial symbionts are passed on from the parent sponge through reproductive stages through vertical transmission (Usher et al. 2001; Ereskovsky et al. 2005; Schmitt et al. 2007; Schmitt et al. 2008a). These symbionts are stable populations which are phylogenetically distinct from transient bacteria and which are filtered out of the surrounding seawater during the feeding process of the sponge. Symbiotic functions that have been attributed include nutrient acquisition, sponge skeleton stabilization, metabolic waste processing and metabolite production (Wilkinson 1992; Unson et al. 1994; Bewley et al. 1996; Schmidt et al. 2000; Hentschel et al. 2006).

Furthermore, the microbial communities of sponges have been documented by electron microscopy studies indicating high morphological diversity as well as the presence of unusual microorganisms (Vacelet and Donadey 1977; Wilkinson 1978). Several studies have been reported on the diversity of the microbial consortia of sponges using cultivation-dependent approaches (Santavy et al. 1990; Webster and Hill 2001). With the advent of molecular tools such as 16S rRNA gene library construction, fluorescence in situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE), it is now possible to gain insights into the complex microbial consortia of sponges, including those that have eluded cultivation efforts (Head et al. 1998; Juretschko et al. 1998; Schmid et al. 2000; Webster et al. 2001; Hentschel et al. 2002; Olson and McCarthy 2005; Hill et al. 2006).

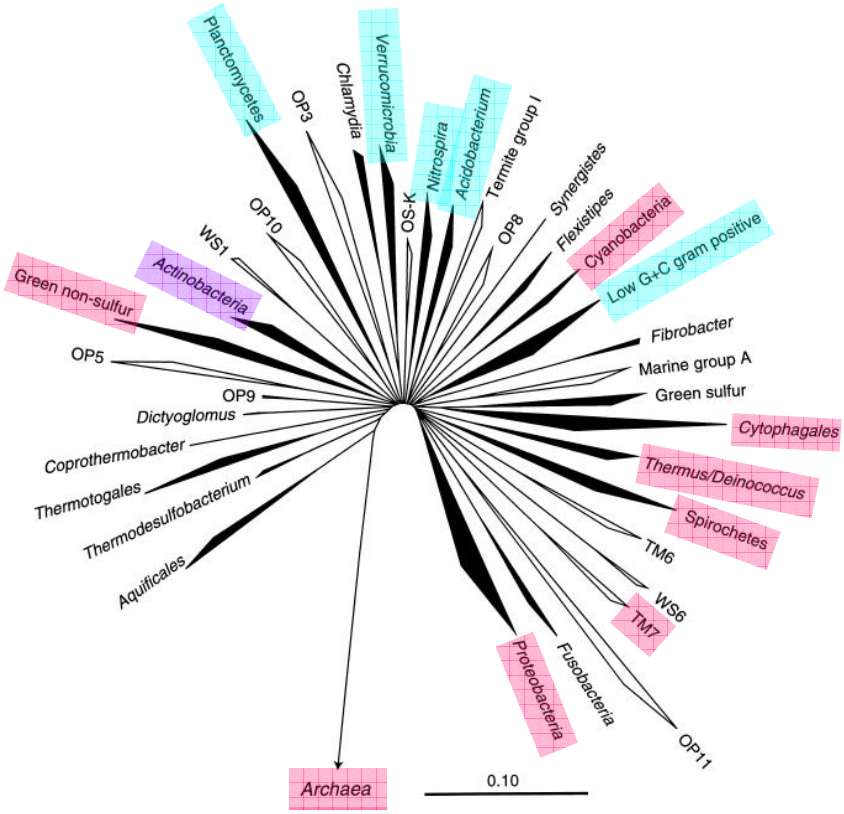


Fig. 1.3 Phylogenetic tree of prokaryotes indicating microbial groups recovered from marine sponges (highlighted in colored boxes) by cultivation-independent (pink boxes) and cultivation-dependent approaches (blue boxes). Members of Actinobacteria have been recovered using both approaches (violet box). Modified from Hugenholtz et al. (1998) and Schmitt et al. (2008b)

For instance, 16S rRNA gene library construction using universal bacterial PCR primers revealed a uniform, yet phylogenetically complex microbial population in sponges from different oceans (Hentschel et al. 2002; Hentschel et al. 2006). These 16S rRNA gene phylotypes are affiliated with the phyla *Chloroflexi, Actinobacteria, Proteobacteria, Nitrospira, Cyanobacteria,* and *Bacteroidetes.* A novel eubacterial candidate phylum *Poribacteria* was also discovered in Verongid sponges (Fieseler et al. 2004). In addition, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, *Proteobacteria*, and *Verrucomicrobia* have also been isolated in pure culture from marine sponges (Burja and Hill 2001; Hentschel et al. 2001; Webster and Hill 2001; Olson et al. 2002; Pimentel-Elardo et al. 2003; Dieckmann et al. 2005; Kim et al. 2005; Montalvo et al.

2005; Enticknap et al. 2006; Kim and Fuerst 2006; Scheuermayer et al. 2006; Zhang et al. 2006; Jiang et al. 2007; Sertan-de Guzman et al. 2007). In contrast, freshwater sponge species have much lower bacterial diversity and abundance and only representatives of *Actinobacteria, Chloroflexi, Alpha-* and *Betaproteobacteria* were recovered (Gernert et al. 2005). Archaeal members have been detected from the phylum *Crenarchaeota* such as the well-studied sponge archaeon "*Candidatus* Cenarchaeum symbiosum" (Preston et al. 1996; Taylor et al. 2007). Fungal strains have also been recovered in several sponges such as *Penicillium* and *Aspergillus*, which are ubiquitous in terrestrial habitats (Höller et al. 2000).

The identification of *Actinobacteria* in marine sponges is interesting as members of this group are known to be prolific producers of pharmacologically important natural products (Munro et al. 1999; Fiedler et al. 2005; Fenical and Jensen 2006; Newman and Hill 2006). For example, the presence of *Actinobacteria* was detected in the sponge *Rhopaloeides odorabile* by culture-independent methods such as FISH and culture-dependent approaches (Webster et al. 2001). Furthermore, sponge-specific actinobacterial clusters were also recovered from *Theonella swinhoei* and *Aplysina aerophoba* (Hentschel et al. 2002; Montalvo et al. 2005). *Actinobacteria* exclusively within the sub-class *Acidimicrobidae* were shown to be the major components of the bacterial community of two *Xestospongia* species, namely *X. muta* and *X. testudinari* (Montalvo et al. 2005). Initial culturing attempts of the sponges also yielded different genera such as *Gordonia, Micrococcus* and *Brachybacterium.* Furthermore, over a hundred of actinomycete strains were cultivated from the marine sponge *Hymeniacidon perleve* and by phylogenetic analysis of the 16S rDNA gene sequences, these isolates belonged to the genera *Actinoalloteichus, Micromonospora, Nocardia, Nocardiopsis, Pseudonocardia, Rhodococcus* and *Streptomyces,* with the latter accounting for 74% of the isolates (Zhang et al. 2006). Using the same culture-dependent approach, several actinomycetes were isolated from the sponge *Haliclona* sp., such as *Streptomyces, Nocardiopsis, Micromonospora* and *Verrucosispora* (Jiang et al. 2007). Interestingly, marine actinomycetes related to the *Salinispora* group previously reported only from marine sediments were isolated from the Great Barrier Reef marine sponge *Pseudoceratina clavata* (Kim et al. 2005). *Salinispora*, the first seawater-requiring marine actinomycete discovered, produce secondary metabolites in a species-specific pattern (Jensen et al. 2007). Interestingly, genome sequencing revealed that *Salinispora tropica* dedicates a large percentage of its genome to natural product assembly, greater than previous *Streptomyces* species and other actinomycetes (Udwary et al. 2007).

1.2 Secondary metabolites

1.2.1 Natural products from marine sponges

Marine organisms produce novel secondary metabolites with a wide array of biological and pharmacological activities. Out of the 194,000 natural compounds listed in the first volume of the 2006 Chapman and Hall Dictionary of Natural Products, approximately 18,000 are derived from marine sources (Singh and Pelaez 2008). The number of marine natural products continues to rise with sponges as the most prolific producers of novel compounds, with more than 200 new metabolites reported annually (Blunt et al. 2003; Blunt et al. 2004; Blunt et al. 2005; Blunt et al. 2006; Blunt et al. 2007; Taylor et al. 2007; Blunt et al. 2008). These compounds have shown promise in treating inflammation, cancer as well as microbial and viral infections (Newman and Cragg 2004b; Fenical 2006; Newman 2008). The wealth of the bioactive compounds isolated especially from soft-bodied, sessile marine organisms such as sponges that lack morphological defense structures stresses the ecological importance of the compounds for these invertebrates (Thoms et al. 2004; Gross and König 2006; Paul et al. 2006; Thoms et al. 2006). Marine sponges accumulate structurally diverse secondary metabolites which have been found to be deterrent to potential fish predators (Pawlik et al. 1995), exhibit anti-fouling activity (Martin and Uriz 1993) and prevent the growth of competing invertebrates (Porter and Targett 1988; Paul et al. 2006).

The majority of the natural products currently in clinical and pre-clinical trials are produced by invertebrates, including marine sponges (Proksch et al. 2002; Haefner 2003; Newman and Cragg 2004a; Fenical 2006). One example is discodermolide, a polyhydroxylated lactone isolated from the sponge *Discodermia dissoluta* (Gunasekera et al. 1991) which is now under phase I clinical trial for pancreatic cancer. The compound KRN7000 from the sponge *Agelas mauritianus* (Kikuchi et al. 2001) and HTI-286, a hemiasterlin derivative from the South African sponge *Hemiasterella minor*, are anticancer drugs under phase II clinical trial (Newman and Cragg 2004b). The contignasterol derivative, IPL-576,092 isolated from the sponge *Petrosia contignata* (Coulson and O'Donnell 2000) is under phase II clinical trial for oral asthma therapy.

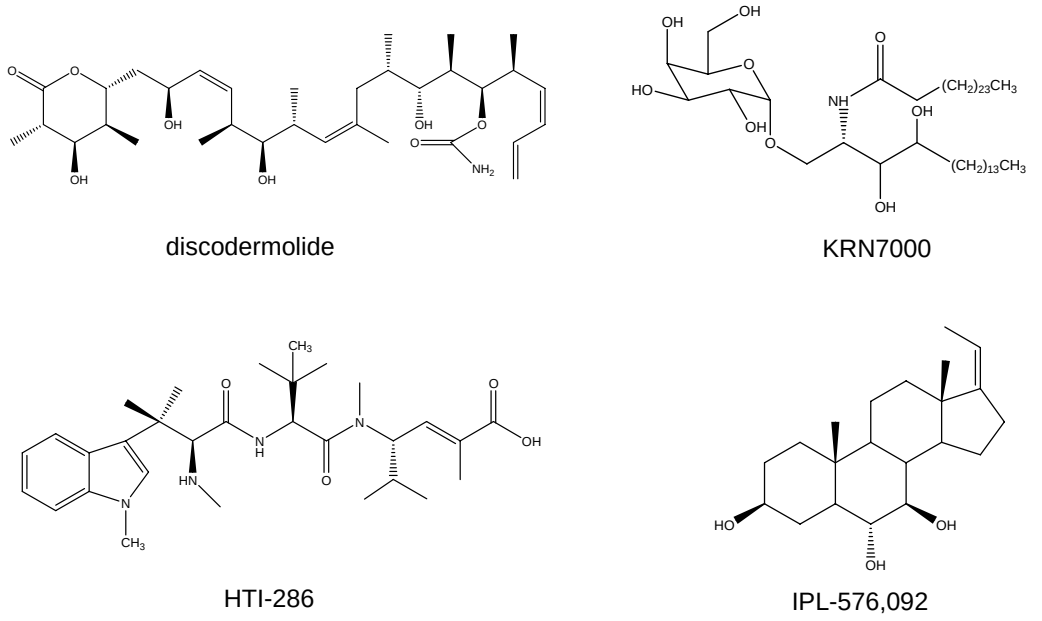


Fig. 1.4 Examples of natural products from sponges in clinical trials

Furthermore, there are several antitumour compounds from marine sponges in preclinical status (Newman and Cragg 2004a) such as laulimalide and isolaulimalide from the Pacific Ocean sponge *Cacospongia mycofijiensis* (Mooberry et al. 1999); peloruside A from the New Zealand sponge *Mycale hentscheli* (West et al. 2000)*;* salicylihalimides A and B from the Western Australian sponge *Haliclona* sp. (Erickson et al. 1987); and the variolins from the Antarctic sponge *Kirckpatrickia variolosa* (Perry et al. 1994; Trimurtulu et al. 1994).

Despite the large number of novel bioactive metabolites reported from marine sponges over the years, not a single compound has been approved as a drug, whether isolated or from total synthesis. A couple of exceptions are the nucleoside analogues Ara-A and Ara-C, now commercialized as antiviral and anticancer agents but these were not directly isolated from sponges but are synthetic derivatives of compounds from the Caribbean sponge *Cryptotethia crypta* (Bergmann and Feeney 1951). A major problem to the ultimate development of these compounds undergoing clinical or pre-clinical trials is the problem of supply. The concentrations of these bioactive compounds in marine invertebrates are minute, accounting typically less than 10^{-6} % of the wet weight (Proksch et al. 2002). This already causes considerable difficulties in clinical studies where gram

quantities of compounds are required. Such is the case of the potent antitumor halichondrins originally isolated from the Japanese sponge *Halichondria okadai* (Hirata and Uemura 1986). Although these macrolides have also been subsequently isolated from a number of other sponges such as *Axinella* sp. from the Western Pacific (Pettit et al. 1991), *Phakellia carteri* from the Eastern Indian Ocean (Pettit et al. 1993), and from *Lissondendoryx* sp. off the East Coast of South Island, New Zealand (Munro et al. 1999), still the demand for clinical trials and annual requirement as a commercial drug is not sufficient (Hart et al. 2000). The synthetic analogue of halichondrin B, E7389 having the same biological properties circumvented the supply problem and is now under phase II clinical trial (Choi et al. 2003; Sipkema et al. 2005).

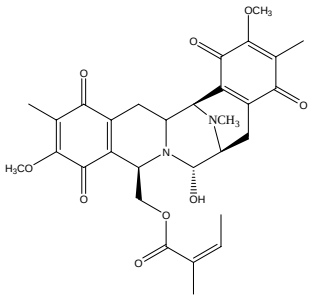
1.2.2 Metabolites from associated microorganisms

The majority of secondary metabolites that have been isolated from marine sponges are hypothesized to be produced by bacterial symbionts (Piel 2004). This speculation was based on the striking structural similarities with bacterial natural products (Table 1.1) belonging to substance classes that are typical for these microorganisms such as polyketides and nonribosomal peptides.

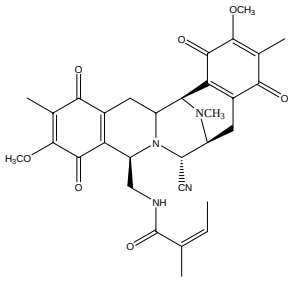
Table 1.1 Natural products from marine sponges that resemble bacterial metabolites (Piel 2004)

Sponge metabolite	Sponge Source	Bacterial metabolite	Bacterial Source
Arenastatin A	*Dysidea arenaria*	Cryptophycin	*Nostoc* sp.
Discodermide	*Discodermia dissoluta*	Alteramide A	*Alteromonas* sp.
		Ikarugamycin	*Streptomyces* sp.
Jaspamide	*Jaspis* spp.	Chondramide D	*Chondromyces crocatus*
Keramamide A	*Theonella* sp.	Ferintoic acid A	*Microcystis aeruginosa*
Mimosamycin	*Petrosia* sp.	Mimosamycin	*Streptomyces lavendulae*
Motuporin	*Theonella swinhoei*	Nodularin	*Nodularia spumigena*
Renieramycin E	*Reniera* sp.	Safracin B	*Pseudomonas fluorescens*
		Saframycin A	*Streptomyces lavendulae*
		Saframycin Mx1	*Myxococcus xanthus*
Salicylihalamide A	*Haliclona* sp.	Apicularen A	*Chondromyces* sp.
Swinholide A	*Theonella swinhoei*	Tolytoxin	*Tolypothrix* sp.
Misakinolide A	*Theonella* sp.	Scytophycin C	*Scytonema* sp.

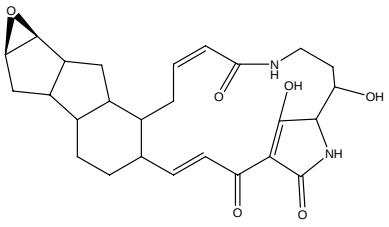
Despite structural complexity, the majority of these compounds exhibits only slight differences in the substitution patterns. Examples are renieramycin from *Reniera* sp. sponge (He and Faulkner 1989) and saframycin A from *Streptomyces lavendulae* (Arai et al. 1977), discodermide from the marine sponge *Discodermia dissoluta* (Gunasekera et al. 1980; Gunasekera et al. 1991) and ikarugamycin from *Streptomyces* sp. (Ito and Hirata 1972).



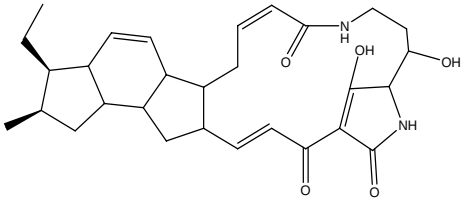
renieramycin E from sponge *Reniera* sp.



saframcyin A from *Streptomyces lavendulae*



discodermide from sponge *Discodermia dissoluta*



ikarugamycin from *Streptomyces* sp.

Fig. 1.5 Examples of sponge metabolites structurally similar to bacterial metabolites

Secondary metabolites found in the tropical sponges *Dysidea herbacea* and *Theonella swinhoei* provide additional evidence to the involvement of microorganisms in natural product synthesis (Proksch et al. 2002). The Great Barrier reef sponge *Dysidea herbacea* was found to contain the sesquiterpenes spirodysin and herbadysidolide as well as the chlorinated amino acid derivative (Unson and Faulkner 1993). These compounds were also found in their cyanobacterial symbiont *Oscillatoria spongeliae* (Berthold et al. 1982) after disruption of the sponge tissue and separation of the cyanobacterial cells. The sponge *Theonella swinhoei* collected from the Philippines and Micronesia produces the cyclic peptide theopalaumide and the macrolide swinholide and these compounds were

detected in cellular fractions containing filamentous bacteria as well as unicellular bacteria, respectively (Bewley et al. 1996; Piel 2004). Using 16S rDNA sequencing, the theopalaumide-containing symbiont was assigned as a novel δ-proteobacterium "*Candidatus* Entotheonella palauensis" (Schmidt et al. 2000).

1.2.3 Metabolites from marine actinomycetes

The class *Actinobacteria*, specifically bacteria belonging to the order *Actinomycetales*, accounts for approximately 7000 of compounds reported in the Dictionary of Natural Products (Jensen et al. 2005). Actinomycetes are responsible for the production of about half of the discovered bioactive secondary metabolites such as antibiotics. Remarkably, the genus *Streptomyces* accounts for 80% of actinomycete natural products reported (Berdy 2005), mostly producing phenazone and lactone amide type of compounds with antibiotic properties (Kelecom 2002). Although the majority of the actinomycetes were previously isolated from terrestrial sources, these bacteria have also been recovered from marine sediments, and even from deepest ocean trenches (Grein and Meyers 1958; Mincer et al. 2002; Maldonado et al. 2005b; Fenical and Jensen 2006; Pathom-Aree et al. 2006; Bredholdt et al. 2007). The isolation of these marine actinomycetes could be accounted for by the cultivation of spores that may have originated from soil-inhabiting strains that were washed into the marine environment. The first marine actinomycete described taxonomically was *Rhodococcus marinonascens* (Helmke and Weyland 1984) and quite recently, new genera have been described including *Salinispora*, the first obligate marine actinomycete isolated from ocean sediments (Han et al. 2003; Yi et al. 2004; Maldonado et al. 2005a). Furthermore, Mincer et al. (2005) demonstrated that *Salinispora* strains are actively growing in sediment samples indicating that these bacteria are metabolically active in the natural marine environment, addressing the question whether actinomycetes only exist as dormant spores. The discovery of numerous new marine actinomycete taxa, their demonstrated metabolic activity, and their ability to form stable populations in different habitats clearly illustrate that indigenous marine actinomycetes exist in the oceans (Lam 2006).

Numerous novel secondary metabolites have been isolated from marine actinomycetes, although exploitation of this group is still at its infancy. Example is the novel β-lactone-γ-lactam, salinisporamide A isolated from *Salinispora tropica* now undergoing phase I clinical trial as proteasome inhibitor for treating multiple myeloma cells (Feling et al. 2003; Chauhan et al. 2005). Another example is abyssomicin C, a novel polyketide antibiotic produced by the marine *Verrucosispora* strain (Riedlinger et al. 2004), is a potent inhibitor

of para-aminobenzoic acid biosynthesis inhibiting folic acid biosynthesis at an earlier stage compared to the well-known sulfa drugs (Bister et al. 2004). Diazepinomicin, produced by a *Micromonospora* strain, is another example (Charan et al. 2004). This unique farnesylated dibenzodiazepenone is now under preclinical development as an anticancer agent.

Table 1.2 Novel metabolites produced by marine actinomycetes during the period 2003-2005 (Lam 2006)

Compound	Source	Activity
Abyssomicins	*Verrucosispora*	Antibacterial
Aureoverticillilactam	*Streptomyces aureoverticillatus*	Anticancer
Bonactin	*Streptomyces* sp.	Antimicrobial
Caprolactones	*Streptomyces* sp.	Anticancer
Chandrananimycins	*Actinomadura* sp.	Antimicrobial; anticancer
Chinikomycins	*Streptomyces* sp.	Anticancer
Diazepinomicin	*Micromonospora* sp.	Antibacterial; anticancer; antiinflammatory
3,6-disubstituted indoles	*Streptomyces* sp.	Anticancer
Frigocyclinone	*Streptomyces griseus*	Antibacterial
Glaciapyrroles	*Streptomyces* sp.	Antibacterial
Gutingimycin	*Streptomyces* sp.	Antibacterial
Helquinoline	*Janibacter limosus*	Antibacterial
Himalomycins	*Streptomyces* sp.	Antibacterial
IB-00208	*Actinomadura* sp.	Anticancer
Komodoquinone A	*Streptomyces* sp.	Neuritogenic
Lajollamycin	*Streptomyces nodosus*	Antibacterial
Marinomycins	*Marinispora* sp.	Antibacterial; anticancer
Mechercharmycins	*Thermoactinomyces* sp.	Anticancer
Salinosporamide A	*Salinispora tropica*	Anticancer
Sporolides	*Salinispora tropica*	Unknown biological activity
Trioxacarcins	*Streptomyces* sp.	Antibacterial; anticancer; antimalarial

1.3 Biosynthetic gene clusters

A growing number of novel, bioactive secondary metabolites produced by marine invertebrates as well as marine bacteria appear to be structurally biosynthesized by polyketide synthases (PKS) and/or nonribosomal peptide synthetases (NRPS). These

enzymes are organized in a modular fashion utilizing specific domains to sequentially catalyze the condensation of simple carboxylic acids for PKS systems or amino acid building blocks for NRPS systems into a growing chain. Each module is a cluster responsible for chain elongation through the recognition, activation and incorporation of specific substrates and structural diversity is introduced through various combinations of the integral and auxiliary domains (Schwarzer and Marahiel 2001; Salomon et al. 2004).

Nonribosomally produced peptides exhibit a remarkable spectrum of biological and pharmacological activities such as the antibiotics vancomycin and penicillin, immunosuppressive agent cyclosporine and the antitumor compound bleomycin. Actinomycetes are known to produce these pharmacologically important peptides. PCR screening efforts for genes associated with secondary metabolism such as NRPS have been used to evaluate the biosynthetic potential of actinomycetes (Ayuso-Sacido and Genilloud 2005; Ayuso et al. 2005). PCR primers tested on a large collection of 210 reference strains encompassing major families and genera in actinomycetes revealed the wide distribution of these genes in *Streptomyces* species as well as in other minor lineages where in some cases only a few compounds have been isolated. The NRPS systems occur more frequently in strains of the main antibiotic producers from the families *Micromonosporaceae, Pseudonocardiaceae,* and *Actinosynnemataceae.* Furthermore, NRPS genes were also detected by PCR amplification in cultivated actinobacteria from the South China Sea sponge *Haliclona* sp. belonging to the genera *Streptomyces, Nocardiopsis, Micromonospora* and *Verrucosispora* (Jiang et al. 2007). Biosynthetic genes have also been recovered from marine invertebrates. However, in another study by Kennedy et al. (2008), NRPS genes were not detected from the microbial metagenome of the sponge *Haliclona simulans*. A remarkable discovery on biosynthetic pathways for investigating marine invertebrate-bacteria associations is the isolation of the peptides patellamide A and C (Schmidt 2005; Schmidt et al. 2005). These cyclic peptides were thought to be synthesized by the cyanobacterial symbiont *Prochloron didemni* of the ascidian *Lissoclinum patella*. Schmidt et al. identified these NRPS genes and confirmed their function by heterologous expression of the whole pathway. This work represents the potential of functional expression of a marine natural product pathway from an obligate symbiont, thus alleviating the problem of supply by genetic manipulation.

Polyketides are classified as aromatic, polyenes and polyethers including macrolides (Rawlings 1997). The PKSs are responsible for the synthesis of several pharmacologically important bacterial polyketides such as antibiotics (i.e. erythromycin, tetracycline), antitumor, immunosuppressive and cholesterol-lowering agents.

Remarkably, a number of important natural products isolated from sponges such as discodermolide, laulimalide and peloruside are of polyketide biosynthetic origin (Fortman and Sherman 2005). Piel et al. (2004) made a milestone discovery on polyketide genes from a marine sponge confirming the long-suspected role of symbiotic bacteria in the production of natural products such as polyketides. Using metagenomics, production of the antitumor onnamide was studied in the marine sponge *Theonella swinhoei* by first investigating the production of pederin subsequently linked to a beetle symbiont closely related to *Pseudomonas aeruginosa* (Piel 2002). The sponge metagenome strongly indicated bacterial origin for the genome fragment corresponding to the entire onnamide polyketide region. PKS systems have also been studied in other sponges by metagenomics. PCR amplification of ketosynthase domains from the microbial consortia of the Caribbean sponge *Discodermia dissoluta* revealed great diversity and a novel group of sponge-specific KS domains (Schirmer et al. 2005). The most abundant modular PKS appeared to be encoded by a bacterial symbiont that made up <1% of the sponge community. Fieseler et al. (2007) systematically investigated 20 demosponge species in different oceans and found that the sponge microbial metagenomes were dominated by an evolutionary distinct and highly sponge-specific group of polyketide synthases. In another study by Kim and Fuerst (2006), direct amplification of KS domains in the Great Barrier reef sponge *Pseudoceratina clavata* using fosmid library construction yielded KS domains falling into a sponge-specific cluster. Quite interestingly, cultivated bacteria from the sponge were also screened for KS domains and these were detected in representatives of *Actinobacteria, Alphaproteobacteria* and *Firmicutes*. This is not surprising since members of the *Actinobacteria*, in particular are known to produce important natural products with polyketide biosynthetic origin. An example is the rifamycin polyketide, known to be produced by the soil actinomycete *Amycolatopsis mediterranei* (August et al. 1998) was also found to be produced by the marine actinomycete *Salinispora* sp. isolated from the sponge *Pseudoceratina clavata*. Phylogenetic analysis of the KS gene sequences revealed that these are closely related to rifamycin B synthase of *A*. *mediterranei* (Kim et al. 2006). Additional examples of PKS gene clusters identified from marine actinomycetes are enterocin PKS from *Streptomyces maritimus* (Piel et al. 2000) and the aromatic polyketide griseorhodin from *Streptomyces* sp. (Li and Piel 2002).

Marine bacterial gene clusters that have been so far identified have demonstrated that while PKS and NRPS biosynthetic routes are conserved between terrestrial and marine systems, interestingly, there are additional novel catalytic enzymes responsible for the unique functional groups found solely in marine natural products (Piel et al. 2000; Chang et al. 2002; Li and Piel 2002; Salomon et al. 2004). Furthermore, recent investigations

focusing on marine actinomycetes have yielded numerous novel biologically active compounds, and more importantly, a surprisingly much more improved rate of discovery of new compounds in contrast to their terrestrial counterparts (Bernan et al. 1997). Thus, given the promising potential of marine actinomycetes, different strategies (Fig.1.6) were employed in this Ph.D. study to fully exploit the capacity of actinomycetes, specifically those associated with marine sponges, to produce bioactive secondary metabolites.

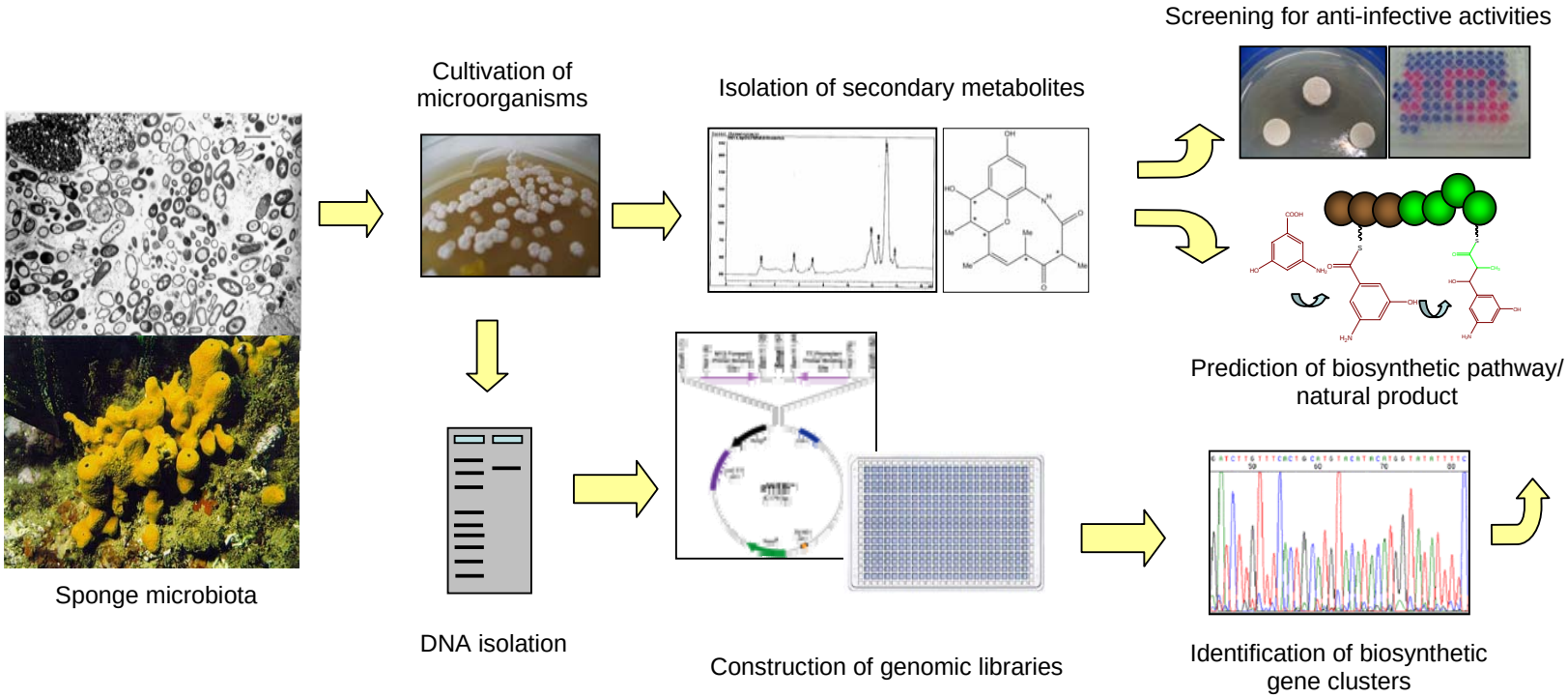


Fig. 1.6 Stategies to access the potential of sponge-associated microorganisms to produce anti-infective metabolites

1.4 Aims

Marine sponges, particularly 'high-microbial-abundance sponges' are known to harbour huge amounts of microorganisms which include members of the order *Actinomycetales.* Actinomycetes are known as prolific producers of pharmacologically important compounds such as antibiotics. The main aim of this Ph.D. study was to investigate the potential of actinomycetes associated with marine sponges to produce novel anti-infective agents.

The first aim of this Ph.D. study (Chapter 2) was to cultivate actinomycetes derived from different marine sponges with a particular focus on strains belonging to novel taxa. The strains were identified by 16S rRNA gene sequence analysis and further characterized using polyphasic techniques.

The second aim of this Ph.D. study (Chapter 3) was to investigate the secondary metabolites produced by the sponge-associated actinomycetes. The metabolites were isolated using a bioassay-guided purification scheme followed by structure elucidation using spectroscopic methods. These compounds were subjected to an elaborate anti-infective screening panel to determine various activities, namely antibacterial, antifungal, antiparasitic, cytotoxic as well as inhibition against different cysteine proteases.

The third aim of this Ph.D. study (Chapter 4) was to identify biosynthetic gene clusters encoding for nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) present in the actinomycete strains. Genomic library construction and sequencing were carried out in order to provide insights into the metabolic potential and biosynthetic pathways of selected strains.

Finally, a general discussion (Chapter 5) of the association of actinomycetes with marine sponges and their biosynthetic potential as well as future perspectives is provided.

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Chapter 2
Isolation and identification of sponge-associated actinomycetes

2.1 Materials and methods

2.1.1 Sponge collection and cultivation

The sponge *Haliclona* sp. was collected by SCUBA diving off Maribago waters (10° 17' 0.97" N, 124° 00' 01.8" E), Cebu, Philippines in February 2003. *Axinella polypoides* was collected offshore Banyuls-sur-mer, France (42° 29' N 03° 08' E) in May 2003. The sponges *Aplysina aerophoba, Chondrosia reniformis, Dysidea avara*, *Tedania* sp. and *Tethya* sp. were also collected by SCUBA diving offshore Rovinj, Croatia (45° 05′ N, 13° 38′ E) in May 2006.

Sponge tissues were excised from the center of individual sponge samples (ca. thumb-sized pieces) using flame-sterilized scalpel. The tissues (2-3 pieces per sponge sample) were rinsed with sterile natural or artificial seawater (ASW) (Lyman and Fleming 1940) and homogenized in seawater. The homogenates were diluted (10^{-1} to 10^{-3}) in seawater and plated out on different actinomycete-selective media: M1 (Mincer et al. 2002), M2 (Mincer et al. 2002), ISP medium 2 (Shirling and Gottlieb 1966), M7 (Webster et al. 2001) and NaSt21Cx (Magarvey et al. 2004). Media formulations are provided in detail in the Annex section. These media were supplemented with the following antibiotics: cycloheximide (100 μg/ml), nystatin (25 μg/ml) and nalidixic acid (25 μg/ml). Undiluted sponge homogenates (in duplicates) were heated at 90°C for 15 min to enrich for spore-forming actinomycetes and likewise plated out. The plates were incubated at 30°C for about 6-8 weeks and inspected regularly for growth. Colonies were picked up and transferred to fresh media for pure culture isolation and glycerol stocks were prepared for long-term storage at -80°C.

2.1.2 Cloning, sequencing and phylogenetic analysis

DNA was extracted from growing cultures either by boiling cells at 95°C for 10 min or using the FastDNA® spin kit for soil (Q-Biogene) following manufacturer's instructions. The broth cultures were centrifuged at 8000 rpm for 5 min and the supernatant was discarded. The resulting pellet was resuspended in 978 µl sodium phosphate buffer and 122 µl MT buffer. The solution was transferred to lysing matrix tubes and these were processed using a FastPrep® instrument (Q-Biogene) for 30 s with speed set at 5.5. The resulting solution was centrifuged at 13000 rpm for 30 s. The supernatant was transferred

to a clean tube and 250 µl of PPS reagent was added. The solution was mixed by manually shaking the tube ten times. Centrifugation at 13000 rpm for 5 min followed to pellet the precipitate. The resulting supernatant was transferred to a clean 2-ml microfuge tube. One milliliter of binding matrix suspension was added to the supernatant and the mixture was mixed for 2 min to allow binding of the DNA to the matrix. The tube was placed in a rack and allowed to stand for 3 min to allow settling of the silica matrix. Five hundred microliters of the supernatant was discarded while 600 µl was transferred to a spin filter provided by the kit. This was then centrifuged at 13000 rpm for 1 min. The catch tube was emptied and the remaining supernatant was added to the spin filter. Centrifugation at 13000 rpm for 1 min was repeated. To the spin filter, 500 µl of SEWS-M was added which was followed by the same centrifugation step. The flow-through was discarded and the spin filter was centrifuged at 13000 rpm for 2 min. The spin filter was removed, placed in a new catch tube and subsequently air-dried for 5 min at room temperature. Fifty microliters of DES (DNAse/pyrogen-free) water was then added and the filter matrix was gently stirred using a pipette tip to resuspend the silica and to efficiently elute the DNA. Centrifugation at 13000 rpm for 1 min followed to transfer the eluted DNA to the catch tube. The resulting DNA extract was stored at -20°C.

PCR amplification using the specific primers S-C-Act-0235-a-S-20 (5'-CGCGGCCTATCAGCTTGTTG-3') and S-C-Act-0878-A-19 (5'-CCGTACTCCCCAGGCGG GG-3') targeting the class *Actinobacteria* was performed to initially screen the isolates (Stach et al. 2003). This was used to minimize bias in selecting isolates based solely on morphological characteristics typical for actinomycetes. Amplification of the 16S rRNA gene was then performed using the universal primers 27f (5'-GAGTTTGATCCTGGCTCA-3') and 1492r (5'-TACGGCTACCTTGTTACGACTT-3') corresponding to *E. coli* numbering (Lane 1991). A standard PCR reaction mix (50 µl) was prepared which consisted of the following: 10x reaction buffer, 5 µl; Q solution, 10 µl; 25 mM $MgCl_2$, 2 µl; 10 mM dNTPs, 1 µl; 100 µM of each primer, 1 µl; 5 U/µl Taq polymerase, 0.25 µl; H_2O, 28.75 µl; DNA template, 1 µl. PCR conditions were as follows: initial denaturation (2 min at 95°C) followed by 30 cycles of denaturation (1 min at 95°C), primer annealing (1 min at 56°C for 27f/1492r and 60°C for S-C-Act-0235-a-S-20/S-C-Act-0878-A-19), primer extension (1.5 min at 72°C) and a final extension step (10 min at 72°C). PCR products were purified using the QIAquick PCR purification kit (Qiagen). Five volumes of buffer PB was added to one volume of PCR product and mixed using a pipette. The mixture was applied to a QIAquick column and centrifuged at 13000 rpm for 1 min. The flow-through was discarded and the column was placed back into the same

tube. Buffer PE (750 µl) was added to the column followed by centrifugation at 13000 rpm for 1 min. The flow-through was discarded and the column was centrifuged for an additional min at 13000 rpm. The column was then placed in a clean 1.5-ml microfuge tube, added with 30 µl of buffer EB and allowed to stand for 1 min. A final centrifugation step at 13000 rpm for 1 min was performed and the resulting purified PCR product was stored at -20 °C.

Ligation of the purified PCR products and transformation in competent *E. coli* XL1-Blue cells were subsequently performed. The following were mixed on ice: 2x T4 DNA ligase buffer, 5 µl; 50 ng/µl pGEM-Teasy vector (Promega), 1 µl; 3 U/µl T4 DNA ligase, 1 µl; PCR product, 4 µl. The ligation solution was incubated overnight at 4°C for maximum number of transformants. For transformation, 2-3 µl of the ligation solution was added to 50 µl of the competent *E. coli* cells. The resulting mixture was transferred to a UV-sterilized electroporation cuvette. Electroporation was applied using an Easyject Prima Electroporator (Equibio) at 2500V. The solution was transferred to a clean 2-ml microfuge tube, added with 1 ml of SOC medium with mixing to resuspend the pellet and incubated with shaking at 37°C for at least 3 hours. After incubation, 20% and 80% of the cells were plated out on LB/amp/IPTG/X-gal agar. The plates were incubated at 37°C overnight and successful transformants were observed using the blue-white colony screening. The plasmid DNA was then isolated using a standard miniprep protocol. Single, white colonies were picked up, inoculated in 2 ml of LB/amp broth and incubated with shaking at 37°C overnight. Following incubation, the culture was centrifuged at 13000 rpm for 5 min and the supernatant was discarded. The pellet was resuspended in 150 µl of buffer P1. Addition of 150 µl of buffer P2 with standing for 5 min at room temperature followed by the addition of 150 µl of buffer P3 on ice for 5 min were subsequently done. The solution was mixed by gentle flicking of the tube after each addition of the buffer. The resulting mixture was centrifuged at 13000 rpm for 10 min. The supernatant was transferred to clean 1.5-ml microfuge tube and the centrifugation step was repeated. The supernatant was again transferred to clean tube, added with 0.7 volume of isopropanol and mixed by gently flicking the tube. This was then centrifuged at 13000 rpm for 15 min. The resulting pellet containing the plasmid DNA was washed with 70% ethanol and allowed to air-dry. Sterile water (50 µl) was added to resuspend the pellet and the plasmid DNA was stored in -20°C until use. To verify the correct clones, the plasmid DNA was digested with a restriction endonuclease. Restriction digestion with *Eco*RI (New England Biolabs) was done by mixing the following: 10x *Eco*RI buffer, 2 µl; water, 12 µl; 20,000 U/ml *Eco*RI, 1 µl; plasmid DNA, 5 µl. The reaction mix was

incubated at 37°C for 2-3 hours and the restriction patterns were analyzed by agarose gel electrophoresis.

For amplification of the pGEM-T easy insert, sequencing PCR was done using the primers SP6 (5'- ATTTAGGTGACACTATAG-3') and T7 (5'- GTAATACGACTCACTATAGGG-3') and the BigDye® terminator cycle sequencing kit (Applied Biosystems). The PCR reaction mix was composed of the following: 5x BigDye® terminator v1.1 sequencing buffer, 2 µl; BigDye® terminator v1.1 premix, 2 µl; plasmid DNA, 2 µl; 25 µM of primer, 1 µl; sterile water, 3 µl. The PCR conditions were as follows: initial denaturation (2 min at 96°C), 25 cycles of denaturation (30 s at 96°C), primer annealing (15 s at 45°C), primer extension (4 min at 72°C) and a final extension step (10 min at 60°C). DNA sequencing was performed using an ABI 377XL automated sequencer (Applied Biosystems). Sequences were assembled using the ContigExpress tool in Vector NTI suite 6.0 (InforMax, Inc) and subsequently aligned using Clustal X. Phylogenetic analysis was done using the ARB software (Strunk and Ludwig 1997).

2.1.3 Morphological, physiological and biochemical characterization of selected strains

The strains were cultivated in liquid medium and the bacterial biomass was subsequently fixed with 2.5% glutaraldehyde and washed with 50 mM cacodylate buffer. The samples were fixed in 2% osmium tetroxide, washed with water five times, followed by dehydration with a series of ethanol solutions (50%, 70%, 90% and 100%) and incubation in propylene oxide. This was followed by overnight incubation in propylene oxide/glycidether (Epon 812, Roth) and polymerization of the resin for three days at 60°C. The samples were sectioned using an ultramicrotome (OM U3, C. Reichert) and contrasted with 1% uranyl acetate and lead citrate. The sections were examined with a Zeiss EM 10 electron microscope operating at 80V.

For scanning electron microscopy, colonies grown on agar plates were stanced out using a sterile cork borer with surrounding material. These were then fixed in 6.25% glutaraldehyde, washed five times with Sörensen-phosphate buffer pH 7.4 and dehydrated with increasing concentrations of acetone (30%, 50%, 75%, 90% and 100%). After critical-point drying and platinum coating of the dried material, colonies were examined with a Zeiss DSM 962 scanning electron microscope.

Cultural characteristics of the strains such as mycelial morphology and production of diffusible pigments were observed on a number of standard International *Streptomyces* Project (ISP) agar media, namely: yeast-malt extract (ISP 2), oatmeal (ISP 3), inorganic salts-starch (ISP 4), glycerol-asparagine (ISP 5) and peptone-yeast extract-iron (ISP 6) and tyrosine (ISP 7) (Shirling and Gottlieb 1966). Growth requirement for seawater and salt tolerance were tested using various amounts of NaCl (0%, 2.5%, 5.0%, 7.5%, 10.0%, 12.5% and 15.0%) as well as artificial seawater (25%, 50%, 75% and 100%) in appropriate media. Optimum temperature for growth was also determined by growing the strains in different temperature conditions (4, 10, 15, 20, 25, 30, 37, 45 and 55 °C). Sensitivity to different antibiotics (100 μg/ml) such as ampicillin, chloramphenicol, gentamicin, kanamycin, lincomycin, oxacillin, penicillin, rifampin, streptomycin, tetracycline and vancomycin was also determined by observing the growth as turbidity in liquid medium supplemented with the antibiotics after incubation at 30°C for 7-10 days. Oxygen requirement for growth was also determined by incubating the agar plates in an anaerobic jar. For phenotypic characterization, API kits (Biomerieux) and Biolog (Biolog Inc.) plates were used following manufacturer's instructions. API CH system and Biolog SF-P2 plates were used to evaluate the utilization of different carbon sources while enzymatic activities were detected using the API Zym kit. The cells were harvested by aseptically scraping the spores and mycelial fragments on the agar media and suspended either in artificial seawater for use in the API kits or in 0.2% phytagel (Sigma) for Biolog SF-P2. Appropriate reagents supplied together with the kit were added to individual cupules following manufacturer's instructions and positive reactions were noted. For the carbon utilization tests, a positive reaction was noted as turbidity on the cupules or wells after incubation at 30°C for 7-10 days. Furthermore, the ability to degrade macromolecules was determined as clearing zones around colonies growing on agar media containing adenine, casein, chitin, hypoxanthine and tyrosine (Korn-Wendisch et al. 1989).

Diagnostic cell wall components, G+C content of the genomic DNA as well as DNA-DNA relatedness of the strains were determined and performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Established procedures were used to determine the diagnostic isomers of the diaminopimelic acid (A_2pm) and the predominant sugars of the whole organism (Staneck and Roberts 1974). A quinone analysis was carried out as described by Kroppenstedt (1985). The presence of mycolic acids was investigated following the procedure of Minnikin *et al.* (1975). The polar lipids were extracted and analyzed following the integrated procedure of Minnikin *et al.* (1984). The composition of the fatty acid pattern was determined by gas chromatography using MIDI

software. The DNA base composition of genomic DNA was determined by HPLC (Tamaoka and Komagata 1984; Mesbah et al. 1989).

2.2 Results

2.2.1 Strain description of new actinomycete species

2.2.1.1 *Saccharopolyspora cebuensis* sp. strain SPE 10-1^T (Pimentel-Elardo et al. 2008b)

Strain SPE 10-1^T was isolated from the marine sponge *Haliclona* sp. (Fig. 2.1A) collected from Cebu, Philippines and was cultivated on M1 agar, a medium specifically designed for the selective isolation of marine actinomycetes (Mincer et al. 2002).

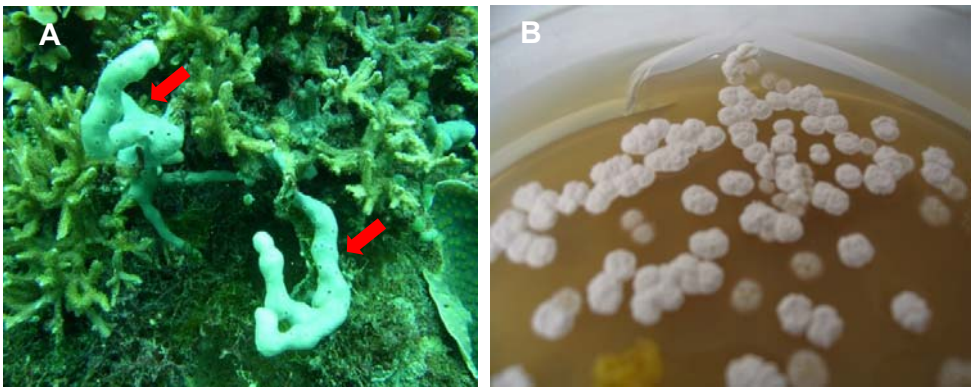

Fig. 2.1 (A) marine sponge *Haliclona* sp. (underwater photography by J. Apurado, University of San Carlos); (B) Strain SPE 10-1^T colonies on M1 agar

An almost complete 16S rDNA sequence (1483 nucleotides) was generated for the strain and compared to the validly described species of the genus *Saccharopolyspora* as its closest neighbors as well as representative genera from the family *Pseudonocardiaceae* (Fig. 2.2). Phylogenetic analysis revealed that the strain SPE 10-1^T has highest sequence similarity (96%) with *Saccharopolyspora gregorii* and 93-95% similarity with all other species of the genus *Saccharopolyspora*.

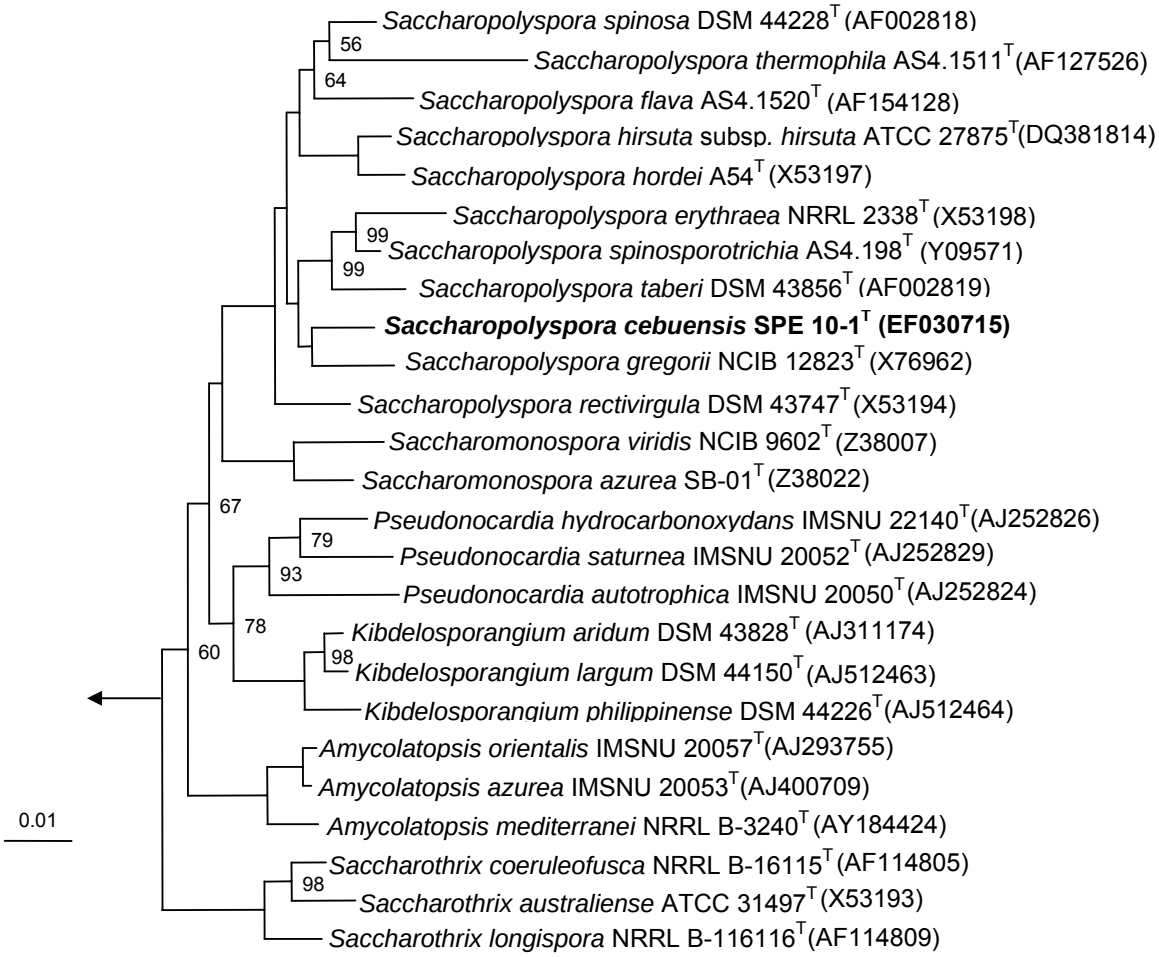

Fig. 2.2 Neighbor-joining tree based on nearly complete 16S rDNA sequences of strain SPE 10-1^T and representative strains of the family *Pseudonocardiaceae* and related taxa. *Escherichia coli* (DQ360844) was used as outgroup. Numbers at the nodes indicate the levels of bootstrap support based on 100 resampled data sets; only values greater than 50% are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

Strain SPE 10-1^T exhibited morphological properties characteristic of *Saccharopolyspora*, forming extensively branched substrate mycelia which fragments into rod-shaped elements (Fig. 2.3A). Scanning electron microscopy showed hyphae bearing short chains of spores as well as single spore cells (Fig. 2.3B). The spores were round to oval and the surface was smooth. Light microscopy of colonies confirmed the presence of spores in aerial mycelia.

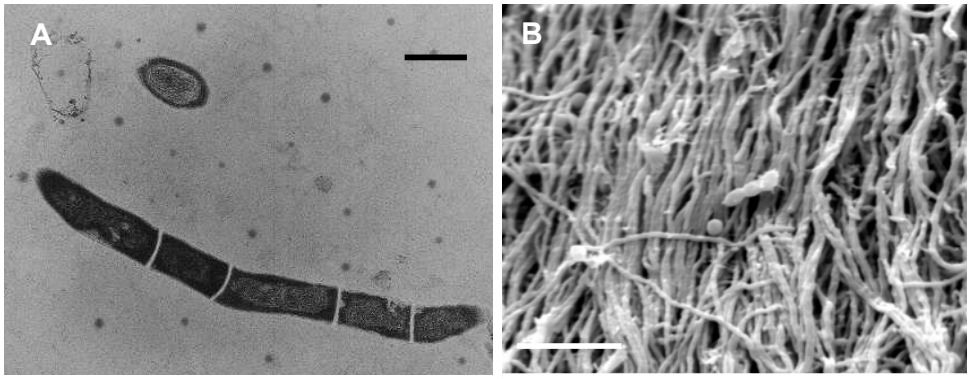

Fig. 2.3 Hyphae and spores of strain SPE 10-1^T grown in M1 broth (A) and on M1 agar (B) at 30°C for 7 days; (A), transmission electron microscopy, bar = 0.5 µm; (B), scanning electron microscopy, bar = 5 µm

Furthermore, strain SPE 10-1^T was able to grow in ISP medium 2 with ASW as well as in Zobell marine medium (Oppenheimer and Zobell 1952). Brown diffusible pigment was also observed. Growth was observed at temperatures from 15 to 37°C, with optimal growth at 25 to 30°C. Colonies displayed chalky-white mycelia with brownish soluble pigment on M1 agar. Cultures of the strain SPE 10-1^T grown in M1 broth for 7-14 days appeared yellowish-brown to brown in color. Strain SPE 10-1^T did not grow in ISP medium 2 without ASW. M1 media supplied with different amounts of ASW or NaCl were used to test for requirement for seawater and salt tolerance. Growth was possible in regular strength M1 (100% ASW) and in M1 containing 75, 50 and 25% but not 0% ASW. Growth was also possible when regular strength ASW was replaced with 12.5, 10, 7.5 or 5% NaCl in distilled water. Growth was poor in M1 with 2.5% NaCl and growth was not observed without NaCl or with 15% NaCl. Furthermore, strain SPE 10-1^T was able to grow in M1 liquid medium supplemented with antibiotics (100 µg/ml) gentamicin and kanamycin but not with rifampin, penicillin, streptomycin, lincomycin, vancomycin, oxacillin, chloramphenicol, ampicillin and tetracycline. No growth was observed on M1

agar plates incubated in an anaerobic jar. Using the API CH system, the strain was able to utilize a variety of organic compounds such as glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, N-acetylglucosamine, amygdalin, esculin, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-trehalose, inulin, D-raffinose, amidon, glycogen, gentibiose, D-fucose, D-arabitol, and potassium gluconate as sole carbon source. Using the API Zym system, the following enzymes were tested positive: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-B1-phosphohydrolase, α-glucosidase, N-acetyl-β-glucosaminidase and α-mannosidase. The strain is able to degrade tyrosine but not adenine, casein, chitin and hypoxanthine. Furthermore, strain SPE 10-1^T stained positive by Gram-staining and was positive for catalase following standard reaction to hydrogen peroxide. Strain SPE 10-1^T tested negative for oxidase activity and for reduction of nitrate to nitrite. The organism can be distinguished from the other type strains of the validly described species of *Saccharopolyspora* by using a combination of phenotypic properties (Table 2.1).

The strain contained *meso*-A_2pm as the wall diamino acid, the diagnostic sugars arabinose and galactose were present but glucose and ribose were found in addition. A menaquinone with a tetra-hydrogenated-isoprenoid side chain of nine units MK-9 (H4) was the principal isoprenoid quinone. Small amounts of MK-8(H4) and MK-10(H4) were found in addition. Mycolic acids were not detected. The phospholipid pattern was composed of phosphatidyl-choline, phosphatidyl-ethanol amine, phosphatidyl-methylethanolamine, diphosphatidyl-glycerol, phosphatidyl-glycerol and phosphatidyl-inositol. Two unknown glycolipids were found in addition. The fatty acid pattern was mainly composed of terminally branched iso- and anteiso-fatty acids but small amounts of diagnostic 10-methyl-branched fatty acids were found in addition while 2-hydroxy fatty acids were missing. The DNA G+C content was 72.6 mol%.

Table 2.1 Selected physiological properties of *S. cebuensis* sp. nov. SPE 10-1^T in comparison to validly described *Saccharopolyspora* type strains

Strains: 1, *S. cebuensis* SPE 10-1^T; 2, *S. gregorii* (DSM 44324^T), 3, *S. spinosporotrichia* (DSM 44350^T), 4, *S. spinosa* (DSM 44228^T); 5, *S. erythraea* (DSM 40517^T); 6, *S. hirsuta* (DSM 43463^T); 7, *S. hordei* (DSM 44065^T); 8, *S. rectivirgula* (DSM 43747^T); 9, *S. flava* (AS4.1520^T); 10, *S. thermophila* (AS4.1511^T); 11, *S. taberi* (DSM 43856^T). Data for the reference strains other than *S. cebuensis* sp. nov. SPE 10-1^T were taken from Lu *et al.* (2001) except for the data on hypoxanthine, G+C content and D-mannitol utilization which were taken from Goodfellow *et al.* (1989), Labeda (1987), Lacey & Goodfellow (Lacey and Goodfellow 1975), Mertz & Yao (1990) and Zhou *et al.* (1998). Abbreviations: Bf, buff; Br, brown; C, colorless; G, grey; O, orange; P, pink; R, red; W, white; Y, yellow; +, positive; -, negative; n/a, no aerial mycelium; nd, not determined. All *Saccharopolyspora* strains were positive for utilization of D-fructose, glycerol and D-mannose as sole carbon source.

[a]: The temperature data from Lu *et al*. (2001) on the type strains differ slightly from those of Korn-Wendisch *et al*. (1989) who reported a temperature range of 20-50°C for *S. hirsuta* and 37-60°C for *S. rectivirgula* species.

[b]: *S. cebuensis* SPE 10-1^T is the only known isolate with a strict requirement for salt.

[c]: Note that the ISP medium 2 was supplemented with ASW and that mycelia color may vary depending on media composition.

Characteristics	*Saccharopolyspora* reference strains										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Carbon source utilization											
L-Arabinose	+	+	-	+	+	-	+	-	-	-	-
D-Galactose	+	+	+	-	+	+	+	+	+	+	+
D-Lactose	+	-	-	-	-	+	+	+	+	+	+
D-Maltose	+	+	+	-	+	+	+	+	+	+	+
D-Mannitol	-	+	+	+	+	+	+	+	+	+	+
D-Raffinose	+	+	+	-	+	+	+	+	+	+	+
L-Rhamnose	+	+	+	-	+	+	+	+	+	+	+
Sucrose	+	+	+	-	+	+	+	+	+	+	+
D-Xylose	+	+	+	-	+	+	+	+	+	-	+
Temp. range (°C)	15-37	10-35	28-37	15-37	20-42	25-50[a]	20-60	37-63[a]	28-37	45-55	20-45
Nitrate reduction	-	-	-	+	+	-	-	+	+	-	+
NaCl tolerance (%)	2.5-12.5[b]	13	<3	<11	<5	<7	<13	<10	7	7	7
Degradation ability											
Adenine	-	-	-	-	+	+	+	-	+	+	+
Casein	-	+	+	-	-	+	+	-	-	-	+
Chitin	-	-	-	-	+	-	+	-	-	-	+
Hypoxanthine	-	+	+	+	+	+	+	+	+	-	+
Tyrosine	+	+	-	+	+	+	+	+	-	+	+
Color of											
Aerial mycelia	W[c]	W-Y	W-G	W-P	P-BrG-W	W	W-Y	W-lightP	W	W	n/a
Substrate mycelia	W[c]	C-Bf	Br-R	G-OY-Br	OY-RBr	C-Bf	C-Bf	Y-O	Y	C-Bf	C-Y
G+C content (mol %)	72.6	74.0	70.4	nd	71.1	71.5	72.0	70.4	67.0	73.1	70.8

2.2.1.2 *Streptomyces axinellae* strain Pol001^T (Pimentel-Elardo et al. 2008a)

Strain Pol001^T was isolated from the marine sponge *Axinella polypoides* (Fig. 2.4A) collected from Banyuls-sur-mer, France (Scheuermayer 2006).

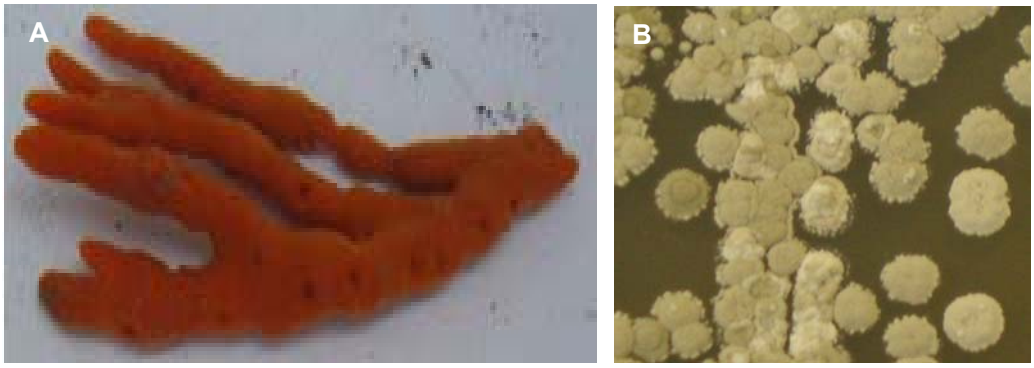

Fig. 2.4 (A) marine sponge *Axinella polypoides* (photography by M. Scheuermayer, University of Würzburg); (B) Strain Pol001^T colonies on ISP medium 2

An almost complete 16S rDNA sequence (1422 nucleotides) was generated for the strain and compared to the validly described species of the genus *Streptomyces* as its closest neighbors. Phylogenetic analysis revealed that the strain Pol001^T exhibits closest sequence similarities with the following: *Streptomyces sclerotialus* DSM 46032^T (97.61%), *Streptomyces rimosus* subsp. *rimosus* DSM 40260^T (97.47%), *Streptomyces niger* DSM 43049^T (97.20%) and *Streptomyces olivaceiscleroticus* DSM 40595^T (97.20%).

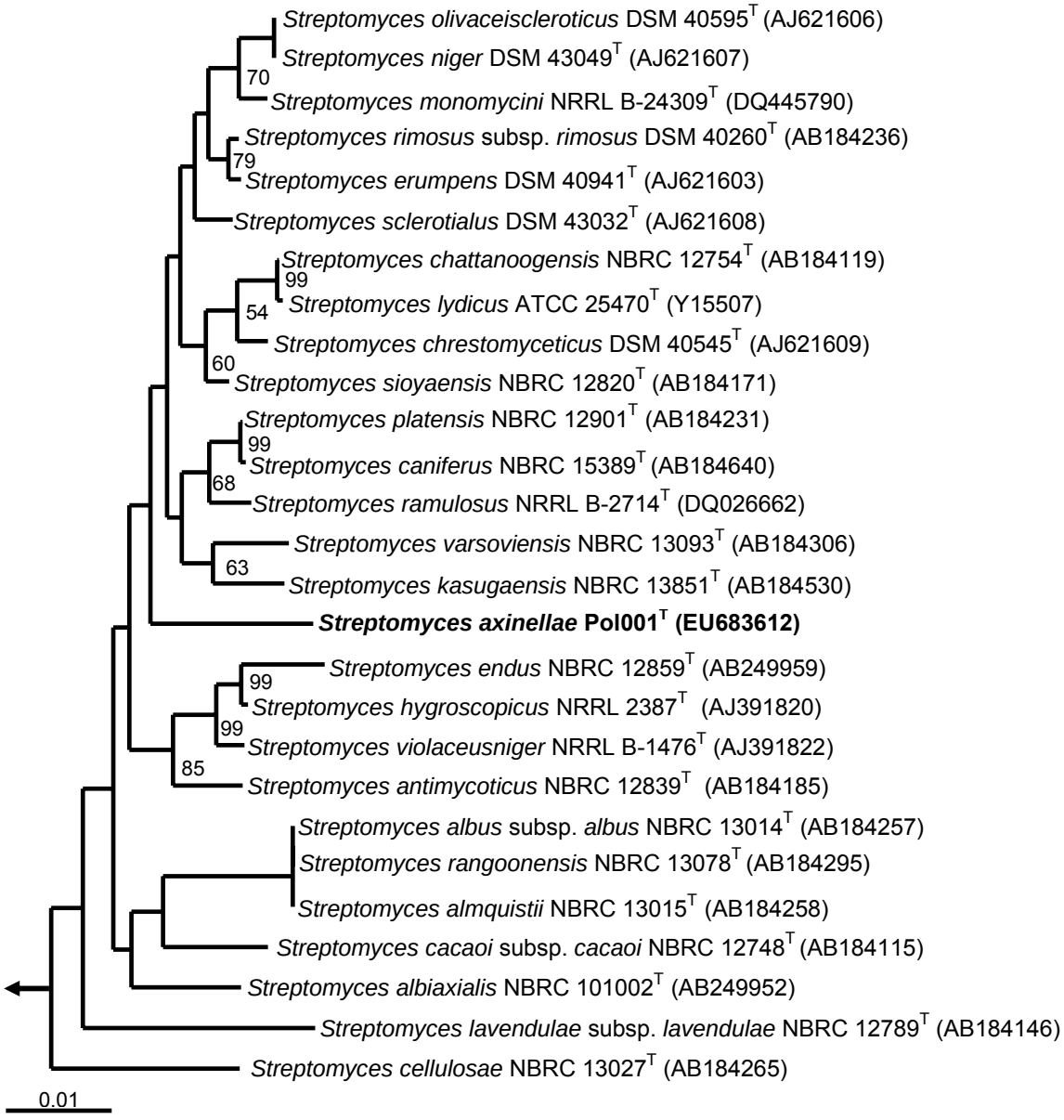

Fig. 2.5 Neighbor-joining tree of strain Pol001^T and representative species of the genus *Streptomyces* based on nearly complete 16S rDNA sequences. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. Arrow points to outgroup, *Salinispora tropica* (AY040618). The scale bar indicates 0.01 substitutions per nucleotide position.

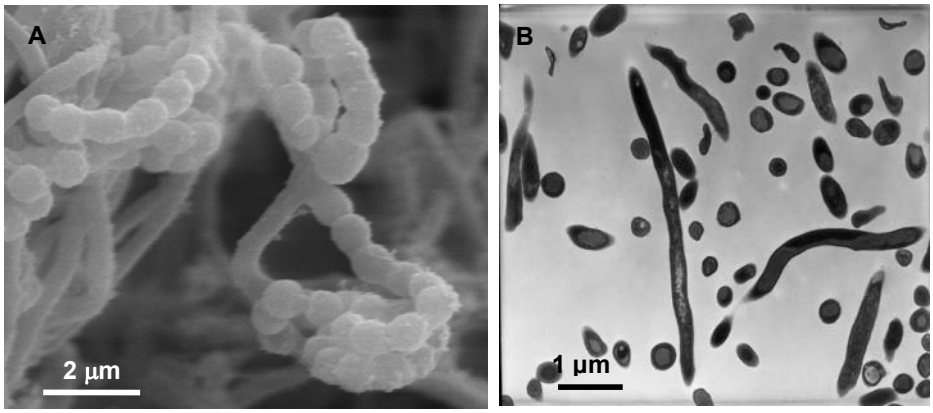

Fig. 2.6 (A) Scanning electron micrograph and (B) transmission electron micrograph of spores and hyphae of strain Pol001^T grown in ISP 2 media

Strain Pol001^T grew well on a variety of standard International *Streptomyces* Project (ISP) agar media after incubation at 30°C for 21 days: yeast-malt extract (ISP 2), oatmeal (ISP 3) and peptone-yeast extract-iron (ISP 6). A diffusible pigment was observed only on tyrosine agar (ISP 7) (Table 2.2). Extensively branched grey aerial and white substrate mycelia were abundant on ISP 2 medium (Fig. 2.4B). At 21 days, the strain produced spiral chains of spores with a smooth surface, elliptical shape and 0.8-0.9 μm in length (Fig. 2.6A). Furthermore, transmission electron microscopy revealed non-fragmenting hyphae (Fig. 2.6B). Strain Pol001^T was able to grow at 20-37°C, with optimum growth at 30°C. Growth was possible in 0, 2.5, 5, 7.5 % NaCl but not in 10, 12.5, and 15 % NaCl, with optimal growth at 0-2.5 % NaCl. Growth was also possible in ISP 2 medium with 25, 50, 75 and 100% ASW. Furthermore, strain Pol001^T was able to grow in medium supplemented with the antibiotics (100 μg/ml) ampicillin, chloramphenicol, nalidixic acid, penicillin and rifampicin but not with erythromycin, gentamicin, kanamycin and vancomycin.

Table 2.2 Cultural characteristics of strain Pol001^T on various agar media

Medium	Growth	Diffusible pigment	Aerial mycelium	Substrate mycelium
Yeast-malt extract (ISP 2)	Abundant	None	Grey	White
Oatmeal (ISP 3)	Abundant	None	Dark Brown	Light Green
Inorganic salts-starch (ISP 4)	Few	None	Yellow Orange	White
Glycerol-asparagine (ISP 5)	Moderate	None	White	Light Yellow
Peptone-yeast extract-iron (ISP 6)	Abundant	None	Light Pink	White
Tyrosine (ISP 7)	Moderate	Red	Light Pink	Light Green
Czapek	Abundant	None	Grey to Black	Light Green
LB agar	Abundant	None	White	White

Physiological tests indicated that strain Pol001^T is able to utilize a variety of organic compounds as carbon sources such as N-acetyl-β-D-mannosamine, N-acetyl-D-glucosamine, N-acetyl-L-glutamic acid, L-alaninamide, L-alanine, L-alanyl-glycine, D-arabitol, D-cellobiose, dextrin, D-fructose, D-galactose, gentiobiose, D-gluconic acid, α-D-glucose, L-glutamic acid, glycerol, DL-α-glycerol phosphate, α-D-lactose, L-malic acid, D-mannitol, D-mannose, propionic acid, L-rhamnose, D-ribose, L-serine, Tween 40, Tween 80, D-xylose but not acetic acid, adenosine, adenosine-5'-monophosphate, D-alanine, amygdalin, L-arabinose, arbutin, L-asparagine, 2,3-butanediol, α-cyclodextrin, β-cyclodextrin, 2'-deoxyadenosine, D-fructose-6-phosphate, L-fucose, D-galacturonic acid, α-D-glucose-1-phosphate, D-glucose-6-phosphate, glycogen, glycyl-L-glutamic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxy-phenylacetic acid, inosine, m-inositol, inulin, α-ketoglutaric acid, α-ketovaleric acid, lactamide, L-lactic acid, D-lactic acid methyl ester, lactulose, D-malic acid, maltose, maltotriose, mannan, D-melezitose, D-melibiose, α-methyl-D-galactoside, β-methyl-D-galactoside, 3-methyl-D-glucose, α-methyl-D-glucoside, β-methyl-D-glucoside, α-methyl-D-mannoside, palatinose, D-psicose, putrescine, L-pyroglutamic acid, pyruvic acid, pyruvic acid methyl ester, D-raffinose, salicin, sedoheptulosan, L-serine, D-sorbitol, stachyose, succinamic acid, succinic acid, succinic acid mono-methyl ester, sucrose, D-tagatose, thymidine, thymidine-5'-monophosphate, D-trehalose, turanose, uridine, uridine-5'-monophosphate and xylitol. Furthermore, the strain is positive for gelatin liquefaction but negative for melanin production, starch hydrolysis, nitrate reduction and hydrogen sulfide production. It is capable of degrading casein, but not adenine, chitin or hypoxanthine.

Levels of DNA-DNA relatedness between strain Pol001^T and four closely related *Streptomyces* species were as follows (average of two values): 26.8% (*S. sclerotialus* DSM 46032^T), 16.9% (*S. olivaceiscleroticus* DSM 40595^T), 8.75% (*S. niger* DSM 43049^T) and 8.65% (S. *rimosus* subsp. *rimosus* DSM 40260^T).

Strain Pol001^T contained LL-diaminopimelic acid in the cell wall. Analysis of the whole-cell sugar composition revealed the presence of glucose and ribose as well as traces of mannose. A menaquinone with a hexahydrogenated-isoprenoid side chain of nine units MK-9 (H_6) were found as the principal isoprenoid quinone. Two additional quinones with nine isoprene units [MK-9 (H_4, H_8)] were also found. Phospholipid pattern consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, some unidentified phospholipids, phosphoglycolipids, glycolipids and an aminolipid. Fatty acid pattern consisted of iso-$C_{16:0}$ (30.78%), anteiso-$C_{15:0}$ (17.77%),

iso-$C_{15:0}$ (12.03%), anteiso-$C_{17:0}$ (9.80%), iso-$C_{16:1}$ (6.92%), iso-$C_{14:0}$ (5.77%) and iso-$C_{17:1}$ (4.58%). The DNA G+C content of strain Pol001^T was 71.0 mol%.

Table 2.3 Selected physiological properties that separate strain Pol001^T from closely related *Streptomyces* species

Strains: 1, Pol001^T; 2, *S. sclerotialus* DSM 46032^T; 3, S. *rimosus* subsp. *rimosus* DSM 40260^T; 4, *S. niger* DSM 43049^T; 5, *S. olivaceiscleroticus* DSM 40595^T. Color of mycelium, reverse side of colony, production of diffusible and melanoid pigments were compared using growth on ISP 2 medium. Smooth spore surface, absence of melanoid pigment and utilization of glucose and fructose as carbon sources were observed for all strains. Data for reference strains were taken from Shirling & Gottlieb (Shirling and Gottlieb 1968a; Shirling and Gottlieb 1968b; Shirling and Gottlieb 1972).

Characteristic	1	2	3	4	5
Spore-chain morphology	SP	SP	SP, RA	SP	SP
Color of aerial mycelium	GW	LYR	R or W	YG	BG
Reverse-side of colony	W	YB	GY	DB	GY
Production of diffusible pigment	-	-	-	+	+
Utilization of					
L-Arabinose	-	+	+	+	+
Inositol	-	+	+	+	+
Mannitol	+	+	+	+	+
Raffinose	-	+	+	+	+
Rhamnose	+	+	-	+	+
Sucrose	-	+	-	+	+
Xylose	+	+	d	+	+

Abbreviations: SP, *Spirales*; RA, *Retinaculiaperti*; G, grey; W, white; Y, yellow; R, red; B, brown; L, light; D, dark; +, positive; -, negative; d, doubtful.

2.2.2 Isolation of other actinomycete strains

Nineteen additional strains cultivated from various marine sponges exhibiting typical actinomycete morphology (Fig. 2.7) were identified by 16S rDNA sequencing (Table 2.4). These strains were found to cluster together with known species of different actinomycete genera such as *Streptomyces, Isoptericola, Micromonospora, Gordonia, Nocardiopsis* and *Saccharopolyspora* (Fig. 2.8). Furthermore, the strains account to about 60% of the total number of isolates positively identified from the strain collection, the majority of which were originally cultivated from M1 and ISP 2 media after 2-3 weeks of incubation at 30°C.

Table 2.4 16S rDNA phylogenetic affiliation of actinomycete strains

Isolate code	16S rDNA closest relative (% homology)	Source sponge
Aer003*	*Streptomyces bingchengensis* 226541 (99.5)	*Aplysina aerophoba*
A188*	Actinomycetales bacterium XJSS-18 (100.0)	*Aplysina aerophoba*
Cr03	*Nocardiopsis* sp. 20052 (99.7)	*Chondrosia reniformis*
Da02	*Streptomyces* sp. CNS-774_SD06 (99.9)	*Dysidea avara*
T02	*Streptomyces* sp. CNS-774_SD06 (99.9)	*Tethya* sp.
T03	*Streptomyces* sp. MP47-91 (99.7)	*Tethya* sp.
4-3	*Micromonospora* sp. HBUM84229 (99.7)	unidentified sponge
8-6	*Saccharopolyspora gregorii* (96.0) ♣	unidentified sponge
9-1	*Streptomyces* sp. C12 (99.0) ♣	unidentified sponge
9-3	*Gordonia terrae* AIST-1 (99.8)	unidentified sponge
10**	*Streptomyces albogriseolus* NBRC 3709 (99.9)	*Aplysina aerophoba*
10-11	*Nocardiopsis* sp. M048 (97.0) ♣	unidentified sponge
11**	*Streptomyces spinoverrucosus* 174464 (99.8)	*Tedania* sp.
11-2	*Streptomyces* sp. 3194 (99.0) ♣	unidentified sponge
11-11	*Isoptericola* sp. TUT1258 (98.3)	unidentified sponge
17**	*Streptomyces variabilis* strain 173733 (99.9)	*Aplysina aerophoba*
22**	*Streptomyces* sp. VTT E-042674 (100.0)	*Aplysina aerophoba*
27**	*Streptomycetaceae* bacterium WBF21 (99.6)	*Aplysina aerophoba*
34**	*Streptomyces* sp. VTT E-042674 (99.9)	*Axinella polypoides*

* Strains from previous study (Scheuermayer 2006).
** Strains isolated by S. Kozitska (AG Hentschel, University of Würzburg).
♣ Based on partial 16S rDNA sequences.

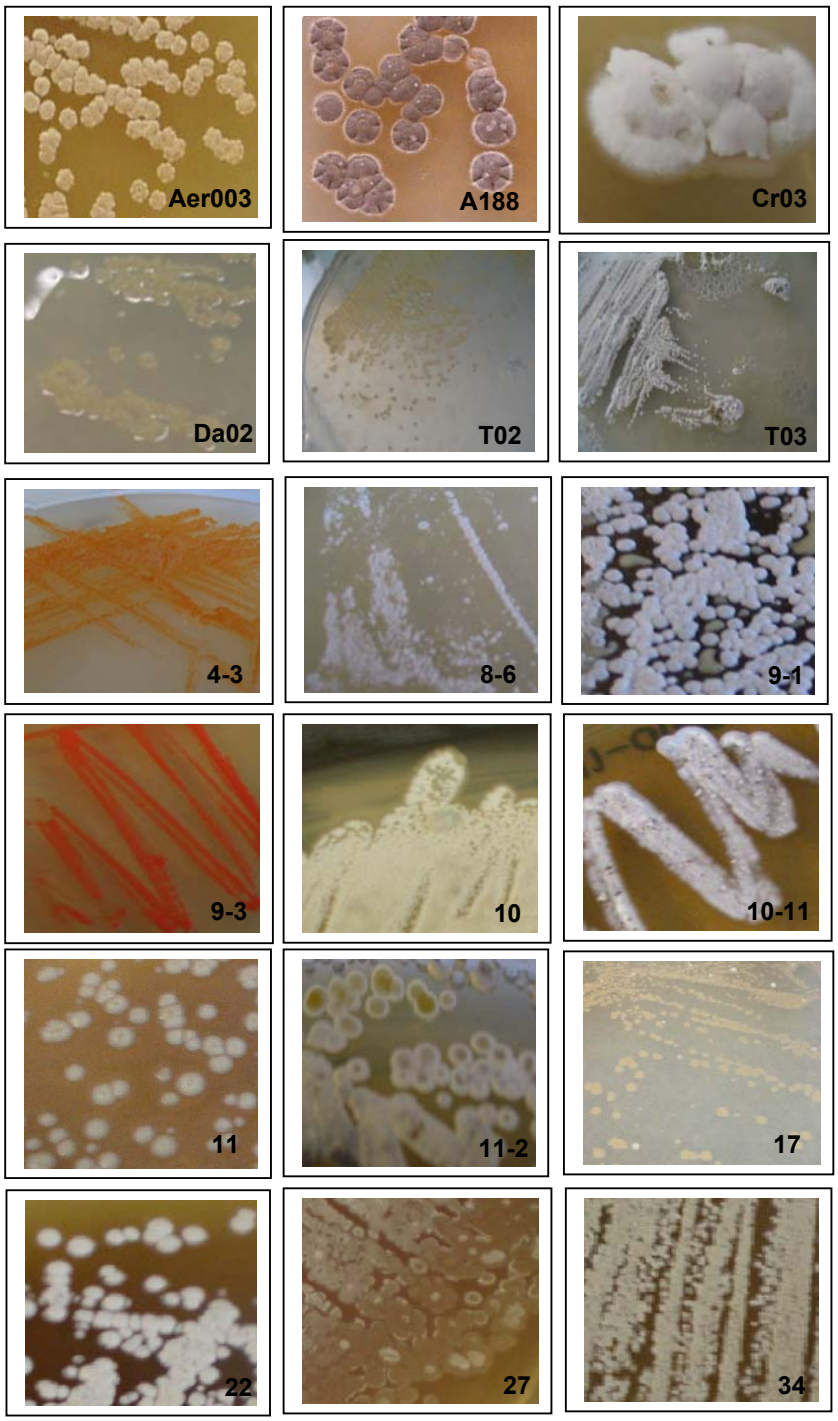

Fig. 2.7 Various colony morphologies of actinomycete strains grown on M1 and ISP 2 media at 30°C for 7-14 days

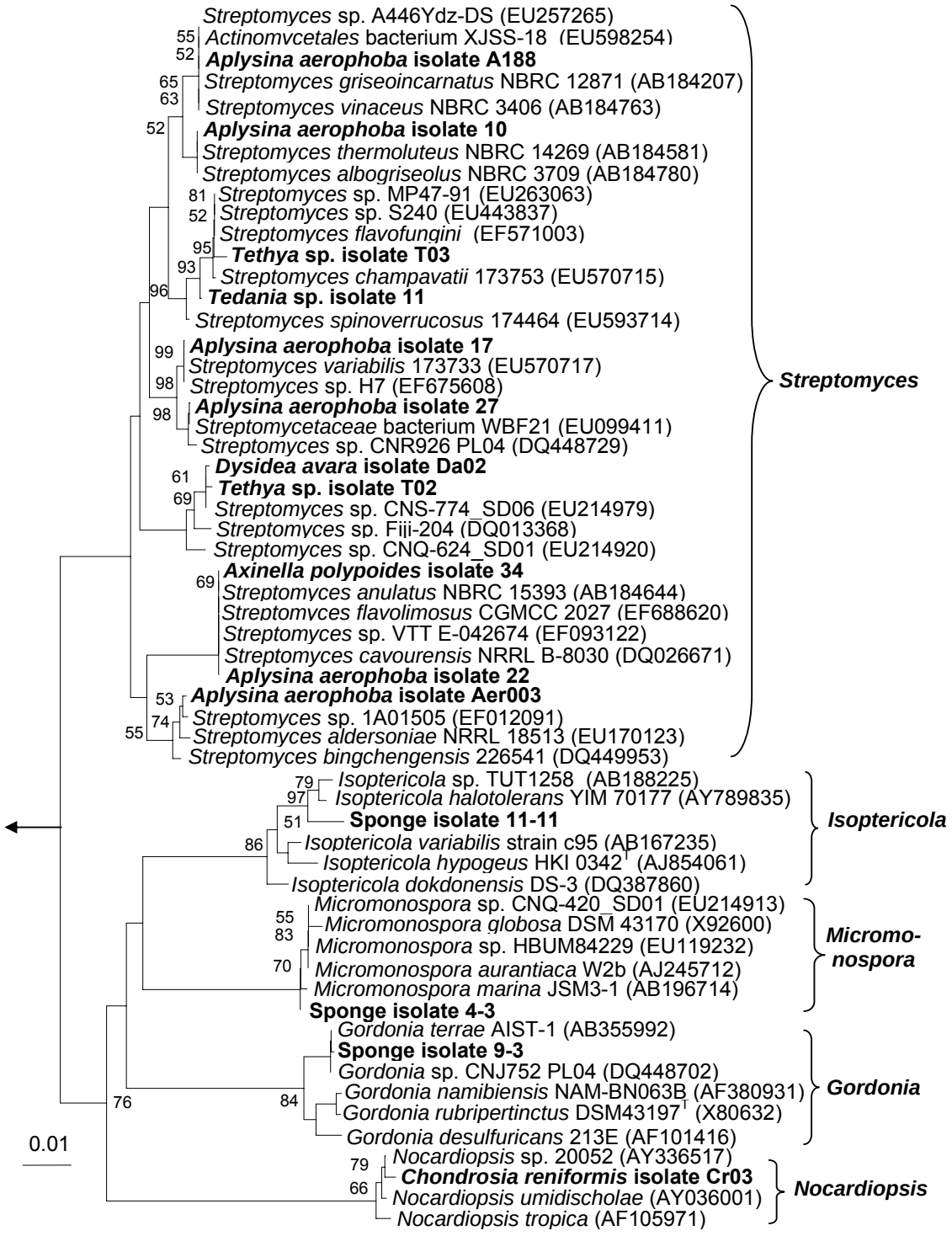

Fig. 2.8 Neighbor-joining tree based on nearly complete 16S rDNA sequences (>1400 bp) of the different sponge isolates. *Escherichia coli* (DQ360844) was used as outgroup. Numbers at the nodes indicate the levels of bootstrap support based on 100 resampled data sets; only values greater than 50% are shown. The scale bar indicates 0.01 substitutions per nucleotide position.

2.3 Discussion

The order *Actinomycetales* is composed of approximately 80 genera, mostly from terrestrial soils (Magarvey et al. 2004). The order encompasses bacteria that are diverse with respect to morphology and biochemistry as well as having G+C-rich DNA. Members of this taxon are of significant interest primarily because these bacteria have consistently produced a considerable amount of bioactive metabolites of pharmacological and biomedical importance such as the antibiotics, erythromycin and streptomycin. As the search for producers of novel compounds continues, the rate of finding the same compounds from existing terrestrial actinomycete genera has increased (Kelecom 2002; Fenical and Jensen 2006) while the chance of finding new bioactive molecules from screening of large actinomycete libraries has decreased (Busti et al. 2006). Hence, efforts are now directed at exploiting new potential sources such as the marine environment. A number of reports have been published describing the isolation of novel actinomycete genera from marine sediments and invertebrates and these actinomycetes have been found to exhibit diverse biological activities, suggesting that the marine environment is an interesting source for bioprospecting (Fiedler et al. 2005; Jensen et al. 2005a; Jensen et al. 2005b; Kim et al. 2005; Montalvo et al. 2005; Fenical and Jensen 2006; Lam 2006).

The aim of this Ph.D. study was to isolate novel actinomycete taxa from marine sponges using selective isolation procedures and polyphasic characterization of selected strains. The ability to order prokaryotic taxa hierarchically has been improved by high quality 16S rDNA sequence analyses (Stackebrandt et al. 2002). The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker. It has been demonstrated that 16S rRNA gene sequence data on an individual strain with a nearest neighbor exhibiting a similarity score of <97% represents a new species (Janda and Abbott 2007). Based on 16S rDNA phylogenetic analysis of all the isolated strains in this study, it was found that 2 out of the 19 identified strains are new actinomycete species belonging to the genera *Saccharopolyspora* and *Streptomyces*.

Strain SPE 10-1^T isolated from the sponge *Haliclona* sp. was the first strain identified by 16S rDNA sequencing belonging to the genus *Saccharopolyspora.* This genus was first described by Lacey & Goodfellow from sugar-cane bagasse (1975) and at present comprises ten validly described species: *Saccharopolyspora hirsuta (*Korn-Wendisch et al. 1989), *Saccharopolyspora erythraea* (Labeda 1987), *Saccharopolyspora taberi* (Korn-Wendisch et al. 1989), *Saccharopolyspora gregorii* (Goodfellow et al. 1989),

Saccharopolyspora hordei (Goodfellow et al. 1989), *Saccharopolyspora rectivirgula* (Korn-Wendisch et al. 1989), *Saccharopolyspora spinosa* (Mertz and Yao 1990), *Saccharopolyspora spinosporotichia* (Zhou et al. 1998), *Saccharopolyspora flava* (Lu et al. 2001) and *Saccharopolyspora thermophila* (Lu et al. 2001). Members of this genus are aerobic, Gram-positive, non-acid fast organisms with substrate hyphae that either fragments into rod-shaped elements, do not fragment or are transformed partially into chains of spores (Korn-Wendisch et al. 1989). They lack mycolic acid but contain *meso*-diaminopimelic acid, arabinose and galactose in the cell wall and predominant amounts of tetra-hydrogenated menaquinones with nine isoprene units. DNA base composition falls within the range of 67-74 mol% G+C for the type strains (Embley et al. 1987; Goodfellow et al. 1989; Korn-Wendisch et al. 1989). Strain SPE10-1^T exhibited morphological and biochemical characteristics consistent with members of the genus *Saccharopolyspora* but can be distinguished from the other type strains of this genus based on a combination of phenotypic properties. The low 16S rDNA sequence similarities of the strain with validly described species of *Saccharopolyspora* further suggest that the strain constitutes a novel species of this genus. Interestingly, the strain is only able to grow in medium with sodium chloride or ASW indicating a strict requirement for salt and hence, suggests that it is an obligate marine bacterium. Taken together, the phenotypic and genotypic data obtained in this study clearly show that strain SPE 10-1^T represents a novel and obligate marine species within the genus *Saccharopolyspora*. Thus, this type strain was assigned the species name as *S. cebuensis* (se.bu.en'sis N.L. fem. adj. *cebuensis*) pertaining to the province of Cebu in the Philippines, where the type strain was collected. It has been recently considered as a validly described species by the *International Journal of Systematic and Evolutionary Microbiology* (Pimentel-Elardo et al. 2008b) and is deposited at two public culture collections (=DSM 45019^T, =CIP 109355^T). It is also interesting that a similar strain 8-6 cultivated in this Ph.D. study from another sponge sample but from the same collection site also exhibited high sequence similarity (based on partial 16S rDNA sequence) with *S. gregorii* (96.0%), the closest relative of *S. cebuensis* suggesting that SPE 10-1 and 8-6 could possibly be the same strains. However, complete 16S rDNA sequencing and phenotypic characterization of the strain 8-6 must be done in order to confirm whether the same strain has been in fact, re-isolated from different sponge species.

The second strain Pol001^T isolated from the sponge *Axinella polypoides* was found by 16S rDNA sequencing to belong to the genus *Streptomyces*. The genus *Streptomyces* was first proposed by Waksman and Henrici (1943) for aerobic, spore-forming actinomycetes. These Gram-positive bacteria have distinct features such as extensive

branching substrate and aerial mycelium, high DNA G+C content (69-78 mol %), presence of LL -diaminopimelic acid and the absence of characteristic sugars in the cell wall (Anderson and Wellington 2001). The strain Pol001^T exhibited morphological, biochemical and chemotaxonomic characteristics consistent with the genus *Streptomyces*. There are currently more than 500 validly described species and subspecies under *Streptomyces*, making this genus to contain the largest number of species in the *Bacteria* domain (Hain et al. 1997). It is not surprising therefore that the 16S rDNA sequence similarity of the strain Pol001^T against reference *Streptomyces* strains was slightly above the 97% cut-off for species delineation (highest similarity value of 97.6%) considering the complexity of *Streptomyces* taxonomy. The 16S rDNA sequence similarity values still suggest that the strain Pol001^T can be considered a new *Streptomyces* taxon and this is also supported by the distinct phyletic line formed by the strain as seen in the neighbor-joining tree shown in Fig. 2.5. To further support this claim, DNA-DNA hybridization was carried out against the four reference *Streptomyces* strains with which strain Pol001^T exhibited the highest sequence similarities. The hypothesis for the species concept in the genus *Streptomyces* is that strains of the same species have DNA relatedness >70% (with a ΔT_m of <5°C) (Wayne et al. 1987; Labeda 1992; Anderson and Wellington 2001). Remarkably, the strain Pol001^T showed very low DNA-DNA relatedness values (highest value at 26.8%), hence further confirming that the strain is indeed a novel taxon of the genus *Streptomyces*. Thus, the type strain was assigned the species name *Streptomyces axinellae* (a.xi.nel'la.e. N.L. gen. n. *axinellae*) pertaining to the marine sponge *Axinella polypoides*, from which the strain was originally isolated. The strain has likewise been recently considered as a validly described species by the *International Journal of Systematic and Evolutionary Microbiology* (Pimentel-Elardo et al. 2008a) and is currently deposited in two internationally recognized culture collections (= DSM 41948^T, = CIP 109838^T).

The remainder of the strains isolated from various marine sponges were found by 16S rDNA sequencing belonging to different actinomycete genera and exhibiting sequence similarities between 98-100% with known species, with the majority belonging to the genus *Streptomyces*. This is not surprising since *Streptomyces* bacteria are easy to cultivate and in fact, generally dominate several strain collections (Fiedler et al. 2005; Maldonado et al. 2005; Busti et al. 2006; Bredholdt et al. 2007). Several studies have also shown that diverse actinomycetes are associated with marine sponges using cultivation-dependent and cultivation-independent approaches (Webster and Hill 2001; Webster et al. 2001; Hentschel et al. 2002; Kim et al. 2005; Montalvo et al. 2005; Zhang et al. 2006; Jiang et al. 2007; Xin et al. 2008). Interestingly, some representatives of the class

Actinobacteria have been found belonging to monophyletic, sponge-specific sequence clusters using 16S rRNA gene-based techniques (Hentschel et al. 2002). However, none of the strains cultivated in this Ph.D. study overlapped with the sponge-specific actinobacterial clusters. These results suggest that the strains are transient bacteria that have been taken up by the host sponge through its aquiferous canal at the time that the sponge samples were collected. It is also highly likely that these isolates are present in the surrounding seawater or sediments since the majority of the strains isolated in this study showed very high sequence similarities with those previously derived from marine sediments. For example, isolates Da02 and T02 cultivated from two different Mediterranean sponges *Dysidea avara* and *Tethya* sp., respectively showed 100% 16S rDNA sequence similarity against each other suggesting that these are the same strains and nearly identical sequences (99.9%) with *Streptomyces* sp. strain CNS-774_SD_06, previously isolated from the marine sediment off the coast of California. Remarkably, several studies have also shown that marine actinomycetes are in fact, abundant in various ocean sediments (Zobell and Upham 1944; Grein and Meyers 1958; Mincer et al. 2002; Fiedler et al. 2005; Jensen et al. 2005a; Maldonado et al. 2005; Fenical and Jensen 2006; Pathom-Aree et al. 2006; Bredholdt et al. 2007).

Thus, the results provided here illustrate further that indeed diverse genera, including taxonomically novel actinomycetes are associated with marine sponges. However, the type of association of actinomycetes with marine sponges and their functions were not the main aims of this Ph.D. study but rather, their potential to produce bioactive secondary metabolites which shall be discussed in the succeeding chapters.

2.4 References

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Chapter 3
Secondary metabolites from sponge-associated actinomycetes

3.1 Materials and methods

3.1.1 Preliminary bioactivity testing by disk diffusion

The cultivated actinomycete strains were initially screened for bioactivity using a disk diffusion assay against the following test organisms: *Staphylococcus epidermidis* RP62A, *Staphylococcus aureus* NCTC 8325, *Pseudomonas aeruginosa, Enterococcus faecalis* JH212, *Escherichia coli* 536 and *Candida albicans.* Strains were grown in M1 culture broths (50 ml) as well as on agar plates (10 plates each) at 30°C for 7-14 days depending on the growth period of the strains. An equal volume of methanol was added to the liquid cultures to effectively lyse the cells and release intracellular metabolites. A volume of 300μl was impregnated on sterile 13-mm disks and the disks were air-dried. Previously prepared LB (for bacteria) and YPD (for *C. albicans*) plates were uniformly inoculated with a lawn of the test bacterial strains on the agar surface. The disks were then pressed lightly on the agar surface in equidistant positions and incubated at 37°C overnight. Zones of inhibition were noted by measuring the diameter (in mm) of the clearing zones around the disks. As for the strains grown on agar plates, the colonies growing on the agar surface together with the rest of the solid media were cut into small pieces and these were macerated separately in methanol and ethyl acetate overnight. The resulting extracts were filtered and then dried using a rotary evaporator (Heidolph, Germany), weighed and re-dissolved in their original extracting solvent to make a 20 mg/ml stock solution. A volume of 300μl was impregnated on sterile disks and the same process of disk diffusion assay described previously was followed.

3.1.2 Bioassay-guided isolation and purification

Strains that were found to produce bioactive metabolites from the preliminary antimicrobial assays were cultured on large scale (2-5 L) using the same media, growth conditions and cell lysis with methanol. Ethyl acetate was used as the extracting solvent and the resulting extracts were dried in vacuo using a rotary evaporator (Heidolph, Germany). The crude extracts were subjected to pre-fractionation with Diaion HP-20ss resin (Mitsubishi Chemical Corporation, Japan) using the following eluents: water (FW); 25% isopropanol: 75% water (F1); 50% isopropanol: 50% water (F2); 75% isopropanol: 25% water (F3); 100% methanol (F4), yielding five fractions with 15 ml each. These fractions were completely dried using a Savant speedvac concentrator (Thermo Scientific,

USA), re-dissolved and subjected to the disk diffusion assay at a dose of 200 μg/6-mm disk. HPLC analysis was performed on an Agilent 1100 series chromatography system (Agilent Technologies, USA) with a photodiode array detector to purify the active fractions. To determine the proper solvent gradient for purifying the fractions, analytical HPLC (Phenomenex Luna Analytical RP18e 4.6 x 250 mm) was first performed using H_2O (A) and CH_3OH (B) as the solvents and the following gradient: flow 1.0 ml/min; 0-5 min 10% B, 35-40 min 100% B. The solvent gradient was adjusted accordingly for improved resolution as well as separation of the peaks and the fractions were purified using semi-preparative HPLC (Phenomenex Luna SemiPrep RP18e 10 x 250 mm).

For *Saccharopolyspora cebuensis* strain SPE 10-1, 100 µl of the glycerol stock was inoculated in 3 x 100 ml of ISP 2 medium, incubated with shaking at 180 rpm at 30˚C for five days. An aliquot of 50 ml of the inoculum was transferred to 6 x 750 ml of fresh ISP 2 medium and the cultures were incubated further under the same conditions for 7 days. After incubation, methanol was added to each culture (1:2) with shaking at 20°C overnight. The resulting mixtures were filtered and twice extracted with half-volume of ethyl acetate. The ethyl acetate layers were separated and dried by rotary evaporation. The same procedure was repeated for *Streptomyces* sp. strain A188 except that the strain was grown in 1.8 L of M1 medium.

The following *Streptomyces* sp. strains 11, 22, 34 and T03 were grown on 100 M1 agar plates and incubated at 30°C for seven days. Mycelial mass together with the agar were cut into small pieces and macerated overnight with sufficient volume of ethyl acetate. The resulting solution was filtered and maceration with ethyl acetate was repeated. Both filtrates were combined and subsequently dried. The same procedure was done for *Streptomyces axinellae* strain Pol001, except that the strain was grown on MS (Hobbs et al. 1989) agar.

3.1.3 Structure elucidation of compounds

^{1}H, ^{13}C, HMBC, HSQC and COSY NMR spectra were recorded on a Varian Inova 500 or 600 MHz and Avance 400 MHz spectrometers. Accurate mass measurements were conducted on a Micromass Q-Tof Micro and microTOF mass spectrometers.

3.1.4 Anti-infective profiling of compounds

Pure compounds were dissolved in DMSO at 20mM concentration and were subjected to an anti-infective panel against bacteria (*Staphylococcus aureus* NCTC 8325,

Staphylococcus epidermidis RP62A, *Enterococcus faecalis* JH212, *Enterococcus faecium* 6413, *Escherichia coli* 536, *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis* 252 01A and *Yersinia pestis* KUMA), fungi (*Candida albicans*) and parasites (*Trypanosoma brucei brucei* 221*, Leishmania major*). Cytotoxity testing against J774.1 macrophages and 293T kidney epithelial cells was also done. Furthermore, inhibitions of selected proteases such as SARS-PL^{PRO} SARS-Coronavirus papain-like protease, SARS-M^{PRO} SARS-Coronavirus main protease, cathepsin B and L mammalian, rhodesain *Trypanosoma brucei rhodesiense* and falcipain-2 *Plasmodium falciparum* were tested. The anti-parasitic, cytotoxicity assays and protease inhibition tests as were performed by the SFB 630 collaboration partners: TP Z1 (T. Ölschläger and H. Bruhn, U. Würzburg) and TP A4 (T. Schirmeister, U. Würzburg), respectively.

3.1.4.1 Antimicrobial assays

Antibacterial assay

Bacterial (*Staphylococcus aureus* NCTC 8325, *Staphylococcus epidermidis* RP62A, *Enterococcus faecalis* JH212, *Enterococcus faecium* 6413, *Escherichia coli* 536, *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis* 252 01A and *Yersinia pestis* KUMA) strains were cultivated overnight at 37°C (30°C for *Yersinia* sp.) in LB medium in a shaking incubator. On the next day, the culture was diluted 1:100 in Müller-Hinton broth (23 g per liter; Fluka) and again cultivated until the cells reached the exponential growth phase. Approximately, 1 x 10^5 cells/ml were incubated with various concentrations of the compounds to make a final volume of 200 µl in a 96-well plate at 37°C for 18 h (at 30°C for 48 h for *Yersinia* sp.). The final concentration of DMSO was 0.8% in each well. After incubation, the optical density of the cultures was determined at 550 nm wavelength using an ELISA microplate reader with respect to the control without bacteria or fungi. The lowest concentration of the compound where no bacterial or fungal growth is detectable was determined as the minimal inhibitory concentration (MIC).

Antifungal assay

A colony of *Candida albicans* 5314 (ATCC 90028) was resuspended in 2 ml of 0.9% NaCl. Four microliters of this suspension was added to 2 ml of HR medium. This medium was prepared by adding 14.67 g HR Medium in 450 ml H_2O, followed by the addition of 1.0 g $NaHCO_3$ in 50 ml H_2O and subsequent incorporation of the following solutions: 420 ml of sodium phosphate (35.60 g $Na_2HPO_4{\cdot}2H2O$ per liter) and 80 ml of potassium dihydrogen phosphate (27.22 g KH_2PO_4 per liter, pH 7.2). The test compounds were diluted in various concentrations in 100 µl of medium in a 96-well microplate with final

DMSO concentration of 0.4%. One hundred microliters of the *Candida* suspension was added to each well followed by incubation at 37°C for 48 h. Optical density was measured at 530 nm with respect to a control well without cells. The minimal concentration of the compound where no growth is detectable was considered as the MIC value.

3.1.4.2 Anti-parasitic assays

Anti-Leishmania assay

Leishmania major promastigotes were seeded at a cell density of 1 x 10^7 cells/ml into 96-well plates in complete medium (RPMI with $NaHCO_3$, 10% FCS, 2mM glutamine, 10 mM Hepes pH 7.2, 100 U/ml penicillin, 50 μg/ml gentamicin, 50 μM 2-mercaptoethanol) without phenol red (200 μl), in the absence or presence of different concentrations of the compounds. These were then incubated for 24 h at 26°C, 5% CO2 and 95% humidity. Following the addition of 20 μl of Alamar Blue, the plates were incubated again and the optical densities (ODs) measured 24 and 48 h later with an enzyme-linked immunosorbent assay (ELISA) reader (Multiskan Ascent, Germany) using a test wavelength of 540 nm and a reference wavelength of 630 nm. Absorbance in the absence of compounds was set as 100% of growth. Amphotericin B was used as a reference compound and positive control. The effects of cell density, incubation time and the concentration of DMSO were examined in control experiments. The final concentration of DMSO in the medium never exceeded 1% vol/vol and had no effect on the proliferation of extracellular or intracellular parasites. For each experiment, each drug concentration was assayed in duplicate wells (Ponte-Sucre et al. 2006).

Anti-Trypanosoma assay

Trypomastigote forms of *Trypanosoma brucei brucei* laboratory strain TC 221 were cultured in complete Baltz medium [80 ml Baltz medium basic solution, 0.8 ml 2 mercaptoethanol stock solution (20 mM), 0.8 ml penicillin/streptomycin (10,000 U/ml), 16 ml FCS (inactivated for 30 min at 56°C). Baltz medium basic solution is composed of the following: 500 ml MEM with Earle's salts and L-glutamine, 3 g Hepes, 0.5 g monohydrate glucose, 0.110 g sodium pyruvate, 0.007 g hypoxanthine, 0.002 g thymidine, 0.0107 g adenosine, 0.0141 g bathocuproine disulfonic acid disodium salt, 0.146 g glutamine, 5 ml sterile non-essential amino acid concentrate (100x, pH 7.5) (Baltz et al. 1985). A defined number of parasites (10^4 trypanosomes per ml) were exposed in test chambers of 96-well plates to various concentrations of the test substances (previously dissolved in DMSO) to make a final volume of 200 µl in duplicates. Positive (trypanosomes in culture medium) and negative controls (test substance without trypanosomes) were run simultaneously

with each plate. The plates were then incubated at 37°C in an atmosphere of 5% CO_2 for a total time period of 72 h. After 24 h, 20 µl of Alamar Blue was added. The activity of the test substances was measured by light absorption using MR 700 Microplate Reader at a wavelength of 550 nm with a reference wavelength of 630 nm. The first reading was done at 48 h and subsequently at 72 h. The effect of the test substances was quantified in IC_{50} values by linear interpolation of three independent measurements (Huber and Koella 1993; Raz et al. 1997).

3.1.4.3 Cytotoxicity assays

J774.1 macrophages were cultured in complete medium (RPMI with $NaHCO_3$, 10% FCS, 2mM glutamine, 10 mM Hepes pH 7.2, 100 U/ml penicillin, 50 µg/ml gentamicin, 50 µM 2-mercaptoethanol) without phenol red in the absence or presence of increasing concentrations of the compounds at a cell density of 1 x 10^5 cells/ ml (200 µl) for 24 h at 37°C, 5% CO2 and 95% humidity. Following the addition of 20 µl of Alamar Blue, the plates were incubated and the ODs measured at 24, 48 and 72 h. The same Alamar blue assay previously described for *Leishmania* was followed. Kidney epithelial 293T cells were also tested in the same manner as the macrophages but using complete DMEM medium (4.5 g/l solution of DMEM high glucose solution with sodium pyruvate but without L-glutamine, FBS superior at final concentration of 20%, 200mM L-glutamine 100x) and cell density (2 x 10^4 cells/ ml).

3.1.4.4 Protease inhibition assays

SARS M^{pro} and Pl^{pro} protease inhibition assay

The fluorometric enzyme assays were performed on a Cary Eclipse fluorescence spectrophotometer (Varian, Darmstadt, Germany) using a microplate reader (excitation 325 nm, emission 425 nm). For the inhibition assays, 96-well microplates (Nunc GmbH, Wiesbaden, Germany) were used. Assays were performed at 25°C in a 20 mM Tris-HCl buffer pH 7.5, containing 0.1 mM EDTA, 1 mM DTT, 200 mM NaCl, and 12.5% DMSO (final concentration) in a total volume of 200 µl. The final substrate (H_2N-Abz-Ser-Val-Thr-Leu-Gln-Ser-Gly-(NO_2)Tyr-Arg-(MTS)-TFA-salt for M^{pro} and Z-Arg-Leu-Arg-Gly-Gly-AMC-acetate salt for Pl^{pro}) concentration for inhibition assays was 50 µM, and the final enzyme concentration was 4.25 µg/mL. Inhibitors were used at 100 µM final concentration for preliminary screening. For determination of *K*m values, the substrate was used in concentrations between 50 and 300 µM. Values were corrected for the inner filter effect. Fluorescence increase was measured over a period of 10 min for *K*m determination and 20 min for inhibition assays. Substrate and inhibitor stock solutions were prepared in

DMSO and were diluted with assay buffer, and the enzyme was dissolved in buffer. The *K*m value was calculated by nonlinear regression analyses using the program GraFit. All values were mean values from at least three independent assays (Kaeppler et al. 2005).

Cathepsin L and B protease inhibition assay

Assays were performed at 25°C in a 20 mM Tris-HCl buffer pH 6.0, containing 5 mm EDTA, 2.5 mm DTT, 200 mm NaCl, 0.005% Brij 35 in a total volume of 285 mL. Substrate (Cbz-Phe-Arg-AMC for both enzymes) and inhibitor stock solutions were prepared in DMSO (10% final concentration) and were diluted with assay buffer. The final substrate concentration for the inhibition assays was between 10.0 and 81.0 mm. The final enzyme concentration was 53 ng/mL for cathepsin L (*P. tetraurelia*) and 58 pg/mL for cathepsin B (recombinant, human liver) (Vicik et al. 2006a). Inhibitors were tested at 100 µM (final concentration). The fluorometric enzyme assays were performed on a Cary Eclipse fluorescence spectrophotometer (Varian, Darmstadt, Germany) using a microplate reader (excitation 365 nm, emission 460 nm).

Rhodesain protease inhibition assay

For inhibition of rhodesain, 96-well microplates were used. Assays were performed at 25°C in 50 mM acetate, pH 5.5, containing 5 mM EDTA, 5 mM DTT, 200 mM NaCl, and 0.005% Brij 35 in a total volume of 285 µL. Substrate (Cbz-Phe-Arg-AMC) and inhibitor stock solutions were prepared in DMSO (10% final concentration) and diluted with the assay buffer. The final substrate concentrations used in the inhibition assays ranged from 12.4 to 81.0 µM and the final enzyme concentration was 41 nM. Inhibitors were used at a concentration of 100 µM (Vicik et al. 2006b). The fluorometric enzyme assay was performed in a Cary Eclipse fluorescence spectrophotometer (Varian, Darmstadt, Germany) using a microplate reader (excitation 365 nm, emission 460 nm).

Falcipain protease inhibition assay

The enzyme assay with the cysteine protease Cbz-Phe-Arg-AMC (falcipain-2, 50 µm) as substrate was performed. The enzyme was incubated with 100 µM concentration of the compounds for 0, 15 or 30 min prior to substrate addition. Inhibitor solutions were prepared from stocks in DMSO. Each assay was performed in 96-well plates in a total volume of 120 or 300 µl (n = 2-6 independent assays) with standard deviations of <10%. The following buffer was used: 100 mM acetate, pH 5.5, 10 mM DTT. The Cary Eclipse fluorescence spectrophotometer (Varian, Darmstadt, Germany) with a microplate reader (excitation 365 nm, emission 460 nm) was used to measure fluorescence.

3.2 Results

3.2.1 Isolation and characterization of metabolites from actinomycete strains

3.2.1.1 Novel macrolactam polyketides from *Saccharopolyspora cebuensis* strain SPE 10-1^T

The crude extract obtained from solvent partitioning with ethyl acetate was purified by preparative HPLC (Merck Chromolith SemiPrep RP18e 10 x 100 mm) using H_2O + 0.05% TFA (A) and CH_3CN + 0.05% TFA (B) as the solvents and the following gradient: flow 10 ml/min; 0 min 75% B, 10 min 75% B. Two novel, constitutionally identical macrolactams (Fig. 3.1) named as cebulactam A1 (5.1 mg; R_t = 2.5 min) and cebulactam A2 (4.1 mg; R_t = 4.3 min) were identified. Their constitutionally identical structures, each bearing a 6-membered cyclic ether as part of the macrocycle and their relative configurations were elucidated by MS methods and by 1D and 2D NMR techniques (Tables 3.1 and 3.2). HPLC-ESI-MS analysis revealed the molecular masses of both compounds to be identical (*m/z* 346.3 for $[M+H]^+$), suggesting the two compounds to be structurally highly similar to each other. These assumptions were further corroborated by HR-ESIMS (TOF) measurements which clearly showed an identical molecular formula of $C_{19}H_{23}NNaO_5$ (*m/z* 368.1478 for $[M+Na]^+$, calculated 368.1468) for cebulactams A1 and A2 (Gulder 2008; Pimentel-Elardo et al. 2008). The relative configurations of the stereocenters of cabulactams A1 and A2 were elucidated by NOESY NMR spectroscopy. This work was done in collaboration with T.A.M. Gulder (AG Bringmann, Institute of Organic Chemistry, University of Würzburg). The absolute configurations of both compounds have yet to be elucidated.

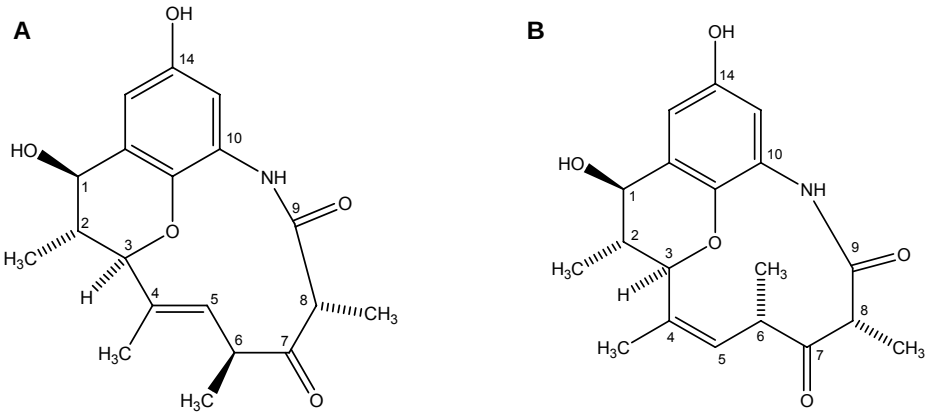

Figure 3.1 Cebulactam A1 (A) and cebulactam A2 (B) polyketides isolated from strain SPE 10-1^T

Table 3.1 NMR spectroscopic data of cebulactam A1 acetone-d_6 (^{1}H: 400 MHz; ^{13}C: 100 MHz)*

Position	δ_C	δ_H, mult (*J* in Hz)	COSY	HMBC
1	70.9	4.38, d (10.1)	2	2, 2-CH_3, 3, 11, 12, 13
2	42.4	1.82, m	2-CH_3, 1, 3	1, 2-CH_3, 3, 12
2-CH_3	16.7	1.19, d (6.6)	2	1, 2, 3
3	87.4	4.27, d (8.5)	2	2, 2-CH_3, 4, 4-CH_3, 5, 11
4	140.5			
4-CH_3	19.3	1.86, s	5	3, 4, 5, 6-CH_3, 7
5	124.7	4.98, d (9.8)	4-CH_3, 6	3, 4-CH_3, 6, 7
6	46.2	3.19, m	6-CH_3, 5	4, 5, 6-CH_3, 6, 7
6-CH_3	17.8	1.00, d (6.3)	6	5, 6, 7
7	207.1			
8	48.2	3.43, q (6.8)	8-CH_3	7, 8-CH_3, 9
8-CH_3	16.6	1.21, d (6.8)	8	7, 8, 9
9	173.9			
10	131.0			
11	142.7			
12	140.9			
13	111.1	7.02, d (2.8)		1, 11, 14, 15
14	154.4			
15	114.0	6.65, d (2.8)		10, 11, 13, 14
NH		7.88, br		

*(Gulder 2008; Pimentel-Elardo et al. 2008)

Table 3.2 NMR spectroscopic data of cebulactam A2 in acetone-d_6 (^{1}H: 400 MHz; ^{13}C: 100 MHz)*

Position	δ_C	δ_H, mult (*J* in Hz)	COSY	HMBC
1	70.8	4.36, d (10.1)	2	2, 2-CH_3, 3, 11, 12, 13
2	46.2	1.51, m	2-CH_3, 1, 3	1, 2-CH_3, 3, 12
2- CH_3	15.0	1.15, d (6.6)	2	1, 2, 3
3	86.2	4.24, d (10.0)	2	2, 2-CH_3, 4, 4-CH_3, 5, 11
4	145.4			
4-CH_3	19.8	2.02, s	5	3, 4, 5
5	125.1	5.07, d (10.5)	4-CH_3, 6	3, 4-CH_3, 6, 7
6	48.3	3.34, m	6-CH_3, 5	4, 5, 6-CH_3, 6, 7
6-CH_3	16.9	0.99, d (6.8)	6	5, 6, 7
7	205.2			
8	55.6	3.97, q (6.7)	8-CH_3	7, 8-CH_3, 9
8-CH_3	15.1	1.27, d (6.7)	8	7, 8, 9
9	173.3			
10	130.4			
11	139.5			
12	138.1			
13	107.3	6.76, d (2.8)		1, 11, 14, 15
14	153.4			
15	110.9	6.83, d (2.8)		10, 11, 13, 14
NH		8.61, br		

*(Gulder 2008; Pimentel-Elardo et al. 2008)

Isolation and structure elucidation of the following compounds (3.2.1.2 to 3.2.1.6) were performed at the Ireland Research Lab, Department of Medicinal Chemistry, University of Utah.

3.2.1.2 Novel tetromycin compounds from *Streptomyces axinellae* strain Pol001^T

The crude extract obtained from maceration of the mycelial mass with ethyl acetate was purified by preparative HPLC (Phenomenex Luna SemiPrep RP18e 10 x 250 mm) using H_2O + 0.1% TFA (A) and CH_3CN (B) as the solvents and the following gradient: flow 4.5 ml/min; 0-10 min 90% B, 11-15 min 100% B. The following compounds were isolated (Fig. 3.2): Pol001-1 (2.7 mg; R_t = 5.938 min); Pol001-3 (4.4 mg; R_t = 7.746 min); Pol001-4 (2.2 mg; R_t = 9.666 min); Pol001-5 (4.4 mg; R_t = 12.240 min); and Pol001-7 (2.1 mg; R_t = 17.468 min).

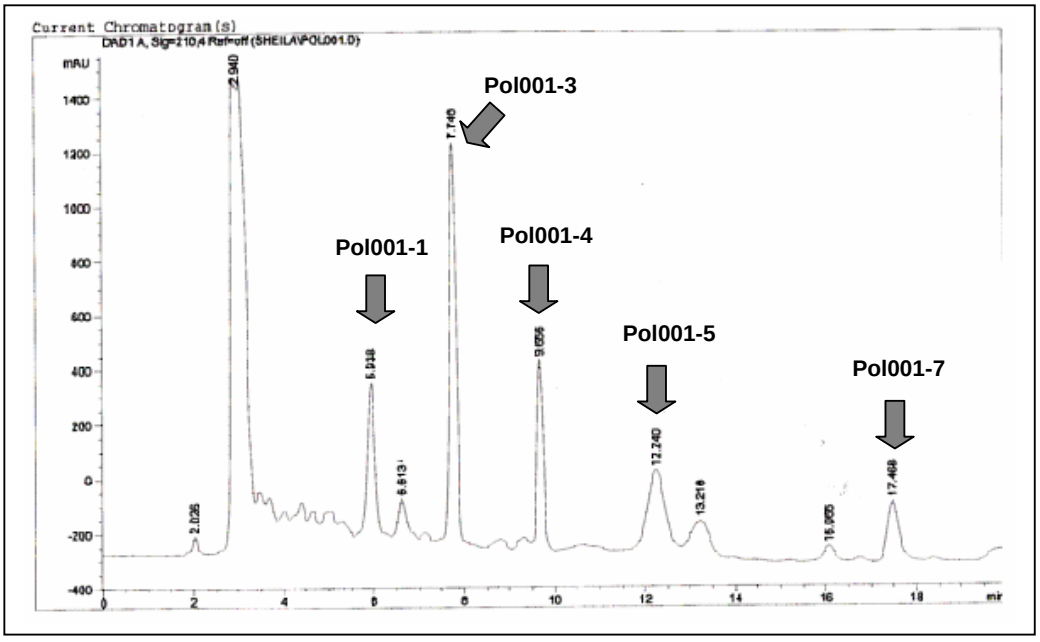

Figure 3.2 HPLC chromatogram of EtOAc extract from *Streptomyces axinellae* strain Pol001^T

Molecular formulas for each of the compounds were established using high-resolution mass spectrometry as follows: Pol001-1 = $C_{50}H_{65}NO_{13}$ (*m/z* 910.4388 for $[M+Na]^+$, calculated 910.4354); Pol001-3 = $C_{50}H_{64}O_{14}$ (*m/z* 911.4232 for $[M+H]^+$, calculated 911.4219); Pol001-4 = $C_{48}H_{60}O_{14}$ (*m/z* 861.4062 for $[M+H]^+$, calculated 861.4062); Pol001-5 = $C_{49}H_{62}O_{14}$ (*m/z* 897.4066 for $[M+Na]^+$, calculated 897.4037); and Pol001-7 = $C_{34}H_{46}O_5$ (*m/z* 557.3229 for $[M+Na]^+$, calculated 557.3243).

Furthermore, the combination of NMR (1D and 2D), and comparison of the spectral data with the database using the SciFinder Scholar tool revealed that the compounds (Pol001-1, Pol001-5, Pol001-7) are identical to the published family of tetromycin compounds previously isolated from *Streptomyces* sp. strain MK67-CF9 (Takeuchi et al. 1989) (Fig. 3.3).

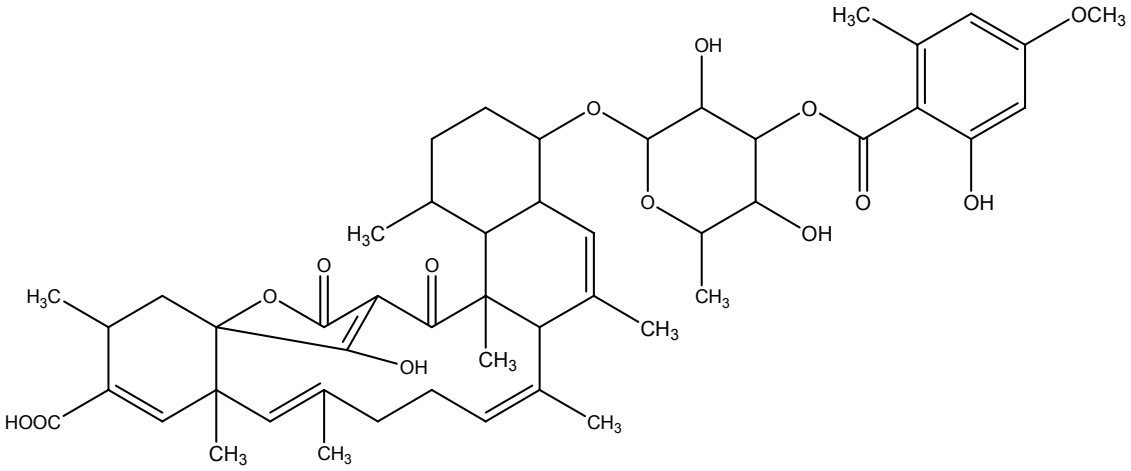

Fig. 3.3 Tetromycin from *Streptomyces* sp. strain MK67-CF9 (Takeuchi et al. 1989)

Interestingly, mass and NMR data of Pol001-3 and Pol001-4 suggest that these compounds are new derivatives of tetromycin. Partial structures (Fig. 3.4, 3.5, 3.6) were elucidated revealing a difference at position 10: hydroxyl function for Pol001-4 (Fig. 3.4) and a methoxy group for Pol001-3 (Fig. 3.5). Furthermore, the additional novelty of these structures is postulated to be found in the decalin ring of the tetromycins (Fig. 3.3) with the difference in the number or position of the methyl groups. Complete elucidation of the structures was not yet possible due to some ambiguous chemical shift assignments in some positions. Nevertheless, preliminary structure analyses still suggest that *Streptomyces axinellae* strain Pol001^T produced novel tetromycin derivatives. Further NMR analysis should therefore be carried out in order to confirm and complete the structure elucidation of these interesting compounds. Stereochemical analysis is also worthwhile to pursue since relative or absolute configurations of the stereocenters in tetromycins have not been reported for any of the compounds.

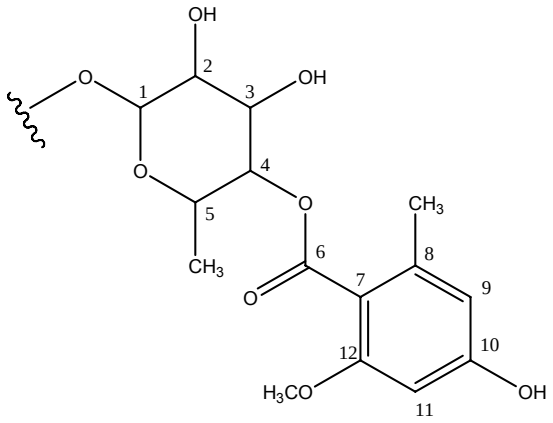

Figure 3.4 Pol001-4 partial structure A

Table 3.3 NMR spectroscopic data of Pol001-4 partial structure A in acetone-d_6 (^{1}H: 500 MHz; ^{13}C: 125 MHz)

Position	δ_C	δ_H, mult (*J* in Hz)	COSY	HMBC
1	102.0	4.80, d (7.9)	2	2
2	71.1	3.54, dd (7.9, 2.9)	1	3
3	68.8	4.38, t (2.9)	4	3
4	76.0	4.75, dd (10.0, 2.9)	3	4
5	66.4	4.16, dq (10.0, 6.3)	5-CH_3	3, 5-CH_3
5-CH_3	17.5	1.25, d (6.3)	5	
6	170.2			4
7	105.3			11, 8-CH_3
8	143.7			8-CH_3
8-CH_3	23.6	2.55, s		
9	110.8	6.37, d (2.1)		8-CH_3
10	165.2			11
11	98.7	6.34, d (2.1)		
12	164.3			
12-OCH_3	54.9	3.83, s		12

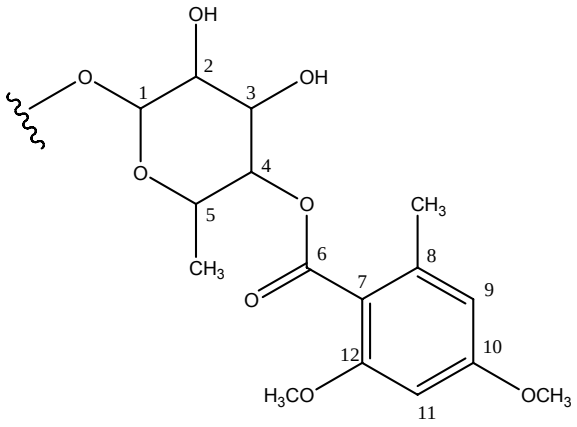

Figure 3.5 Pol001-3 partial structure A

Table 3.4 NMR spectroscopic data of Pol001-3 partial structure A in acetone-d_6 (^{1}H: 500 MHz; ^{13}C: 125 MHz)

Position	δ_C	δ_H, mult	COSY	HMBC
1	102.4	4.79,d	2	
2	71.4	3.54, dd	1	
3	69.5	4.34, t		5-CH_3
4	75.0	4.69, dd	5	5-CH_3
5	66.6	4.03, dq	4, 5-CH_3	5-CH_3
5-CH_3	17.2	1.26, d	5	
6	170.0			
7	106.9			8-CH_3, 11
8	143.0			8-CH_3
8-CH_3	23.8	2.56, s		8
9	110.8	6.37, d		8-CH_3, 11
10	161.6			10-OCH_3
10-OCH_3	54.9	3.84, s		
11	96.0	6.46, d		9
12	158.0			12-OCH_3
12-OCH_3	55.4	3.83, s		

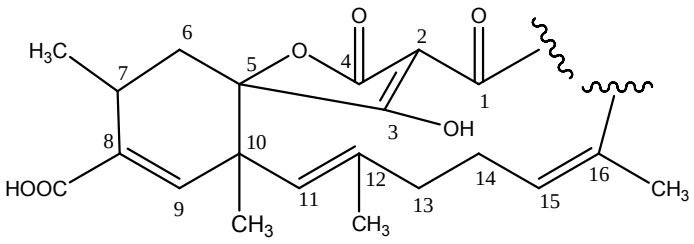

Figure 3.6 Pol001-4 and Pol001-3 partial structure B

Table 3.5 NMR spectroscopic data of partial structure B of Pol001-4 and Pol001-3 in acetone-d_6 (^{1}H: 500 MHz; ^{13}C: 125 MHz)

	Pol001-4		Pol001-3	
Position	δ_C	δ_H, mult	δ_C	δ_H, mult
1	201.0		200.0	
2	135.0		138.0	
3	206.0		205.0	
4	166.6		168.0	
5	81.8		84.6	
6	34.9	2.29, br; 1.77, br	33.6	2.26, br; 1.67, br
7	27.4	2.86, br	27.0	2.94, br
7-CH_3	20.2	1.31, d	20.9	1.32, d
8	120.9		119.8	
8-COOH	166.7		167.0	
9	138.8	6.67, s	147.6	6.93, s
10	68.8		68.0	
10-CH_3	22.6	1.51, s	23.3	1.48, s
11	122.0	6.15, s	122.1	6.18, s
12	124.6		116.5	
12-CH_3	22.4	1.49, s	24.8	1.47, s
13	24.6	2.23, br; 1.93, br	24.6	2.23, br; 1.92, br
14	24.8	2.26, br; 1.95, br	24.8	2.24, br; 1.94, br
15	130.0	5.06, d	130.5	5.14, d
16	135.0		134.9	
16-CH_3	13.1	1.38, s	12.4	1.35, s

3.2.1.3 Valinomycin from *Streptomyces* sp. strains 34 and 22

The crude ethyl extract obtained from maceration of the mycelial mass of *Streptomyces* sp. strain 34 with ethyl acetate was found to exhibit activities (zones of inhibition in mm, 600 µg/ 6-mm disk) against the following organisms: *Staphylococcus aureus* (20 mm), *Staphylococcus epidermidis* (23 mm), and *Escherichia coli* (8 mm). No activities were found against *Pseudomonas aeruginosa* and *Candida albicans*. The ethyl acetate extract was further pre-fractionated using Diaion HP-20ss resin. Out of the five fractions obtained (FW, F1, F2, F3, F4), secondary antibacterial assay against *Staphylococcus aureus* and *Escherichia coli* revealed that fractions F2 and F3 were found to exhibit activity (200 µg/ 6-mm disk) against *S. aureus* with zones of inhibition values of 9 and 8 mm, respectively. All other Diaion fractions were not active against *E. coli*. HPLC chromatograms of both fractions also revealed the same profile. Thus, fraction 11-F3 (20 mg) was further purified by HPLC (Phenomenex Luna SemiPrep RP18e 10 x 250 mm) using H_2O (A) and CH_3OH (B) as the solvents and the following gradient: flow 4.5 ml/min; 0-5 min 90% B, 11-15 min 100% B. The major peak, 34-F3-3 (8.4 mg; R_t = 13.085 min) (Fig. 3.7) yielded the cyclic peptide, valinomycin (Fig. 3.8; Table 3.6).

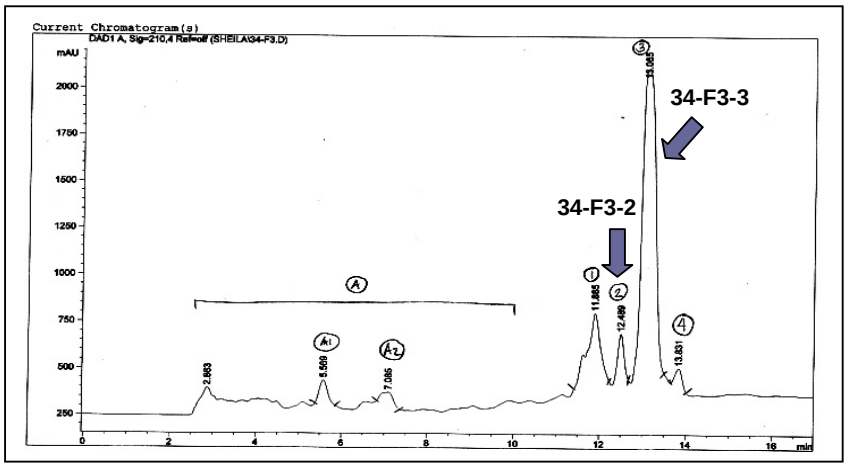

Fig. 3.7 HPLC chromatogram of fraction 34-F3 from *Streptomyces* sp. strain 34

High-resolution mass spectrometry of the purified compound established a molecular formula of $C_{54}H_{90}N_6NaO_{18}$ (*m/z* 1133.6385 for $[M+Na]^+$, calculated 1133.6394). A combination of NMR (1D and 2D), MS-MS fragmentation and comparison of the spectral data with the database, Dictionary of Natural Products (www.chemnetbase.com) suggested that 34-F3-3 contains one α-hydroxyisovaleryl unit (Hiv), a lactoyl group (Lac), and two valines (Val), thus confirming the identity of the compound as valinomycin. However, the stereochemical properties of this compound were not determined.

Furthermore, the structure was also confirmed by comparison of the ^{1}H NMR and ^{13}C NMR data with published data (Brockmann and Schmidt-Kastner 1955; Heisey et al. 1988).

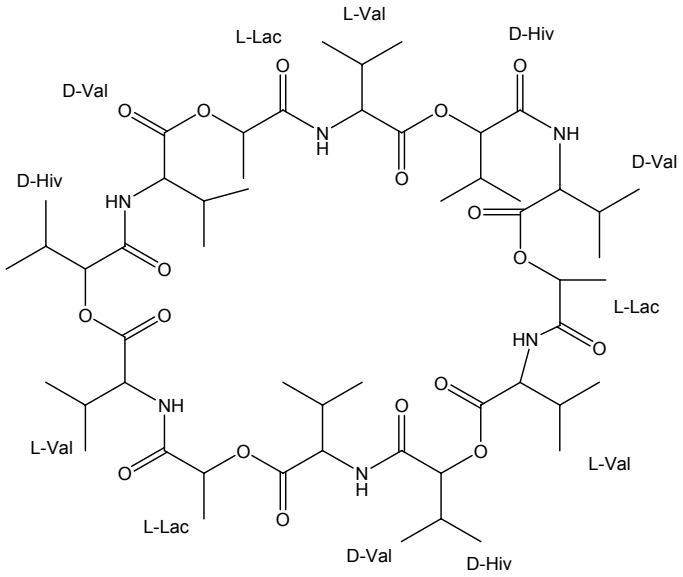

Fig. 3.8 Chemical structure of 34-F3-3 (valinomycin)

Table 3.6 NMR spectroscopic data of 34-F3-3 (valinomycin) in $CDCl_3$ (^{1}H: 500 MHz; ^{13}C: 125 MHz)

Unit	Position	δ_C	δ_H, mult	COSY	HMBC
	1-CO	171.0			2
	2-CH	78.8	5.02, d	3	4, 5
Hiv	3-CH	28.8	2.30, m	4, 5	4, 5
	4-CH_3	22.9	0.85, d		
	5- CH_3	22.8	0.86, d		
	6-CO	170.4			
	7-CH	59.1	4.10, t	8	9, 10
Val	8-CH	28.6	2.25, m	9, 10	9, 10
	9-CH_3	19.5	0.96, d		
	10-CH_3	16.8	0.97, d		
	NH		7.85, d	7	
	11-CO	172.6			13
Lac	12-CH	70.4	5.30, q	13	13
	13-CH_3	17.2	1.46, d		12
	14-CO	171.9			15
	15-CH	60.5	4.00, t	16	17, 18
Val	16-CH	27.4	2.04, m	17, 18	17, 18
	17-CH_3	19.2	0.98, d		
	18-CH_3	18.4	0.99, d		
	NH		7.73, d	15	

This cyclodepsipeptide contains a three-repeat sequence of a tetradepsipeptide basic unit, D-α-hydroxyisovaleryl-D-valyl-L-lactoyl-L-valyl to form a symmetric 36-membered ring molecule. Interestingly, the same compound was isolated from *Streptomyces* sp. strain 22. The fractions in fact, obtained from this strain exhibited exactly the same chromatographic and NMR profiles suggesting that *Streptomyces* sp. strains 22 and 34 produced exactly the same metabolites when cultivated under the same conditions. This is not surprising since a close look at their 16S rDNA sequence revealed that they exhibited 99.93% sequence similarity (1409/1410) with only one nucleotide difference indicating that these are the same strains regardless of the fact that these were isolated from different sponge sources (*Axinella polypoides* for strain 34 and *Aplysina aerophoba* for strain 22).

A derivative of valinomycin was also detected in the fraction 34-F3-2 (1.5 mg; R_t = 12.489 min) (Fig.3.7) with a molecular formula of $C_{53}H_{88}N_6NaO_{18}$ (*m/z* 1119.6064 for $[M+Na]^+$, calculated 1119.6053). This could possibly be a new analog of valinomycin exhibiting a difference of a CH_2 unit but complete spectroscopic analysis should be done to confirm the structure of this potentially novel compound.

3.2.1.4 Staurosporine from *Streptomyces* sp. strain 11

Preliminary disk diffusion assay of the crude ethyl extract obtained from maceration of the mycelial mass of *Streptomyces* sp. strain 11 with ethyl acetate revealed the following activities (zones of inhibition in mm, 600 µg/ 6-mm disk) against the following organisms: *Staphylococcus epidermidis* (8 mm), and *Candida albicans* (9 mm). No activities were found against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*. The ethyl acetate extract was further pre-fractionated using Diaion HP-20ss resin. For a bioassay-guided isolation, the five fractions obtained (FW, F1, F2, F3, F4) were subjected to secondary antibacterial assay. Fractions F2 and F3 were found to exhibit activity (200 µg/ 6-mm disk) against *S. aureus* (7 mm) and no activity against *Escherichia coli*. ^1H NMR of both fractions revealed the same profile. Fraction 11-F3 (13.9 mg) was then further purified by HPLC (Phenomenex Luna SemiPrep RP18e 10 x 250 mm) using H_2O + 0.1% TFA (A) and CH_3OH (B) as the solvents and the following gradient: flow 4.5 ml/min; 0-5 min 70% B, 10 min 80% B, 20-25 min 100% B.

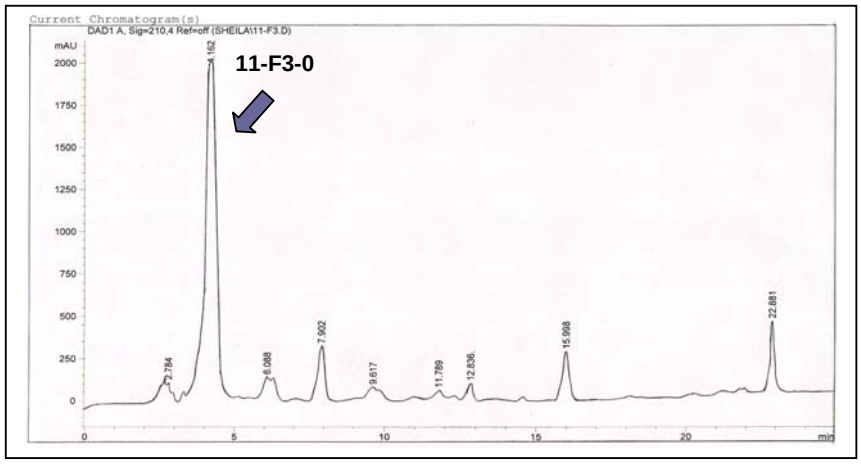

Fig. 3.9 HPLC chromatogram of fraction 11-F3 from *Streptomyces* sp. strain 11

The major peak, 11-F3-0 (1.4 mg; R_t = 4.162 min) (Fig. 3.9) yielded the indolocarbazole, staurosporine (Fig. 3.10). This was confirmed by mass spectrometry revealing the compound to have a molecular formula of $C_{28}H_{26}N_4O_3$ (*m/z* 467.2078 for $[M+H]^+$, calculated 467.2083) coupled with NMR (1D and 2D) analysis (Table 3.7) and comparison of the spectral data with the database, Dictionary of Natural Products (www.chemnetbase.com). Furthermore, the structure was also confirmed by comparison of the ^1H NMR and ^{13}C NMR data with published data (Meksuriyen and Cordell 1988; Schupp et al. 1999). However, the stereochemical properties of this compound were not determined.

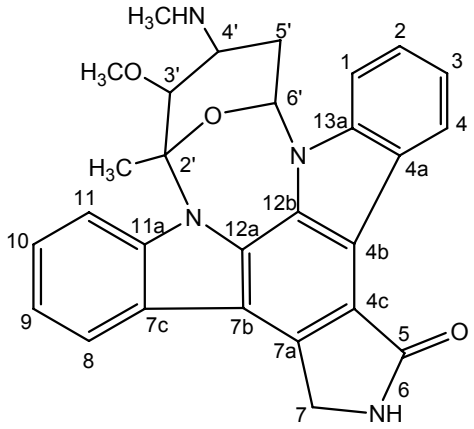

Fig. 3.10 Chemical structure of 11-F3-0 (staurosporine)

Table 3.7 NMR spectroscopic data of 11-F3-0 (staurosporine) in CD_3OD (^1H: 500 MHz; ^{13}C: 125 MHz)

Position	δ_C	δ_H, (H, mult)	COSY	HMBC
1	109.3	7.16 (1H, d)	2	4
2	126.5	7.52 (1H, t)	3	4
3	126.6	7.41 (1H, t)	2	1
4	127.2	9.22 (1H, d)	3	1, 4a, 12b, 13a
4a	127.6			4
4b	115.8			4
4c	120.2			7A, 7B
5	175.1			7A, 7B
7	46.8	7A: 4.73 (1H) 7B: 4.44 1H)		4b, 4c, 5, 7a
7a	133.8			7A, 7B
7b	115.8			
7c	124.5			11
8	122.7	7.81 (1H, d)	9	9
9	120.7	7.26 (1H, t)	10	
10	122.0	7.38 (1H, t)	9	11
11	113.4	7.96 (1H, d)	10	2'-CH_3
11a	139.4			11
12a	131.4			7A, 7B
12b	125.9			6'
13a	137.7			4
2'	94.2			2'-CH_3
3'	81.4	4.21 (1H, s)	4'	2', 2'-CH_3, 3', 4'
4'	55.9	3.90 (1H, dd)	3', 5'A, 5'B	3', 4'-NCH_3
5'	28.8	5'A: 2.13 (1H) 5'B: 3.20 (1H)	4', 6'	6'
6'	82.0	6.44 (1H, dd)	5'A, 5'B	
2'-CH_3	28.7	2.53 (3H, s)		2', 6', 11
3'-OCH_3	60.6	2.14 (3H, s)		3'
4'-NCH_3	31.3	2.78 (3H, s)		4'

3.2.1.5 Cycloisoleucylprolyl from *Streptomyces* sp. strain A188

Initial bioassay testing of the metabolites produced by *Streptomyces* sp. strain A188 after growing in M1 broth indicated activity against *Staphylococcus aureus* (300 µl/ 13-mm disk) with a zone of inhibition of 26 mm. No activity was found against *Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans.* The crude extract obtained from solvent partitioning with ethyl acetate was pre-fractioned with Diaion HP-20ss. The fraction F1 was found to exhibit antibacterial activities against *S. aureus* and *E. coli* with zones of inhibition (200 µg/ 6-mm disk) of 1.0 and 2.2 mm, respectively. Thus, fraction A188-F1 (22 mg) was further purified by HPLC (Phenomenex Luna SemiPrep RP18e 10 x 250 mm) using H_2O (A) and CH_3OH (B) as the solvents and the following gradient: flow 4.5 ml/min; 0-5 min 15% B, 25 min 60% B, 30-35 min 100% B.

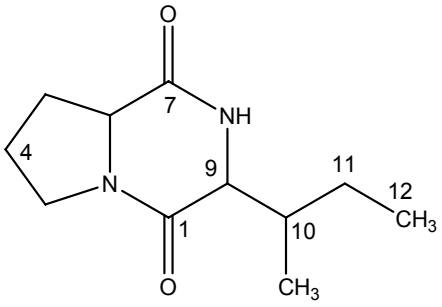

Fig. 3.11 Chemical structure of A188-F1-3 (cycloisoleucylprolyl)

The fraction A188-F1-3 obtained from HPLC yielded the diketopiperazine, cycloisoleucylprolyl (Fig 3.11) (2.2 mg; R_t = 20.295 min). Mass analysis revealed a molecular formula of $C_{11}H_{18}N_2NaO_2$ (*m/z* 233.1262 for $[M+Na]^+$, calculated 233.1266). The structure was confirmed by comparison of NMR (1D and 2D) analysis (Table 3.8) with published spectral data of the compound (Adamczeski et al. 1995; Fdhila et al. 2003) but the stereochemical properties of this compound were not further investigated.

Table 3.8 NMR spectroscopic data of A188-F1-3 (cycloisoleucylprolyl) in CD_3OD (^1H: 500 MHz; ^{13}C: 125 MHz)

Position	δ_C	δ_H, (H, mult)	COSY	HMBC
1	167.3			11
3	46.4	3A: 3.50 (1H, m)	4	
		3B: 3.51 (1H, m)		
4	25.8	4A: 1.94 (1H, m)	3	
		4B: 2.00 (1H, m)		3
5	29.1	5A: 2.02 (1H, m)	5B, 6	3
		5B: 2.30 (1H, m)	5A, 6	
6	60.3	4.26 (1H, t)	5A, 5B	
7	167.8			
9	54.6	4.13 (1H, m)	10	11
10	23.6	1.89 (1H, m)		11B, 10-CH_3
11	39.4	11A: 1.91 (1H, m)		10-CH_3
		11B: 1.53 (1H, m)	11A	9, 12
12	22.2	0.96 (3H, d)	11A	11B
10-CH_3	23.3	0.97 (3H, d)	10	12

3.2.1.6 Butenolide from *Streptomyces* sp. strain T03

The crude ethyl extract obtained from maceration of the mycelial biomass of *Streptomyces* sp. strain T03 was further fractioned with Diaion HP-20ss resin. Fraction T03-F1 which was found to be active against *Staphylococcus aureus* with a zone of inhibition (200 µg/ 6-mm disk) was further purified by HPLC (Phenomenex Luna SemiPrep RP18e 10 x 250 mm) using H_2O (A) and CH_3OH (B) as the solvents and the following gradient: flow 4.5 ml/min; 0-5 min 40% B, 25 min 60% B, 15 min 70% B, 20 min 85% B, 21-26 min 100% B. The fraction T03-F1-2A yielded a compound (1.0 mg; R_t = 17.168 min) with a molecular formula of $C_{13}H_{22}O_3Na$ (*m/z* 249.1447 for $[M+Na]^+$, calculated 249.1467). NMR analysis (Table 3.9) confirmed the compound to be a butenolide (Fig. 3.12), exhibiting identical spectral data with published literature (Mukku et al. 2000; Cho et al. 2001). However, the stereochemical properties of this compound were not determined.

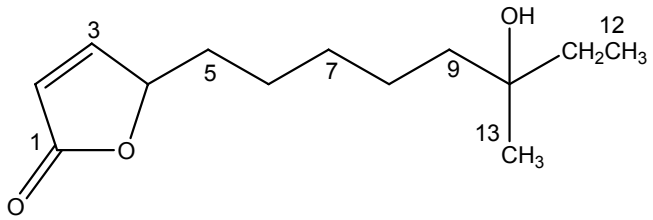

Fig. 3.12 Chemical structure of T03-F1-2A (butenolide)

Table 3.9 NMR spectroscopic data of T03-F1-2A (butenolide) in CD_3OD (^1H: 500 MHz; ^{13}C: 125 MHz)

Position	δ_C	δ_H, (H, mult)	COSY	HMBC
1	175.8			2
2	121.6	6.11 (1H, ddd)		3
3	159.6	7.71 (1H, ddd)	2, 4	2, 5A
4	85.6	5.13 (1H, m)	2, 5A	2, 3, 5A
5	34.9	5A: 1.81 (1H, m)	4, 6	
		5B: 1.64 (1H, m)		
6	25.8	1.45 (2H, m)	5A	
7	30.7	1.35 (2H, m)		5A, 6
8	24.3	1.34 (2H, m)		9
9	42.0	1.42 (2H, m)		11
10	72.2			11, 12, 13
11	41.1	1.46 (2H, q)	12	12, 13
12	8.5	0.89 (3H, t)		11
13	25.9	1.11 (3H, s)		11

3.2.2 Bioactivity profile of compounds

3.2.2.1 Antimicrobial activity

The compounds were subjected to a panel of antimicrobial tests to determine the minimum inhibitory concentration against various Gram-positive and Gram-negative bacteria as well as fungi (Table 3.10). Among the compounds tested, only four exhibited antibacterial activities as follows: the novel tetromycin derivative Pol001-3 exhibited MIC values of 100 µM and 50 µM against *S. aureus* and *S. epidermidis*, respectively; Pol001-4 exhibited slight activity with MIC value of 200 µM against these two bacteria; Pol001-7 exhibited MIC of 200 and 100 µM against *S. aureus, S. epidermidis* respectively; and staurosporine against *S. aureus* with MIC of 100 µM. The novel derivative Pol001-4 was the only compound that exhibited slight activity against *E. faecalis* (MIC of 200 µM). Valinomycin and staurosporine were the only compounds that exhibited significant anti-*Candida* activity with MIC values of 0.63 and 0.31 µM, respectively.

Surprisingly, neither the compounds cebulactam A1 and cebulactam A2 nor the combination of these 2 compounds exhibited any antimicrobial activities, despite the fact that the crude ethyl acetate extract showed antibacterial activity from the preliminary screen. The other tetromycin compounds Pol001-1 and Pol001-5 as well as butenolide also did not show any activity. The compound cycloisoleucylprolyl was not tested due to the lack of material.

Table 3.10 Antimicrobial activity testing of compounds (Minimum inhibitory concentration, µM)

Compound	Sa	Se	Efa	Efe	Ec	Pa	Yps	Ype	Ca
Cebulactam A1	>160	>160	>160	>160	>160	>160	>160	>160	>80
Cebulactam A2	>160	>160	>160	>160	>160	>160	>160	>160	>80
Cebulactam A1+A2	>160	>160	>160	>160	>160	>160	>160	>160	>80
Pol001-1	>200	>200	>200	nd	>200	>200	nd	nd	>80
Pol001-3	**100**	**50**	>200	nd	>200	>200	nd	nd	>80
Pol001-4	**200**	**200**	**200**	nd	>200	>200	nd	nd	>80
Pol001-5	>200	>200	>200	nd	>200	>200	nd	nd	>80
Pol001-7	**200**	**100**	>200	nd	>200	>200	nd	nd	>80
Valinomycin	>200	>200	>200	nd	>200	>200	nd	nd	**0.63**
Staurosporine	**100**	>200	>200	nd	>200	>200	nd	nd	**0.31**
Butenolide	>200	>200	>200	nd	>200	>200	nd	nd	>80

Legend:

Sa = *Staphylococcus aureus* NCTC 8325
Se = *Staphylococcus epidermidis* RP62A
Efa = *Enterococcus faecalis* JH212
Efe = *Enterococcus faecium* 6413
Ec = *Escherichia coli* 536
Pa = *Pseudomonas aeruginosa*
Yps = *Yersinia pseudotuberculosis*
Yps = *Yersinia pestis*
Ca = *Candida albicans*
nd = not determined

3.2.2.2 Anti-parasitic and cytotoxic activities

The compounds were further tested against the parasites *Leishmania major* and *Trypanosoma brucei* subsp. *brucei* as well as their cytotoxicities against the two cell lines, 293T kidney epithelial cells and J774.1 macrophages. All of the compounds, except for the cebulactams A1 and A2 exhibited significant anti-*Trypanosoma* activities exhibiting IC_{50} values below 100 µM with valinomycin and staurosporine showing the lowest IC_{50} values of 0.0032 and 0.022 µM, respectively. Only three compounds showed activity against the parasite *Leishmania major*: Pol001-4 (IC_{50}, 36.80 µM), valinomycin (IC_{50}, <0.11 µM) and staurosporine (IC_{50}, 5,30 µM). The majority of the compounds that showed significant anti-parasitic activities also showed cytotoxicities (<100 µM) with the exception of the compounds, Pol001-1 and butenolide (Table 3.11).

It is interesting to note that among the tetromycin compounds isolated in this Ph.D. study, the novel derivative Pol001-4 exhibited significant anti-*Leishmania* activity. The cebulactams A1 and A2 including the mixture of both substances did not exhibit any activities at all.

Table 3.11 Antiparasitic and cytototoxic activities (IC_{50}, µM) of compounds

Compound	LM	TB, 48 h	TB, 72 h	KE	JM
Cebulactam A1	>100	>100	>100	nd	>100
Cebulactam A2	>100	>100	>100	nd	>100
Cebulactam A1+A2	>100	>100	>100	nd	>100
Pol001-1	>100	**29.30**	**31.69**	**123.38**	>100
Pol001-3	>100	**45.39**	**80.27**	**160.00**	**50.21**
Pol001-4	**36.80**	**26.90**	**30.35**	**33.38**	**25.72**
Pol001-5	>100	**35.85**	**41.61**	**58.58**	**27.54**
Pol001-7	>100	**30.87**	**34.22**	**71.77**	**20.20**
Valinomycin	**<0.11**	**0.0032**	**0.0036**	**11.24**	**<0.1**
Staurosporine	**5.30**	**0.022**	**0.035**	**1.30**	**<0.13**
Butenolide	>100	**31.77**	**33.08**	>100	>100

*Activity not determined for cycloisoleucylprolyl due to lack of material.

Legend:

KE = 293T kidney epithelial cells
JM = J774.1 macrophages
LM = *Leishmania major*
TB = *Trypanosoma brucei brucei* 221
nd = not determined

3.2.2.3 Protease inhibition

Different cysteine proteases were tested to evaluate the ability of the compounds to inhibit these enzymes found in the parasites *Plasmodium falciparum* (falcipain-2) and *Trypanosoma brucei rhodesiense* (rhodesain), mammalian cells (cathepsin B and L), and SARS coronavirus (SARS-PL^{pro} and SARS-M^{pro}) (Table 3.12). A percent inhibition value above 70% at 100 µM concentration was considered as significant protease inhibition activity (T. Schirmeister, personal communication). Among the compounds tested, the tetromycin family of compounds (Pol001 series) showed interesting activities. For example, inhibition of the malarial protease, falcipain-2 was exhibited by three of the tetromycin compounds namely, Pol001-4, Pol001-5 and Pol001-7 at 91.2, 92.9 and 81.4 % inhibition, respectively. Furthermore, only two compounds showed rhodesain inhibition: Pol001-4 (79.9%) and Pol001-5 (77.5%). Inhibition against the mammalian protease, cathepsin B, was found to be exhibited by the compounds Pol001-3 (70.6%), Pol001-5 (77.9%) and Pol001-7 (79.2%), while Pol001-5 was the only compound that inhibited cathepsin L at 72.7% and SARS-Pl^{pro} at 73.4%. Interestingly, the novel tetromycin derivative Pol001-4 displayed specific inhibition against only the parasite proteases, falcipain-2 and rhodesain. On the other hand, the compounds Pol001-1, cebulactam A1 and A2, valinomycin and staurosporine did not show any inhibition against a single protease in these assays. The compound cycloisoleucylprolyl was not tested due to the lack of material.

Table 3.12 Percent inhibition values* (100 µM) against different proteases

Compound	Falcipain -2	Rhodesain	SARS Pl^{pro}	SARS M^{pro}	Cathepsin B	Cathepsin L
Cebulactam A1	51.7	8.3	3.8	5.5	5.8	7.5
Cebulactam A2	51.7	15.0	20.5	10.9	8.3	ni
Cebulactam A1+A2	53.7	17.2	9.4	3.3	7.8	ni
Pol001-1	nd	7.1	16.4	4.8	68.9	nd
Pol001-3	50.7	43.8	34.5	ni	**70.6**	50.3
Pol001-4	**91.2**	**79.9**	59.6	8.2	67.5	62.9
Pol001-5	**92.9**	**77.5**	**73.4**	9.5	**77.9**	**72.7**
Pol001-7	**81.4**	53.6	32.1	9.5	**79.2**	37.8
Valinomycin	nd	13.6	ni	ni	4.9	ni
Butenolide	nd	ni	2.6	ni	2.5	2.0

*****Mean values of two independent assays; nd = not determined; ni = no inhibition

3.3 Discussion

The actinomycetes isolated in this study were found to produce diverse compounds with interesting chemistry and biological activities. The strains belonging to the new actinomycete taxa namely, *Saccharopolyspora cebuensis* strain SPE 10-1^T and *Streptomyces axinellae* strain Pol001^T produced the novel secondary metabolites, cebulactams and the tetromycin family of compounds including novel derivatives, respectively. These results clearly indicate that the cultivation of novel actinomycetes proves to be a good strategy for isolating new chemical entities and minimizes the exhaustive effort of re-isolating known compounds. The cebulactams A1 and A2 represent constitutionally identical macrolactams with the unique cyclic ether connecting the aromatic ring system to the highly functionalized, polyketide-derived carbon chain (Pimentel-Elardo et al. 2008). There are structurally related secondary metabolites that resemble these macrolactams, but are lacking the characteristic cyclic ether. Examples are the anti-oxidative compounds from *Pseudonocardia* Q-1047 isolated from a soil sample from the Zamami Islands, Okinawa Prefecture, Japan (Imai et al. 1989a; Imai et al. 1989b; Yazawa et al. 1990) and the ansamycin antibiotics geldanamycin (Rascher et al. 2003) and herbimycin (Rascher et al. 2005) isolated from *Streptomyces hygroscopicus*. Despite the fact that the cebulactam polyketides produced by *Saccharopolyspora cebuensis* sp. strain SPE10-1^T are structurally related to the ansamycin antibiotics and that the crude extract from which these polyketides were isolated showed preliminary activity, these compounds, still did not exhibit any bioactivities using the different anti-infective screening panel used in this study. The question of synergistic action of the two compounds has also been addressed since a cocktail of these substances also did not show any activity. In contrast to the other structurally related bioactive metabolites, the aromatic ring via the cyclic ether in the cebulactams might possibly be protected from oxidation, preventing the formation of the benzoquinone moeity which appears to be the putative bioactive part of the molecule. In this regard, conversion of the compounds to its oxidized form (i.e. benzophenonic) has been attempted (T.A.M Gulder, University of Würzburg) but the testing of the resulting oxidation products also did not yield bioactivities. Therefore, this semi-synthetic work warrants further investigation to improve the bioactivity potential of the compounds.

The compounds isolated from *Streptomyces axinellae* strain Pol001^T exhibited structural resemblance to tetromycins isolated from *Streptomyces* sp. strain MK67-CF9 (Takeuchi et al. 1989) with the exception of the novel derivatives Pol001-3 and Pol001-4. The compounds reported in this Japanese patent were shown to exhibit activity against

methicillin-resistant *Staphylococcus aureus*. However, the compounds are reported in this study to have a wider spectrum of activity that includes anti-parasitic and protease inhibition activities. Moreover, it is worthwhile to examine whether *Streptomyces axinellae* strain Pol001^T as well as *Saccharopolyspora cebuensis* sp. strain SPE10-1^T produce other compounds when cultivated under different conditions given the fact that these strains belong to novel actinomycete taxa and as shown in this PhD study, have a great potential of producing new secondary metabolites.

Additional interesting yet previously known compounds were also isolated in this Ph.D. study from the different actinomycete strains namely, valinomycin, staurosporine, cycloisoleucylprolyl and butenolide. Valinomycin was isolated from *Streptomyces* sp. strains 34 and 22 cultivated from the Mediterranean sponges *Axinella polypoides* and *Aplysina aerophoba.* This cyclodepsipetide was originally recovered from various soil-derived actinomycetes: *Streptomyces fulvissimus* (Brockmann and Schmidt-Kastner 1955; Brown et al. 1962), *Streptomyces roseochromogenes* (Patterson and Wright 1970) and *Streptomyces griseus* var. *flexipartum* (Heisey et al. 1988). To date, this is the first report of valinomycin isolated from a marine organism. This cyclic depsipeptide is an oligomer similar to peptides but in which some of the amino acids are replaced by hydroxyl acids, so that amide and ester bonds are present along the chain. Furthermore, valinomycin is structurally similar to onchidin B isolated from a mollusc *Onchidinium* sp. (Fernández et al. 1996) and montanastatin, an antineoplastic compound from a soil-derived *Streptomyces annulatus* (Pettit et al. 1999) possessing a cyclic structure with polar groups oriented toward the central cavity, whereas the rest of the molecule is relatively nonpolar. The structure behaves as an ionophore that modulates transport of ions such as potassium across biological membranes (Haynes et al. 1969). It is for this reason that several studies have reported on the chemical synthesis of valinomycin and its analogs (Fonina et al. 1971; Smith et al. 1975; Vinogradova et al. 1975; Kuisle et al. 1999). Valinomycin has also been reported to exhibit other biological activities such as insecticidal (Patterson and Wright 1970; Heisey et al. 1988), nematocidal (Patterson and Wright 1970) and antifungal (Park et al. 2008). In this Ph.D. study, valinomycin is found to exhibit not only antifungal activity but also anti-parasitic activities thus extending the pharmacological value of this known compound.

Another interesting compound isolated in this study is staurosporine from *Streptomyces* sp. strain 11 cultivated from the sponge *Tedania* sp. which apparently has been identified from various sources. This indolocarbazole alkaloid was first isolated from *Streptomyces staurosporeus* Awaya AM-2282 (Omura et al. 1977) and subsequently from various

terrestrial actinomycetes: *Streptomyces actuosus* (Morioka et al. 1985), *Streptomyces* sp. strain M-193 (Oka et al. 1986), *Streptomyces platensis* subsp*. malvinus* RK-1409 (Osada et al. 1992), *Streptomyces* sp. AB 1869R-359 (McAlpine et al. 1994) and *Streptomyces longisporoflavus* (Cai et al. 1995; Cai et al. 1996). Interestingly, staurosporine and its derivatives have been reported to be produced by the marine ascidian *Eudistoma toealensis* and its predatory flatworm *Pseudoceros* sp. (Schupp et al. 1999; Schupp et al. 2002). Furthermore, staurosporine and its derivatives have aroused considerable interest as these compounds exhibit strong inhibitory activities against protein kinase C (Tamaoki et al. 1986) as well as inhibition of platelet aggregation (Oka et al. 1986), blocking of growth phases in cancer cells (Beltran et al. 1997) and reversal of multidrug resistance (Utz et al. 1998). Additionally, staurosporine was found in this Ph.D. study to exhibit significant inhibitory activity against the parasites *Leishmania major* and *Trypanosoma brucei* which has not been previously reported for this compound.

Another compound isolated in this Ph.D. study is cycloisoleucylprolyl from *Streptomyces* sp. strain A188 cultivated from the Mediterranean sponge *Aplysina aerophoba*. This diketopiperazine has also been previously isolated from the Caribbean sponge *Calyx* cf. *podatypa* (Adamczeski et al. 1995) and a structurally similar diketopiperazine from *Pseudomonas aeruginosa* isolated from an Antarctic sponge *Isodictya setifera* (Jayatilake et al. 1996). Diketopiperazines are the smallest peptides known, commonly biosynthesized from amino acids by different organisms and are considered to be secondary functional metabolites or side products of terminal peptide cleavage (Martins and Carvalho 2007). It is common to find diketopiperazines in fermentation mixtures as microorganisms are capable of protein hydrolysis and that a certain proportion of diketopiperazines present may arise during the fermentation to replace or supplement the quantity supplied in the medium (Mitscher et al. 1963). These compounds were in fact found to be produced by the majority of the strains cultivated in this study.

Furthermore, butenolide was isolated from *Streptomyces* sp. strain T03 from *Tethya* sp. sponge and exhibited significant activity against *Trypanosoma brucei*. This lactone-containing metabolite has also been previously isolated from a marine sediment-derived *Streptomyces* sp. strain M027750 (Cho et al. 2001). Butenolides are a family of α,β-unsaturated lactones often encountered among fungi, bacteria and gorgonians (Rodriguez and Ramirez 1994; Braun et al. 1995; Smith et al. 2000). Their saturated analogues act as signaling substances in bacteria and enhance spore formation of *Streptomyces* sp. or induce metabolite formation (Mukku et al. 2000).

Bioassay data of the secondary metabolites isolated in this study revealed that the majority of the compounds exhibited significant anti-parasitic and protease inhibitory activities. The specificity of the various screening panels employed here provides further insights into the mechanisms of action against particular pathogens and underscores the anti-infective potential of the compounds. The parasites *Leishmania major* and *Trypanosoma brucei* are clinically important pathogens as these are known to cause leishmaniasis and the African trypanosomiasis, respectively (Fenwick 2006; Caffrey et al. 2007). Furthermore, cysteine proteases play pivotal roles in the growth, differentiation and pathogenicity of various pathogens (Otto and Schirmeister 1997; Mahmoudzadeh-Niknam and McKerrow 2004). Therefore, inhibition of cysteine proteases presents a promising strategy for combating infection. For example, the enzyme rhodesain in *Trypanosoma brucei rhodesiense* has been the target in developing new anti-trypanosomal drugs. Furthermore, proteases of *Plasmodium falciparum* parasites play important roles in the processes of host erythrocyte rupture, erythrocyte invasion and hemoglobin degradation (Rosenthal 2004). Treatment with cysteine protease falcipain inhibitors blocks hemoglobin hydrolysis and development of the parasite (Schulz et al. 2007). Additionally, the coronavirus SARS Co-V is another important pathogen causing the severe acute respiratory syndrome (SARS) which was responsible for the severe epidemic in 2002-2003 with more than 800 reported deaths worldwide (Peiris et al. 2003). Coronaviruses are plus-strand RNA viruses where the genome RNA is translated to produce two large replicase polyproteins that are autocatalytically cleaved by viral proteases (Kaeppler et al. 2005). The coronavirus main protease M^{pro} is the key enzyme in the proteolytic process and is therefore also considered as an attractive target for new antiviral drugs against SARS and other coronavirus infections (Anand et al. 2003).

Therefore, the novelty of the chemical structures as well as the bioactivities exhibited by the different compounds isolated in this study clearly shows the potential of marine sponge-associated actinomycetes to produce pharmacologically important secondary metabolites. Expanding the anti-infective screening panel to include more clinically relevant pathogens and improving the bioactivity of the compounds by structure-activity studies are thus worthwhile pursuing.

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Chapter 4
Identification of biosynthetic gene clusters in actinomycete strains

4.1 Materials and methods

4.1.1 Screening of actinomycete strains for NRPS and PKS genes

Cultivated actinomycete strains were screened for the presence of biosynthetic gene clusters specifically nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) using the following degenerated primers: NRPS A7R (5'-SASGTCVCCSGTSCGGTAS-3') and A3 (5'-GCSTACSYSATSTACACSTCSGG-3') (Ayuso-Sacido and Genilloud 2005); type 1 PKS (PKS-1): K1 (5'-TSAAGTCSAACATCGGBCA-3') and M6R (5'-CGCAGGTTSCSGTACCAGTA-3') (Ayuso-Sacido and Genilloud 2005); type 2 PKS (PKS-II): KSα (5'-TSGRCTACRTCAACGGSCACGG-3') and KSβ (5'-TACSAGTCSWTCGCCTGGTTC-3') (Ayuso et al. 2005), KS1 (5'-TSGCSTGCTTCGAYGCSATC-3') and KS2 (5'-TGGAANCCGCCGAABCCGTC-3') (Metsa-Ketela et al. 1999), 540F (5'-GGITGCACSTCIGGIMTSGAC-3') and 1100R (5'-CCGATSGCICCSAGIGAGTG-3') (Wawrik et al. 2005); type 3 PKS (PKS-III): Fwd (5'-TCGCTSCTSTCGAACGGCCTSTTCGGCGACGCSCTSTCGGC-3') and Rev (5'-CTCSGCGGTGATSCCGGGSCCGAAGCCSGCGATSAGGC-3') (Cortes et al. 2002). A standard PCR reaction mix (50 µl) was prepared which consisted of the following: 10x reaction buffer, 5 µl; Q solution, 10 µl; 25 mM $MgCl_2$, 2 µl; 10 mM dNTPs, 1 µl; 100 µM of each primer, 1 µl; 5 U/µl Taq polymerase, 0.25 µl; H_2O, 28.75 µl; DNA template, 1 µl. PCR conditions were as follows: initial denaturation (5 min at 95°C) followed by 35 cycles of denaturation (30 s at 95°C), primer annealing (2 min at 59°C for A7R/A3, 55°C for K1/M6R, 58°C for KSα/KSβ, KS1/KS2 and PKS-IIIFwd/Rev and 64°C for 540F/1100R), primer extension (4 min at 72°C) and final extension step (10 min at 72°C).

4.1.2 Cloning, sequencing and phylogenetic analysis of NRPS adenylation domains

The PCR amplification products obtained with the NRPS adenylation primers A7R and A3 were purified using the QIAquick PCR purification kit (Qiagen). Five volumes of buffer PB was added to one volume of PCR product and mixed using a pipette. The mixture was applied to a QIAquick column and centrifuged at 13000 rpm for 1 min. The flow-through was discarded and the column was placed back into the same tube. Buffer PE (750 µl) was added to the column followed by centrifugation at 13000 rpm for 1 min. The flow-through was discarded and the column was centrifuged for an additional min at 13000

rpm. The column was then placed in a clean 1.5-ml microfuge tube, added with 30 µl of buffer EB and allowed to stand for 1 min. A final centrifugation step at 13000 rpm for 1 min was performed and the resulting purified PCR product was stored at -20°C.

Ligation of the purified PCR products and transformation in competent *E. coli* XL1-Blue cells were subsequently performed. The following were mixed on ice: 2x T4 DNA ligase buffer, 5 µl; 50 ng/µl pGEM-Teasy vector (Promega), 1 µl; 3 U/µl T4 DNA ligase, 1 µl; PCR product, 4 µl. The ligation solution was incubated overnight at 4°C for maximum number of transformants. For transformation, 2-3 µl of the ligation solution was added to 50 µl of the competent *E. coli* cells. The resulting mixture was transferred to a UV-sterilized electroporation cuvette. Electroporation was applied using an Easyject Prima Electroporator (Equibio) at 2500V. The solution was transferred to a clean 2-ml microfuge tube, added with 1 ml of SOC medium with mixing to resuspend the pellet and incubated with shaking at 37°C for at least 3 hours. After incubation, 20% and 80% of the cells were plated out on LB/amp/IPTG/X-gal agar. The plates were incubated at 37°C overnight and successful transformants were observed using the blue-white colony screening. The plasmid DNA was isolated using a standard miniprep protocol. Single, white colonies were picked up, inoculated in 2 ml of LB/amp broth and incubated with shaking at 37°C overnight. Following incubation, the culture was centrifuged at 13000 rpm for 5 min and the supernatant was discarded. The pellet was resuspended in 150 µl of buffer P1. Addition of 150 µl of buffer P2 with standing for 5 min at room temperature followed by the addition of 150 µl of buffer P3 on ice for 5 min were subsequently done. The solution was mixed by gentle flicking of the tube after each addition of the buffer. The resulting mixture was centrifuged at 13000 rpm for 10 min. The supernatant was transferred to clean 1.5-ml microfuge tube and the centrifugation step was repeated. The supernatant was again transferred to clean tube, added with 0.7 volume of isopropanol and mixed by gently flicking the tube. This was then centrifuged at 13000 rpm for 15 min. The resulting pellet containing the plasmid DNA was washed with 70% ethanol and allowed to air-dry. Sterile water (50 µl) was added to resuspend the pellet and the plasmid DNA was stored in -20°C until use. To verify the correct clones, the plasmid DNA was digested with a restriction endonuclease. Restriction digestion with *Eco*RI (New England Biolabs) was done by mixing the following: 10x *Eco*RI buffer, 2 µl; water, 12 µl; 20,000 U/ml *Eco*RI, 1 µl; plasmid DNA, 5 µl. The reaction mix was incubated at 37°C for 2-3 hours and the restriction patterns were analyzed by agarose gel electrophoresis.

For amplification of the pGEM-T easy insert, sequencing PCR was done using the primers SP6 (5'- ATTTAGGTGACACTATAG-3') and T7 (5'- GTAATACGACTCACTATAGGG-3') and the BigDye® terminator cycle sequencing kit (Applied Biosystems). The PCR reaction mix was composed of the following: 5x BigDye® terminator v1.1 sequencing buffer, 2 µl; BigDye® terminator v1.1 premix, 2 µl; plasmid DNA, 2 µl; 25 µM of primer, 1 µl; sterile water, 3 µl. The PCR conditions were as follows: initial denaturation (2 min at 96°C), 25 cycles of denaturation (30 s at 96°C), primer annealing (15 s at 45°C), primer extension (4 min at 72°C) and a final extension step (10 min at 60°C). DNA sequencing was performed using an ABI 377XL automated sequencer (Applied Biosystems). Sequences were assembled using the ContigExpress tool in Vector NTI Advance™ 10.0 (InforMax, Inc) and subsequently aligned using Clustal X. Phylogenetic analysis was done using the ARB software (Strunk and Ludwig 1997).

4.1.3 Genomic library construction

Genomic libraries were constructed for the following strains: *Streptomyces* sp. strain Aer003 and *Saccharopolyspora cebuensis* strain SPE10-1 using the pWEB™ cosmid cloning kit (Epicentre, Madison) and the libraries were designated as Aer003pWEB and SPE10-1pWEB, respectively.

4.1.3.1 Genomic DNA isolation

Saccharopolyspora cebuensis strain SPE 10-1^T and *Streptomyces axinellae* strain Pol001^T were grown in M1 and ISP 2 broth, respectively at 30°C for 7-10 days until sufficient biomass was obtained. Cells were centrifuged at 8000 rpm for 5 min. The salting-out procedure for the isolation of genomic DNA was followed (Pospiech and Neumann 1995; Kieser et al. 2000). The cell pellet was washed with SET buffer to remove adhering media components and resuspended in 500 µl SET buffer. Addition of 10 µl of lysozyme solution (50 mg/ml) and incubation at 37°C for 30 min followed. After incubation, 14 µl of proteinase K solution (20 mg/ml) and 60 µl of 10% SDS were added with mixing by inversion. The solution was incubated at 55°C for 2 hours with occasional inversion. An additional amount of proteinase K was added, if necessary, until the solution was clear. Following incubation, 200 µl of 5M NaCl was added and allowed to cool to 37°C. Five hundred microliters of chloroform was then added and the solution was thoroughly mixed by shaking for 30 min at room temperature. The resulting solution was centrifuged at 6000 rpm for 15 min. The supernatant was transferred to a fresh Eppendorf tube and 0.6 volume of isopropanol was added followed by mixing with inversion. The

DNA was spooled from the solution using a flame-bent Pasteur pipet. The DNA was rinsed with 70% ethanol, air-dried and dissolved in 100 µl water. DNA concentration was then measured using a NanoDrop ND1000 spectrophotometer (PeqLab, Erlangen).

4.1.3.2 Insert DNA end-repair reaction

Following extraction of genomic DNA, end-repair reaction was done using the end-repair mix (Epicentre, Madison) to generate blunt ends for cloning into the vector. The following reagents were combined on ice: 8 µl 10X End-Repair buffer, 8 µl 2.5 mM dNTP Mix, 8 µl 10 mM ATP, 20 µg insert DNA, 4 µl end-repair enzyme and sufficient volume of sterile water to make a total reaction volume of 80 µl. The resulting mixture was incubated at room temperature for 45 min. This was then followed by incubation at 70˚C for 10 min to inactivate the end-repair enzyme Mix. DNA was extracted using standard phenol-chloroform procedure. Phenol (10 µl) was added and the solution was mixed by gentle inversion. Centrifugation at 9000 rpm for 10 min followed and the supernatant was then transferred to a clean Eppendorf tube. Chloroform (10 µl) was added, followed again by centrifugation at 9000 rpm for 10 min. The resulting supernatant was transferred to a clean Eppendorf tube and 0.1 volume of 3M sodium acetate and 2.5 volumes of ice-cold 100% ethanol were subsequently added. The whole solution was centrifuged at 9000 rpm for 10 min followed by washing of the DNA with 70% ethanol and air-drying. The resulting DNA was re-dissolved in 20 µl water and stored at 4°C.

4.1.3.3 Ligation of insert DNA into the pWEB vector

The following reagents were combined in the order as listed and mixed thoroughly after each addition: 5 µl sterile water, 2 µl 10X fast-link ligation buffer (Epicentre, Madison), 1 µl 10 mM ATP (Epicentre, Madison), 1 µl pWEB vector (0.5 µg, ~8 kb) (Epicentre, Madison), 10 µl concentrated insert DNA (from previous step) and 1 µl fast-link DNA ligase (Epicentre, Madison) for a total reaction volume of 20 µl. The resulting solution was incubated at room temperature for 2 hours and further incubated at 70°C for 10 min to inactivate the fast-link ligase.

4.1.3.4 *In Vitro* packaging

An overnight culture of EPI100-T1^R (Epicentre, Madison) was prepared by inoculating 10 µl of EPI100-T1^R Phage T1-Resistant *E. coli* Plating Strain in 5 ml of LB broth supplemented with 10 mM $MgSO_4$ and shaking at 37°C. Fifty milliliters of LB supplemented with 10 mM $MgSO_4$ was inoculated with 100 µl of the overnight culture and

was shaken at 37°C until the cell density reached an OD_{600} of 0.8-1.0. MaxPlax Lambda Packaging Extracts (Epicentre, Madison) were thawed on ice. Half of each packaging extract (25 µl) was transferred to a 1.5-ml eppendorf tube. Ten microliters of the ligated cosmid DNA was added. The mixture was mixed by pipetting several times with care not to introduce air bubbles in the process. The tube was then centrifuged briefly to pool again all the contents of the tube. The reaction was then incubated at 30°C for 90 min. At the end of this incubation, the remaining 25 µl of the packaging extract was added to the reaction and incubated further for an additional 90 min at 30°C. Five hundred microliters of the phage dilution buffer (10 mM Tris-HCl pH 8.3, 100 mM NaCl, 10 mM $MgCl_2$) was added and mixed by gentle vortexing, followed by the addition of 25 µl of chloroform and gentle vortexing again. The resulting solution was stored at 4°C until use. For every 100 µl of the previously prepared EPI100-$T1^R$ host cells, 10 µl of the packaged cosmids was added. Adsorption by incubation at 37°C for 20 min was subsequently done. After incubation, the transfected bacteria (100 µl per plate) were spread on LB-ampicillin (100 µg/ml) agar plates and incubated overnight at 37°C. Single colonies were then picked up, streaked on LB-ampicillin agar plates in an organized fashion corresponding to a 384-well format arrangement and incubated further at 37°C. The grown colonies were then stamped from the plates using a flame-sterilized pin replicator into a 384-well microplate containing LB-ampicillin medium with 15% glycerol. The plates were incubated again at 37°C overnight and subsequently stored at -80°C.

4.1.3.5 Genomic library screening

Clones generated from the genomic library were screened for the biosynthetic gene of interest by PCR. Pools of clones were generated by scraping colonies according to their arrangement by columns and rows on the plates. The colonies were resuspended in 100 µl sterile water and heated at 90°C for 10 min. The corresponding suspension was then used as template for subsequent PCR reactions. The following primer pairs were used: sebNRPS-1 (5'-GGCAGGCTGGTTGACGTAG-3') and sebNRPS-2 (5'-GTGTGGTGGA GCTGGCTTT-3') (Proksch 2008); degAH-F2 (5'-ATCATGCCSGTSCAYATGGCSGG-3') and degAH-R2 (5'-CKRTGRTGSARCCASTKRCARTC-3') (Rascher et al. 2003) to screen Aer003pWEB and SPE10-1pWEB libraries, respectively. A standard PCR reaction mix (25 µl) was prepared which consisted of the following: RedTaq™ ReadyMix™ PCR reaction mix (Sigma), 12.5 µl; 100 µM of each primer, 0.5 µl; H_2O, 11.5 µl; DNA template, 1 µl. PCR conditions were as follows: initial denaturation (3 min at 94°C) followed by 35 cycles of denaturation (30 s at 94°C), primer annealing (1 min at 59°C for sebNRPS-1/-2 and 2 min at 52°C for degAH-F2/-R2 primers), primer extension (1.5 min at 72°C) and

final extension step (10 min at 72°C). Individual clones from the positive pools were again screened by PCR using the same set of primers. The cosmid DNA from the positive clones were extracted using a standard miniprep protocol described previously. The same PCR reactions were repeated using the extracted cosmid DNA as template and the PCR products were purified using the QIAgen PCR purification kit. Sequencing PCR was then done to verify the PCR products. Restriction digestion of the cosmid DNA was performed using the restriction enzymes *Eco*RI and *Not*I (New England Biolabs) to check insert sizes. To ensure that the cosmid DNA from each clone was not a mixture of different cosmid DNA, these were transformed in heat-competent *E. coli* XL1-Blue cells. Five microliters of cosmid DNA was added to 50 µl of *E. coli* cells and incubated for 30 min on ice. The mixture was incubated further at 42°C for 1 min 15 s and subsequently transferred on ice for an additional 5 min. One milliliter of SOC broth was added followed by incubation with shaking at 37°C for at least 3 hours. After incubation, 20% and 80% of the cells were plated out on LB/amp/IPTG/X-gal agar. The plates were incubated at 37°C overnight and successful transformants were observed using the blue-white colony screening. Transformants were screened by colony PCR and the cosmids verified again by restriction digestion and PCR.

4.1.3.6 Cosmid sequencing and annotation

Clones harboring the positive cosmid DNA from each library were sent to Macrogen, South Korea for end sequencing and shotgun sequencing of the entire cosmid. Assembled contigs sent by Macrogen were then analyzed and annotated using Vector NTI 10 (Invitrogen).

4.2 Results

4.2.1 NRPS and PKS clusters identified by PCR and phylogenetic analysis

Biosynthetic gene clusters encoding for nonribosomal peptide synthetases were detected in the majority of the actinomycete strains as well as polyketide synthases in some of the strains cultivated in this study (Table 4.1). Degenerated primers designed specifically to amplify the conserved regions of the adenylation (NRPS) and ketosynthase domains (PKS) in actinomycetes (Ayuso-Sacido and Genilloud 2005; Ayuso et al. 2005) were used. The majority of the strains (14 out of 20) were positive for NRPS and five strains possessed both NRPS and PKS systems. Type I PKS was detected in five strains while four strains exhibited type II PKS. However, all of the strains were negative for type III PKS.

Table 4.1 PCR screening results of actinomycete isolates for the presence of NRPS and PKS clusters

Isolate code	16S rDNA closest homology/identity	NRPS	Type I PKS	Type II PKS
SPE 10-1	*Saccharopolyspora cebuensis*	-	+	-
A188*	Actinomycetales bacterium XJSS-18	+	-	-
10	*Streptomyces albogriseolus* NBRC 3709	-	-	-
T03	*Streptomyces* sp. MP47-91	+	-	+
11	*Streptomyces spinoverrucosus* strain 174464	-	-	-
17	*Streptomyces variabilis* strain 173733	+	-	-
27	*Streptomycetaceae* bacterium WBF21	-	-	-
Da02	*Streptomyces* sp. CNS-774_SD06	+	-	-
T02	*Streptomyces* sp. CNS-774_SD06	+	-	-
34	*Streptomyces* sp. VTT E-042674	+	-	-
22	*Streptomyces* sp. VTT E-042674	+	-	-
Aer003*	*Streptomyces bingchengensis* strain 226541	+	-	-
11-11	*Isoptericola* sp. TUT1258	-	+	-
4-3	*Micromonospora* sp. HBUM84229	+	+	-
9-3	*Gordonia terrae* strain AIST-1	+	+	-
Cr03	*Nocardiopsis* sp. 20052	+	-	-
Pol013*	*Streptomyces albiaxialis* NBRC 101002	+	+	+
Pol014*	*Streptomyces* sp. VTT E-99-1334	-	-	+
A233*	Actinomycetales bacterium HPA66	+	-	-
SB182*	*Streptomyces* sp. VTT E-99-1334	+	-	+

*Strains isolated from previous study by Scheuermayer (2006).
All strains were negative for type III PKS.

The adenylation (A) domains of five NRPS-positive strains were cloned in pGEM-T easy vector and sequence analysis of the 700bp-PCR products was performed. In a Master's thesis (Proksch 2008), the diversity and distribution of NRPS systems in the microbial

consortia of different sponge species, including seawater and an ascidian (*Ecteinascidia turbinata*) collected from Patch Reef, Bahamas and Banyuls-sur-Mer, France were investigated. Proksch (2008) screened for the presence of NRPS genes by PCR and sequencing using the same set of degenerated primers (A7R/A3) used in this study. Analysis of the adenylation domains revealed a single NRPS cluster present in majority of the sponge samples as well as from the sequences obtained from the seawater and ascidian samples. In another study, Grozdanov (unpublished data, AG Hentschel, University of Würzburg) constructed a metagenomic library from the microbial consortia of the Mediterranean sponge *Aplysina aerophoba*. Screening of the library revealed the presence of NRPS gene clusters (termed lubA1 and A2) speculated to encode for the *Verongida* brominated alkaloids. Phylogenetic analysis of the adenylation domains of the five strains from this study together with those obtained from the previously mentioned studies was thus carried out to investigate further the diversity of the NRPS clusters detected in the actinomycete strains. Interestingly, phylogenetic analysis (Fig. 4.1) revealed that the *Streptomyces* sp. strain Aer003 forms one large distinct cluster (further on termed sebNRPS cluster) together with the majority of the sequences obtained from different sponge samples, seawater as well as from the ascidian. Their high amino acid sequence similarities (97-99%) also provide further evidence of the sebNRPS cluster. Moreover, the sequences in this clade share high amino acid sequence similarity with the enduracidin EndB peptide cluster of *Streptomyces fungicidus* ABD65957 (Identities: 65%, Positives: 73%) (Yin and Zabriskie 2006). The detection of such a cluster suggests a widely distributed NRPS system and that the corresponding A domain could be part of one and the same, or at least very similar, NRPS gene cluster present in microorganisms from seawater, sponges and ascidian animals. The sequence similarities of the A domains at the amino acid level are 97-99% indicating further that these are probably identical genes. On the other hand, *Streptomyces* sp. strains A188, Da02, T02 and T03 formed separate clusters from strain Aer003 and the adenylation domains of the metagenomic clones from *Aplysina aerophoba* lubA1 and lubA2. It is important to note that the phylogenetic tree (Fig. 4.1) presented here was constructed using the ARB software (Strunk and Ludwig 1997). The high bootstrap values from neighbor-joining and maximum parsimony algorithms as well as the logical clustering of the different A domains based on their sequence similarities provided sufficient basis for considering such phylogenetic tree. Nevertheless, other suitable programs for phylogenetic analysis of functional genes such as MrBayes (Bayesian inference of phylogeny) can also be used for improved resolution of the tree.

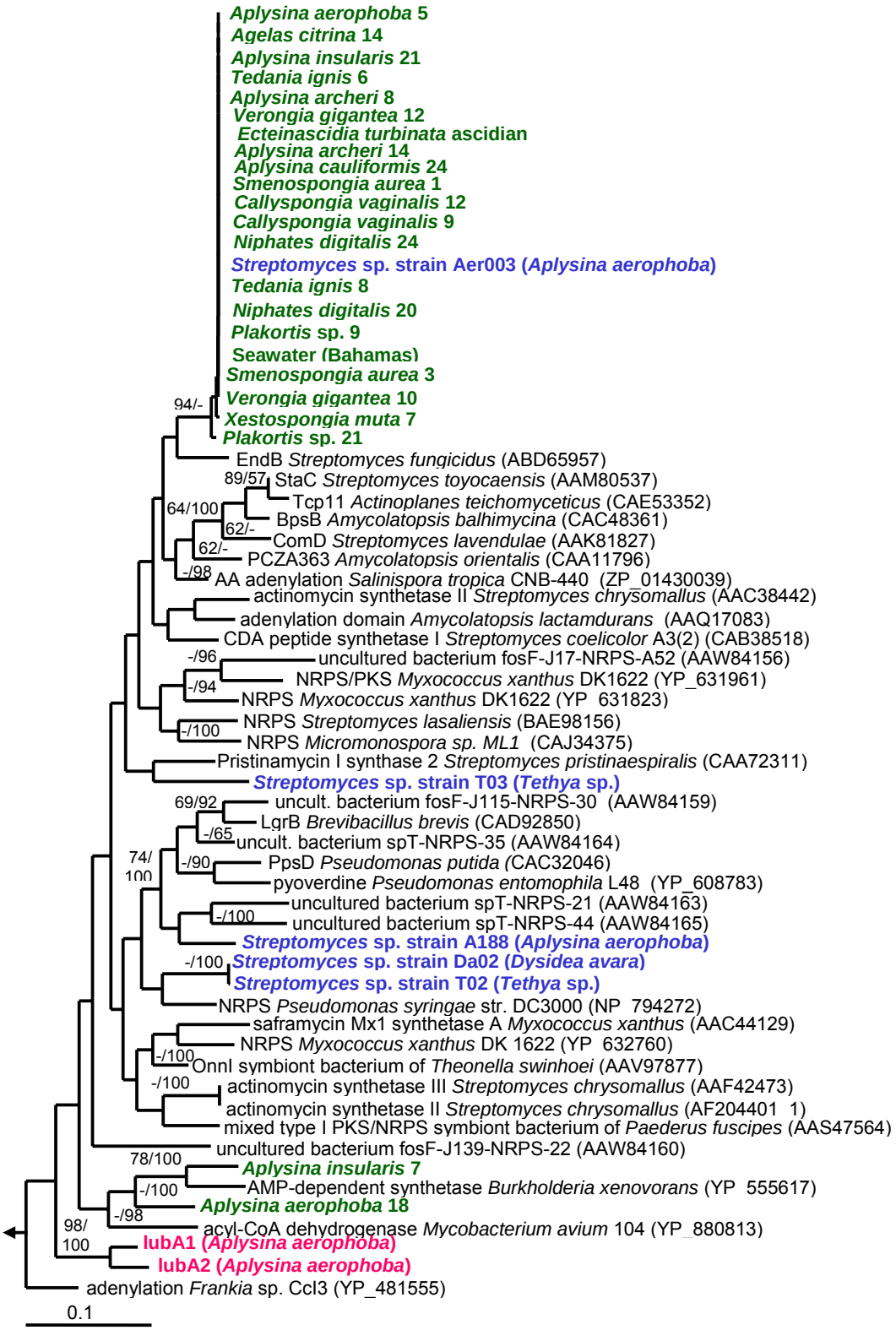


Fig 4.1 Phylogenetic tree of adenylation domains from cultivated *Streptomyces* strains obtained in this study (blue font); from different sponges, seawater, *E. turbinata* ascidian (green font); and from metagenomic clones (red). Arrow points to outgroup, LysI *E. coli* (P40976). Numbers at the nodes indicate the levels of bootstrap support (neighbor-joining/maximum parsimony) based on 100 resampled data sets; only values greater than 50% are shown. The scale bar indicates 0.1 substitutions per nucleotide position.

Furthermore, prediction of adenylation domain specificity was carried out using the NRPS predictor tool (Rausch et al. 2005). The analysis is based on computational data processing that leads to identification of the amino acid substrates of the nonribosomal peptide synthetases, according to the specificity-conferring code of their adenylation domains. The specificity of the A domains dictates the composition of the corresponding peptide product (Stachelhaus et al. 1999). Using this tool, the predicted substrate specificity of the adenylation domains of strain Aer003 as well as the other sequences from the distinct sebNRPS cluster was determined to be 4-hydroxy-phenylglycine (Hpg). Moreover, the predicted substrates for the other strains were alanine (*Streptomyces* sp. strain A188), valine (*Streptomyces* sp. strain T03) and glycine (*Streptomyces* sp. strains Da02 and T02). The substrate specificities of the adenylation domains were in agreement with the functional clustering of the sequences in the phylogenetic tree.

4.2.2 NRPS gene cluster obtained from a genomic library (Aer003pWEB) of *Streptomyces* sp. strain Aer003

The intriguing similarity of the adenylation domain of strain Aer003 with the majority of the sequences obtained from the different sponge samples as well as from seawater and an ascidian prompted the construction of a genomic library to further examine the gene organization of the NRPS cluster found in this strain. A cosmid genomic library (Aer003pWEB) of 706 clones was generated and screened with primers (sebNRPS-1 and sebNRPS-2*)* targeting the widely distributed sebNRPS cluster. Two PCR-positive clones were identified and hereafter named 1/C13 and 2/E12. The presence of the sebNRPS-specific A domain sequence was verified by PCR and direct sequencing of the PCR products using the primers mentioned above. Restriction fragment length analysis using the enzymes *Not*I and *Eco*RI suggested that these two cosmids do not overlap since no common bands were observed. Nevertheless, these cosmids were subjected to shotgun library construction and complete sequence analysis by the company Macrogen.

Sequence analysis of the cosmid inserts of 1/C13 (34090 bp) and 2/E12 (42315 bp) revealed the presence of several putative open reading frames (ORF) (Fig. 2.2). The predicted function and the presence of conserved domains for each ORF was assigned by comparing the translated product with known proteins in the NCBI Genbank database using the BlastX tool (Tables 2.1 and 2.2). Organization of NRPS domains (Fig.2.2) and substrate recognition sequences (Table 2.3) were predicted using the NRPS analysis online tool (http://www.tigr.org/jravel/nrps/).

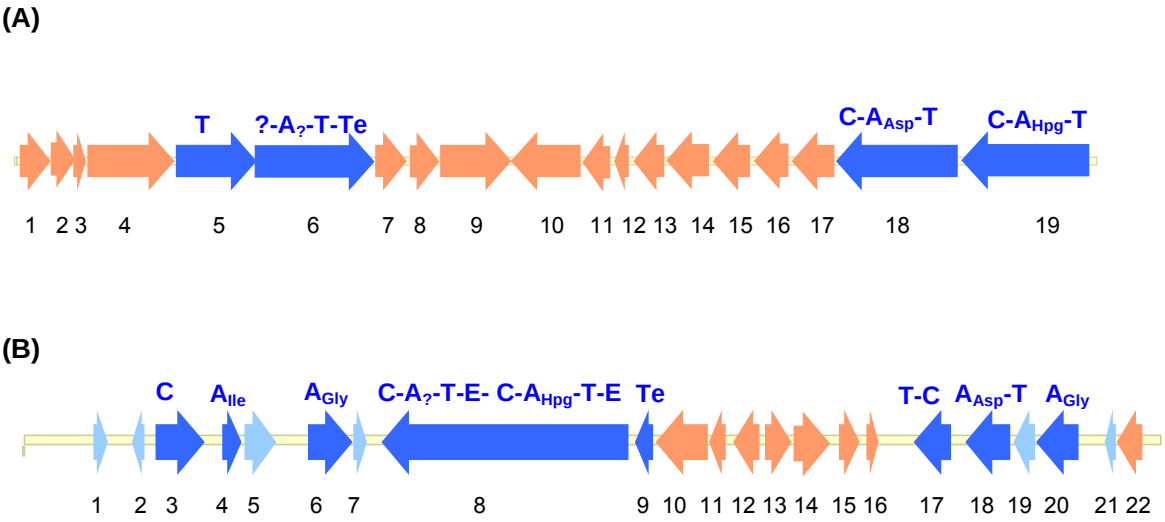


Fig. 4.2 Genetic organization (not drawn to scale) of the DNA regions isolated from the strain Aer003 (A) cosmid 1/C13 (34090 bp) and (B) 2/E12 (42315 bp). Genes putatively belonging to the NRPS cluster are shaded in dark blue and NRPS-related genes in light blue. Modules and domain organization are indicated above each ORF: C, condensation; A, adenylation; T, thiolation; Te, thioesterase; E, epimerization; ?, unknown.

There are four ORFs identified in cosmid 1/C13 (Fig. 4.2A) harboring putative NRPS genes. ORF 5 bears a single thiolation domain while ORF 6 harbors one module containing the following: adenylation domain with a novel A domain signature; thiolation; thioesterase; and an extra domain at the N-terminus spanning 394 amino acids. These two ORFS overlap by 453 nucleotides. Both ORFs 18 and 19 harbor a complete module each with condensation, adenylation and thiolation domains but with different substrate specificities (Table 4.2): aspartic acid (Asp) and 4-hydroxy-phenylglycine (Hpg), respectively. These modules seem to function as the elongation modules of the NRPS cluster. Apparently, the closest homolog (71%) of the Hpg-specific domain of ORF 19 is the A domain of CDA peptide synthetase I (module 6; CAB38518) of *Streptomyces coelicolor* A3(2). This particular domain exhibits also 65% similarity to that of the enduracidin gene cluster of *Streptomyces fungicidus* (ABD65957), which is expected since the cosmid was positively identified from the library using primers that amplify Hpg-specific A domains of the sebNRPS cluster. ORF 19 is located at the end of this cosmid suggesting that the cluster is disrupted at this end. Furthermore, a loading module that normally consists of adenylation and thiolation domains could not be identified in the cosmid sequence, confirming that the cluster is not complete. ORF 6 bears a thioesterase domain which is usually found at the extreme C-terminal module with the function of catalyzing the release of the biosynthesized peptide. This indicates therefore that this is the final module of the entire cluster.

Several NRPS ORFs have been identified in the second cosmid 2/E12 mostly consisting of single domains as well as a large ORF with complete NRPS domain organization (Fig. 4.2B). ORF 8 bears two complete modules, each bearing condensation, adenylation, thiolation and epimerization, an additional auxiliary domain. Prediction of the substrate specificities of the A domains based on the residues found in the binding pockets revealed that the first A domain exhibits a novel sequence whereas Hpg is the predicted amino acid for the second A domain (Table 4.3). This ORF is followed by a thioesterase domain signaling the end of this cluster. The residues in the binding pockets of this ORF (DAYHLGVG) are the same as that found in ORF 19 of cosmid 1/C13 suggesting Hpg as its predicted substrate. Comparison of their A domains revealed a high sequence similarity at the amino acid level (Identities=91%; Positives=93%). Their similarities were not high enough to speculate that the ORFs could possibly overlap to cover an entire biosynthetic cluster from both cosmids. Nevertheless, these Hpg-specific domains from both cosmids exhibited 98-99% amino acid sequence similarities with the A domains of the sebNRPS cluster. Although domains were not predicted in some of the ORFs in cosmid 2/E12 (ORF 1, 2, 5, 7, 19, 21) despite displaying high sequence similarities with known NRPS clusters, these ORFs still play a role in the peptide synthesis. The sequences of the small ORFs are short enough such that domain prediction is not possible.

Other genes necessary for regulation and export could be identified as well. Export of the peptide from the cell is likely to involve ORF 9 (1/C13) and ORF 10 (2/E12) predicted to function as components of ABC transporters similar to those found in other antibiotic biosynthetic gene clusters. Regulatory genes such that encoded by ORFs 7 and 14 (1/C13) and ORF 11 (2/E12) are also identified. Interestingly, genes involved in polyketide synthesis are present in cosmid 2/E12, particularly ORF 12 to 14 encoding for the acyl carrier protein (ACP), an essential domain in PKS systems. This could be part of another biosynthetic cluster involving polyketide synthesis or the strain probably encodes a mixed NRPS/PKS product.

Table 4.2 Summary of ORFs identified in cosmid 1/C13

ORF	Putative function	Most similar homolog (protein, accession no., origin)	Identities/ Positives (%)	No. of aa*	DNA coordinates
1	Dihydroxyacetone kinase subunit I	SAV_1285 (NP_822460), *Streptomyces avermitilis* MA-4680)	90/93	330	86..1075
2	Dihydroxyacetone kinase subunit 2	SAML0927 CAJ89913), *Streptomyces ambofaciens* ATCC 23877	73/79	264	1003..1794
3	Dihydroxyacetone kinase phosphotransfer protein	SGR_6660(YP_001828172), *Streptomyces griseus* subsp. *griseus* NBRC 13350	81/89	137	1794..2204
4	Polysaccharide lyase family protein 8	CPF_0394 (YP_694851), *Clostridium perfringens* ATCC 13124	29/49	919	2336..5092
5	NRPS	SAV_7165 (NP_828341), *Streptomyces avermitilis* MA-4680	72/81	852	5130..7685
6	NRPS	SACE_3015 (YP_001105218), *Saccharopolyspora erythraea* NRRL 2338	65/75	1262	7232..11017
7	Syrp-like protein	SAV_7164 (NP_828340), *Streptomyces avermitilis* MA-4680	73/83	334	11064..12065
8	Ornithine cyclodeaminase	SAV_7163 (NP_828339), *Streptomyces avermitilis* MA-4680	67/75	319	12065..13021
9	Transporter	SAV_1418 (NP_822593), *Streptomyces avermitilis* MA-4680	83/90	746	13484..15721
10	Hypothetical protein	SAV_1419 (NP_822594), *Streptomyces avermitilis MA-4680*	75/81	739	15773..17989
11	3-hydroxybutyrate dehydrogenase	SAV_1420 (NP_822595), *Streptomyces avermitilis* MA-4680	80/86	292	17923..18798
12	Mut-like protein	SCO1013 (NP_625309), *Streptomyces coelicolor* A3(2)	71/82	159	18857..19333
13	Secreted protein	SCO1016 (NP_625312), *Streptomyces coelicolor* A3(2)	52/57	327	19753..20733
14	GntR transcriptional regulator	Sare_2660 (YP_001537488), *Salinispora arenicola* CNS-205	60/75	452	20720..22075
15	FMN-dependent α-hydroxy acid dehydrogenase	Strop_2478 (YP_001159302), *Salinispora tropica* CNB-440	58/72	393	22075..23253
16	p-hydroxymandelate synthase	NocF (AAT09803), *Nocardia uniformis* subsp. *tsuyamanensis*	52/63	373	23253..24371
17	L-threonine synthase	Mbar_A3541 (YP_306990), *Methanosarcina barkeri* str. Fusaro	43/60	446	24451..25788
18	NRPS	SCO3230 (NP_627443), *Streptomyces coelicolor* A3(2)	48/59	1282	25864..29709
19	NRPS	Francci3_2461 (YP_481557), Frankia sp. CcI3	51/64	1344	29684..33715

*aa = amino acids

Table 4.3 Summary of ORFs identified in cosmid 2/E12

ORF	Putative function	Most similar homolog (protein, accession no., origin)	Identities/ Positives (%)	No. of aa*	DNA coordinates
1	NRPS	snbDE (CAA67249), *Streptomyces pristinaespiralis*	70/81	181	2558..3100
2	NRPS	EndC (ABD65958), *Streptomyces fungicidus*	69/78	159	4006..4482
3	NRPS	SnbDE (CAA72310), *Streptomyces virginiae*	58/70	613	4877..6715
4	NRPS	EndC (ABD65958), *Streptomyces fungicidus*	60/72	242	7370..8095
5	NRPS	SACE_4288 (YP_001106482), *Saccharopolyspora erythraea* NRRL 2338	53/68	388	8201..9364
6	NRPS	SACE_4288 (YP_001106482), *Saccharopolyspora erythraea* NRRL 2338	51/59	553	10562..12220
7	NRPS	SGR_3264 (YP_001824776), *Streptomyces griseus* NBRC 13350	43/61	167	12249..12749
8	NRPS	Francci3_2461 (YP_481557), *Frankia* sp. CcI3	45/58	3067	13300..22500
9	Thioesterase	SAV_3639 (NP_824816), *Streptomyces avermitilis* MA-4680	49/66	228	22738..23421
10	ABC transporter	SAV_5680 (NP_826857), *Streptomyces avermitilis* MA-4680	76/86	645	23508..25442
11	SyrP-like protein	SAV_3638 (NP_824815), *Streptomyces avermitilis* MA-4680	69/80	213	25482..26120
12	Acyl carrier protein	SGR_3255 (YP_001824767), *Streptomyces griseus* NBRC 13350	41/68	213	26386..27384
13	3-oxoacyl-ACP synthase II	SGR_3254 (YP_001824766), *Streptomyces griseus* NBRC 13350	65/80	324	27442..28413
14	3-oxoacyl-ACP synthase I	SGR_3249 (YP_001824761), *Streptomyces griseus* NBRC 13350	46/59	433	28287..29585
15	Dihydroxybenzoate synthesis	DhbF (NP_391076), *Bacillus subtilis* str. 168	38/59	256	30324..31091
16	NRPS	SACE_1305 (YP_001103553), *Saccharopolyspora erythr*aea NRRL 2338	59/72	148	31365..31808
17	NRPS	SnbDE (CAA67249), *Streptomyces pristinaespiralis*	58/71	460	33121..34500
18	NRPS	PstC (CAM56770), *Actinoplanes friuliensis*	54/64	560	35032..36711
19	NRPS	SACE_4288 (YP_001106482), *Saccharopolyspora erythr*aea NRRL 2338	69/81	262	36835..37620
20	NRPS	SACE_4288 (YP_001106482), *Saccharopolyspora erythr*aea NRRL 2338	51/59	524	37678..39249
21	NRPS	SnbDE (CAA72310), *Streptomyces virginiae*	64/78	140	40296..40715
22	Dihydroxybenzoate synthesis	DhbF (NP_391076), *Bacillus subtilis* str. 168	49/67	321	40675..41637

*aa = amino acids

Table 4.4 Derived substrate signature sequences for NRPS adenylation domains

Cosmid	ORF	Substrate recognition sequence	Predicted amino acid	Closest homolog (accession number)	Identities/ Positives (%)
	6	DIWQST-	No hit	---	---
1/C13	18	DITKIGHI	Asp	SrfAB-M2 (BAA08983)	75/100
	19	DAYHLGVG	Hpg	CdaI-M6 (CAB38518)	71/85
	4	DAYELGVG	Ile	Adp-M1 (CAC01606)	71/85
	6	DILQVGCI	Gly	NosC-M2 (AAF17280)	75/87
2/E12	8	DITKIASV	No hit	---	---
		DAYHLGVG	Hpg	Cda1-M6 (CAB38518)	71/85
	18	DATKMGHV	Asp	SrfAB-M2 (BAA08983)	62/87
	20	DILQVGCI	Gly	NosC-M2 (AAF17280)	75/87

4.2.3 PKS gene cluster from a genomic library (SPE10-1pWEB) of *Saccharopolyspora cebuensis* strain SPE 10-1^T

The strain *Saccharopolyspora cebuensis* SPE 10-1^T was found to produce the novel polyketides named as cebulactam A1 and A2 (Pimentel-Elardo et al. 2008). The chemical structure of these compounds suggests that these are type I polyketides structurally related to ansamycins, compounds which utilize 3-amino-5-hydroxybenzoic acid synthase (AHBA) as a starter unit. In order to gain insight into the biosynthesis of these polyketides, a genomic library was thus constructed. A total of 1,152 clones were generated. Preliminary screening of the library using degenerated primers (K1 and M6R) that amplify the conserved ketosynthase domain for type I PKS revealed six PCR-positive clones. In order to target the specific polyketide synthase encoding for the cebulactam compounds, these clones were screened again using degenerated primers (degAH-F2 and degAH-R2) that amplify the ansamycin starter unit, AHBA. PCR reaction yielded three PCR-positive clones namely, 1/J17, 2/I16 and 3/B3 which was further confirmed by subsequent sequencing of the PCR products. Restriction fragment length analysis of the cosmids revealed common bands (10 and 11 kb) indicating the presence of overlapping regions. These cosmids were subjected to shotgun library construction and complete sequence analysis by Macrogen (South Korea). However, only cosmid clone 3/B3 was successfully completely sequenced since trimmed sequences were found in 1/J17 and 2/I16 and hence primer walking to assemble into final contig sequences was therefore not possible despite efforts on the reconstruction of the shotgun library (Macrogen, personal communication).

Sequence analysis of the cosmid insert of 3/B3 (36355 bp) revealed the presence of several putative open reading frames (ORF) (Fig. 4.3A, Table 4.5) for polyketide synthase. The PKS analysis online tool (http://www.nii.res.in/searchall.html) was used to predict the domain organization in each of the putative PKS ORFs (Ansari et al. 2004). Only two ORFs were found to contain the essential PKS domains. ORF 1 was found to contain the acyl carrier protein (ACP) domain while ORF 5 contained the domains in the following order: ketoreductase (KR); acyl carrier protein (ACP); ketosynthase (KS); and acyltransferase (AT). The KR and ACP domains seem to belong to a separate module attached to another elongation module containing KS and AT domains. Closest homologs of these domains are as follows: KR domain with rapamycin-module 4 (Identities: 45%, Positives: 52%), ACP domain with ascomycin-loading module (Identities: 73%, Positives: 81%), KS domain with rifamycin-module 3 (Identities: 74%, Positives: 85%) and AT domain with pimaricin-module 7 (Identities: 86%, Positives: 86%). The specificity of the

AT domain was predicted to be methylmalonyl-CoA with an active site motif QQGHSQGRSHTNV.

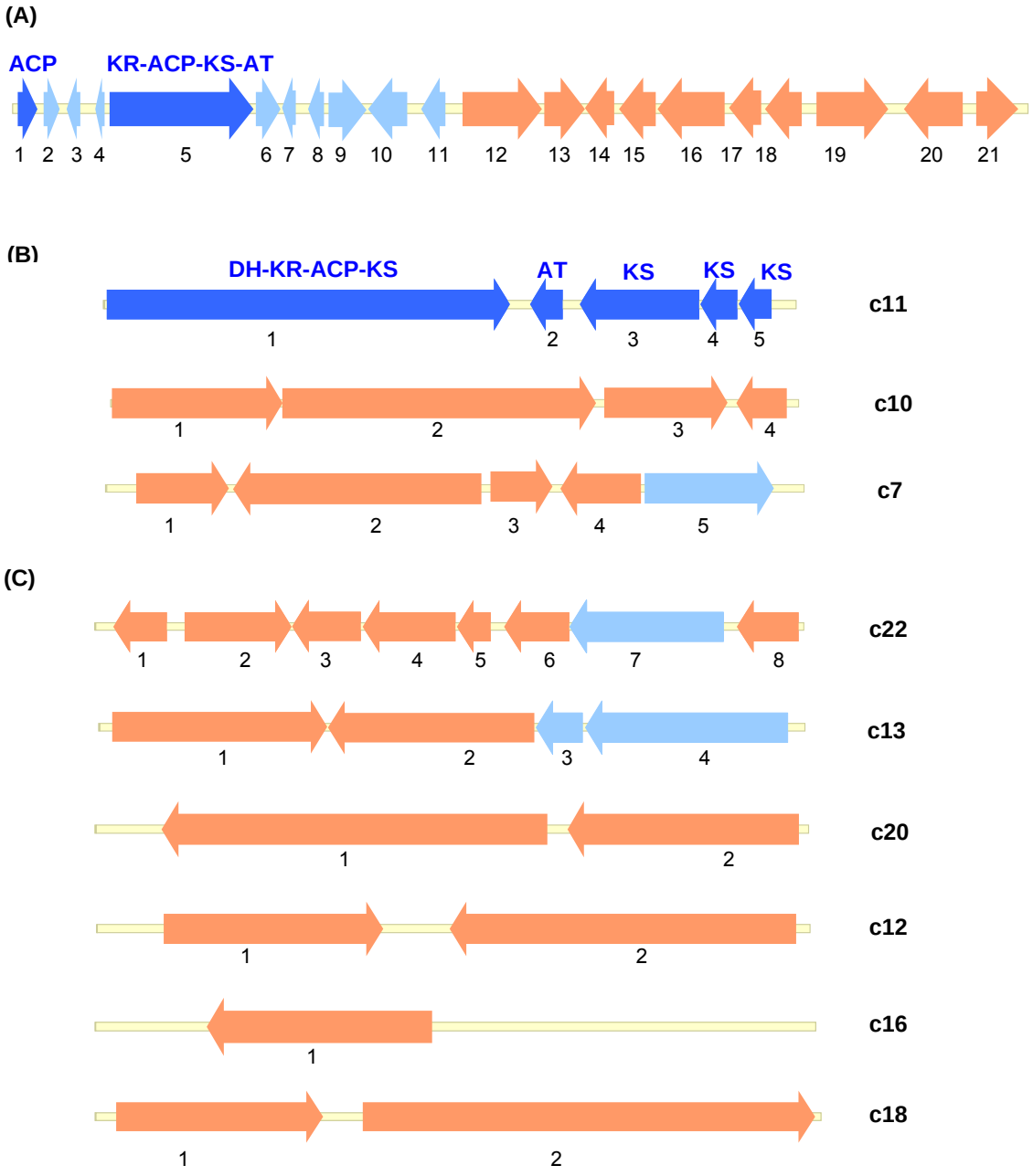


Fig. 4.3 Genetic organization (not drawn to scale) of the DNA regions isolated from the strain SPE 10-1: (A) cosmid 3/B3 (36355 bp); (B) cosmid 1/J17 contigs: c11 (6797 bp), c10 (3718 bp), c7 (2887 bp); (C) cosmid 2/I16 contigs: c22 (9331 bp), c13 (6650 bp), c20 (3136 bp), c12 (1061 bp), c16 (1054 bp), c18 (1721 bp). Genes putatively belonging to the PKS cluster are shaded in dark blue and PKS-related genes in light blue. Modules and domain organization are indicated above each ORF: ACP, acyl carrier protein; DH, dehydratase; KR, ketoreductase; KS, ketosynthase; AT, acyltransferase.

Other PKS-related genes are also found, namely those that are involved in the synthesis of 3-amino-5-hydroxybenzoic acid (AHBA) as seen in ORFs 7 to 11. This is not surprising since AHBA is the starter unit of the cebulactam polyketides and that analysis of the DNA regions surrounding the AHBA synthase gene also revealed the presence of other biosynthesis genes, confirming that indeed, the AHBA synthase gene is essential for cebulactam formation.

Among the three contigs sequenced for cosmid 1/J17 (Fig. 4.3B, Table 4.6), only contig c11 bears five PKS ORFs. In particular, ORF 1 bears a module that contains DH, KR, ACP, KS domains. Closest homologs of these domains are as follows: DH domain with amphotericin-module 3 (Identities: 47%, Positives: 59%); KR domain with ascomycin-module 2 (Identities: 67%, Positives: 75%); ACP domain with ascomycin-module 2 (Identities: 67%, Positives: 75%); KS domain with spinosad-module 7 (Identities: 71%, Positives: 83%). ORF 2 bears the AT domain with methylmalonate as predicted substrate and closest similarity with nystatin (Identities: 62%, Positives: 75%). ORFs 3-5 harbor the ketosynthase domain with following sequence similarities: amphotericin-module 1 (Identities: 87%, Positives: 88%), rifamycin-module 3 (Identities: 72%, Positives: 84%) and avermectin-module 11 (Identities: 74%, Positives: 81%), respectively. ORF 5 of contig c7 bears a beta-ketoacyl synthase which is believed to be involved in polyketide synthesis but the sequence is disrupted at the end of this contig, thus a domain was not successfully predicted. On the other hand, cosmid 2/I16 (Fig.4.3C, Table 4.7) contains only two contigs encoding for PKS-related genes. ORF 7 in contig c22 encodes for acyl CoA-transferase which is most probably involved in the loading or elongation module of the polyketide where CoA is transferred to a propionate unit into the growing ketide chain. ORFs 3 and 4 in contig c13 also encode for enzymes that are involved in the biosynthetic pathway of AHBA. Furthermore, other genes necessary for regulation and export could be identified as well. Export of the polyketide from the cell is likely to involve ORFs 16 and 17 (3/B3) and ORF 1 (2/I16_c16) predicted to function as components of ABC transporters while regulation of antibiotic production is likely to involve ORFs 2 and 3 (1/J17_c10).

Overlapping regions with cosmid 3/B3 were found by comparing nucleotide sequence similarities (99-100 %) of both ends of the cosmid with each of the contigs in cosmid 1/J17 and 2/I16. Contigs c7 and c11 (1/J17) appear to overlap with the 5'-end of 3/B3 while contig c13 (2/I16) overlaps with the 3' end of 3/B3, thus confirming results from restriction fragment length analysis. Since only 3/B3 was fully sequenced, assembly of the three cosmids to identify the whole gene cluster was unfortunately not possible.

Table 4.5 Summary of ORFs identified in cosmid 3/B3

ORF	Putative function	Most similar homolog (protein, accession no., origin)	Identities/ Positives (%)	No. of aa*	DNA coordinates
1	Beta-ketoacyl synthase	Strop_2768 (YP_001159588), *Salinispora tropica* CNB-440	58/72	234	181..882
2	PKS	LipPks2 (ABB05103), *Streptomyces aureofaciens*	48/56	191	1105..1677
3	Beta-ketoacyl synthase	Franean1_4838 (YP_001509110), *Frankia* sp. EAN1pec	47/55	161	1925..2407
4	PKS	CONC (AAZ94388), *Streptomyces neyagawaensis*	62/74	107	2957..3277
5	PKS	SACE_2875 (YP_001105078), *Saccharopolyspora erythraea* NRRL 2338	59/68	1725	3453..8627
6	Unknown function	MbcB (ACF35444), *Actinosynnema pretiosum* subsp. *pretiosum*	38/45	293	8715..9593
7	Aminohydroquinate synthase	MbcO (ACF35443), *Actinosynnema pretiosum* subsp. *pretiosum*	76/86	165	9470..9964
8	Phosphatase-like protein	SACE_2872 (YP_001105075), *Saccharopolyspora erythraea* NRRL 2338	72/82	192	10682..11257
9	Oxidoreductase	Francci3_0430 (YP_479546), *Frankia* sp. CcI3	64/71	454	11310..12671
10	AHBA synthase	FRAAL0914 (YP_711176), *Frankia* alni ACN14a	77/88	464	12488..13879
11	RifN AHBA kinase	SACE_3869 (YP_001105072), *Saccharopolyspora erythraea* NRRL 2338	64/71	292	14623..15498
12	LuxR transcriptional regulator	SACE_2866 (YP_001105069), *Saccharopolyspora erythraea* NRRL 2338	65/76	947	16107..18947
13	MFS transporter	FRAAL0341 (YP_710628), *Frankia* sp. alni ACN14a	55/69	483	19218..20666
14	Monooxygenase	Nfa23860 (YP_118597), *Nocardia farcinica* IFM 10152	48/62	347	20734..21774
15	Monooxygenase	CMS_1261 (YP_001709999), *Clavibacter michiganensis* subsp. *sepedonicus*	44/56	432	21725..23020
16	ABC transporter	Francci3_2764 (YP_481853), *Frankia* sp. CcI3	53/64	799	23096..25492
17	Membrane transporter	Francci3_2766 (YP_481855), *Frankia* sp. CcI3	56/73	380	25482..26621
18	Extracellular solute-binding protein	Francci3_2767 (YP_481856), *Frankia* sp. CcI3	50/66	437	26754..28064
19	SARP transcriptional regulator	Franean1_4842 (YP_001509114), *Frankia* sp. EAN1pec	43/53	859	28788..31364
20	H+/citrate symporter	SACE_1977 (YP_001104212), *Saccharopolyspora erythraea* NRRL 2338	80/87	701	31928..34030
21	Response regulator	SACE_1975 (YP_001104210), *Saccharopolyspora erythraea* NRRL 2338	73/85	318	35062..36015

*aa = amino acids

Table 4.6 Summary of ORFs identified in contigs of cosmid 1/J17

Contig	ORF	Putative function	Most similar homolog (protein, accession no., origin)	Identities/ Positives (%)	No. of aa*	DNA coordinates
c11	1	PKS	SSAG_05828 (EDX26173), *Streptomyces* sp. Mgl	52/63	1321	24..3986
	2	PKS	CONC (AAZ94388), *Streptomyces neyagawaensis*	60/69	107	4184..4504
	3	PKS	GelA (ABB86408), *Streptomyces hygroscopicus*	64/75	1176	4672..5847
	4	PKS	PKS1 (ABB88522), *Streptomyces aculeolatus*	76/84	122	5857..6222
	5	PKS	NemA3 (BAF85843), *Streptomyces cyaneogriseus*	74/82	109	6149..6475
c10	1	H+/citrate symporter	SACE_1977 (YP_001104212), *Saccharopolyspora erythraea* NRRL 2338	76/84	308	1..924
	2	Two-component sensor kinase	SACE_1976 (YP_001104211), *Saccharopolyspora erythraea* NRRL 2338	71/83	566	1007..2704
	3	Response regulator	SACE_1975 (YP_001104210), *Saccharopolyspora erythraea* NRRL 2338	73/85	223	2704..3372
	4	Transcriptional regulator	SSAG_04054 (EDX24263), *Streptomyces* sp. Mgl	47/62	92	3379..3654
c7	1	HxlR transcriptional regulator	Franean1_2255 (YP_001506596), *Frankia* sp. EAN1pec	69/80	128	122..505
	2	SARP transcriptional regulator	Franean1_4842 (YP_001509114), *Frankia* sp. EAN1pec	43/55	342	484..1509
	3	Transcriptional activator	Asm18 (AAM54096), *Actinosynnema pretiosum*	55/63	86	1660..1917
	4	Transcriptional activator	Asm18 (AAM54096), *Actinosynnema pretiosum*	57/65	111	1894..2226
	5	Beta-ketoacyl synthase	Franean1_4838 (YP_001509110), *Frankia* sp. EAN1pec	58/66	178	2197..2730

*aa = amino acids

Table 4.7 Summary of ORFs identified in contigs of cosmid 2/I16

Contig	ORF	Putative function	Most similar homolog (protein, accession no., origin)	Identities/ Positives (%)	No. of aa*	DNA coordinates
c22	1	Urease subunit	Mpop_3208 (YP_001925895), *Methylobacterium populi* BJ001	56/71	236	233..940
	2	Urease subunit	UreC (YP_001851021), *Mycobacterium marinum*	65/76	472	1203..2618
	3	Urease accessory	UreF (NP_828283), *Streptomyces avermitilis* MA-4680	53/67	302	2609..3514
	4	Urease accessory	UreG (YP_481754), *Frankia* sp. CcI3	74/83	407	3518..4738
	5	Hypothetical protein	BURPS1710b_1934 (YP_333333), *Burkholderia pseudomallei*	38/42	149	4757..5203
	6	Transcriptional regulator	SACE_3039 (YP_001105242), *Saccharopolyspora erythraea* NRRL 2338	69/77	286	5381..6238
	7	Acyl CoA-transferase	SACE_3040 (YP_001105243), *Saccharopolyspora erythraea* NRRL 2338	77/86	675	6247..8271
	8	Dicarboxylate carrier	SACE_3042 (YP_001105245), *Saccharopolyspora erythraea* NRRL 2338	75/84	271	8445..9257
c13	1	SARP transcriptional regulator	Franean1_4842 (YP_001509114), *Frankia* sp. EAN1pec	45/56	675	166..2190
	2	Extracellular solute-binding protein	Francci3_2767 (YP_481856), *Frankia* sp. CcI3	46/62	648	2154..4097
	3	Phosphatase	RifM (AAC01721), *Amycolatopsis mediterranei*	78/88	146	4115..4552
	4	3-Dehydroquinate synthase	SACE_2686 (YP_001105071), *Saccharopolyspora erythraea* NRRL 2338	75/83	636	4581..6488
c20	1	Two-component sensor kinase	SACE_1976 (YP_001104211), *Saccharopolyspora erythraea* NRRL 2338	65/76	566	294..1991
	2	H+/citrate symporter	SACE_1977 (YP_001104212), *Saccharopolyspora erythraea* NRRL 2338	77/85	340	2074..3093
c12	1	HxlR transcriptional regulator	Franean1_2255 (YP_001506596), *Frankia* sp. EAN1pec	72/83	99..425	99..425
	2	SARP Transcriptional regulator	Franean1_4842 (YP_001509114), *Frankia* sp. EAN1pec	44/55	172	525..1040
c16	1	Mannitol dehydrogenase	SAMR1012 (CAJ88721), *Streptomyces ambofaciens* ATCC 23877	57/65	110	163..492
c18	1	Membrane transport	SAMR1013 (CAJ88722), *Streptomyces ambofaciens* ATCC 23877	73/84	164	48..539
	2	L-idonate 5-dehydrogenase	SAMR1014 (CAJ88723), *Streptomyces ambofaciens* ATCC 23877	74/82	358	634..1707

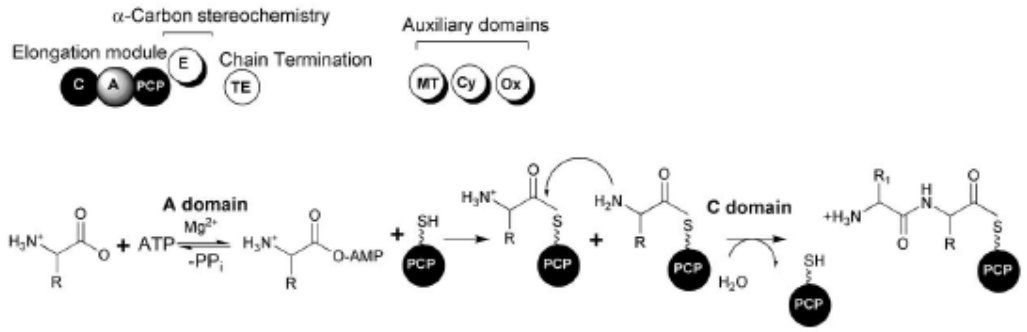
*aa = amino acids

4.3 Discussion

The majority of bioactive secondary metabolites produced by bacteria appear to be structurally biosynthesized by nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS). PCR primers designed for screening NRPS and PKS systems in actinomycetes have been used to identify isolates with metabolic potential (Ayuso-Sacido and Genilloud 2005; Ayuso et al. 2005). Several actinomycete strains cultivated in this Ph.D. study showed the presence of NRPS and PKS systems clearly indicating the potential of these isolates to produce peptides and/or polyketides provided that these are cultivated under appropriate conditions that allows expression of their respective biosynthetic genes. Despite the fact that majority of the strains cultivated in this study are taxonomically known actinomycetes, these strains still hold great potential in producing peptides and/or polyketides with novel chemistry and significant biological activities.

Interestingly, the biosynthesis of peptides and polyketides by enzymes are organized in a modular fashion utilizing specific domains to sequentially catalyze the condensation of simple carboxylic acids for PKS systems or amino acid building blocks for NRPS systems into a growing chain. Nonribosomal peptide synthetase systems, for example consists of a minimal chain elongation module with three essential domains: adenylation, thiolation and condensation. The adenylation domain (A) selects a specific amino acid activating it as an amino acyl adenylate which is then transferred to the phosphopantethiene group of the post-translationally modified peptidyl carrier protein (PCP) or thiolation domain (T). The condensation domain (C) catalyzes the peptide bond formation between amino acids in adjacent modules. The chain is elongated successively and released finally by an integrated thioesterase (TE) domain or a separate TE producing either a linear or cyclic peptide. Additional structural diversity is introduced by modification of the growing chain catalyzed by various auxiliary domains such as epimerization (E), *N*-methylation (MT), cyclization (Cy) and oxidoreductase (Ox) domains (Stachelhaus and Marahiel 1995; Marahiel et al. 1997; Konz and Marahiel 1999; Mootz et al. 2002; Challis and Naismith 2004; Salomon et al. 2004) (Fig. 4.4A).

A. NRPS assembly system



B. PKS assembly system

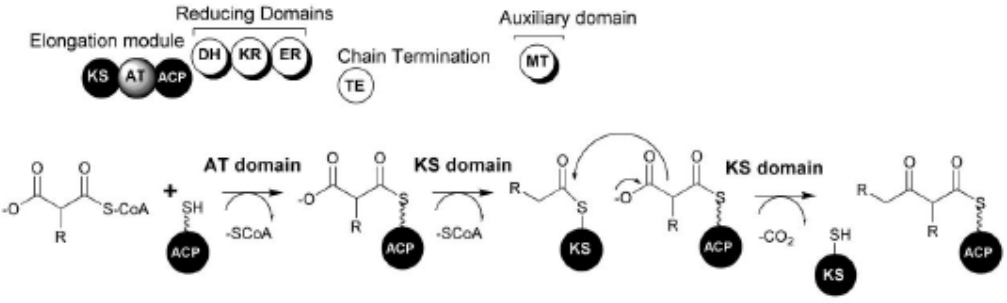


Fig. 4.4 Domain organization and mechanisms for modular biosynthetic pathway systems (A) Nonribosomal peptide synthetase (NRPS) and (B) Polyketide synthase (PKS). C, condensation; A, adenylation; PCP, peptidyl carrier protein; E, epimerase; Cy, cyclization; Ox, oxidoreductase; KS, ketosynthase; AT, acyl transferase; ACP, acyl carrier protein; DH, dehydratase; KR, ketoreductase; ER, enoyl reductase; TE, thioesterase; MT, methyl transferase. (Salomon et al. 2004)

Furthermore, polyketides are also synthesized on multi-modular enzymes called polyketide synthases. Each PKS module is responsible for the incorporation of one acetate or propionate unit into the growing ketide chain (Schwarzer and Marahiel 2001). The initiation module starts with the selection of the acyltransferase domain (AT) of an activated acyl-CoA monomer which then transfers the acyl-CoA to the adjacent acyl carrier protein (ACP). This chain is transferred from the ACP to the upstream ketosynthase domain (KS) required for catalyzing the decarboxylation of the carboxyclic acid and subsequent Claisen condensation between the growing chain and the downstream ACP extender unit. Structural diversity is introduced by optional reductive domains such as: keto-reductase (KR) reducing the carbonyl to hydroxyl group; dehydratase (DH) dehydrating alcohol to form a form a double bond; and enoyl reductase (ER) reducing the double bond to a fully saturated methylene. Other unusual starter molecule such as amino acids and fatty acid derivatives are also incorporated to produce diverse polyketide structures. Additional auxiliary domains include methyl transferase

(MT). The complete elongated and functionalized chain is often transferred to a final thioesterase domain (TE) capable of catalyzing the hydrolytic release of a linear compound or a hydrolytic release coupled with cyclization to generate a macrolactone structure (Hopwood 1997; Rawlings 1997; Hutchinson 1999; Salomon et al. 2004; Sherman and Smith 2006; Ridley et al. 2008) (Fig. 4.4B). There are three major classes of PKS systems (type I, II and III) according to their mode of synthesis and structural type of product. Type I PKSs in bacteria are multi-enzyme complexes organized into individual linear modules responsible for a single, specific chain elongation process and β-carbonyl post condensation modification. Type II, or aromatic PKSs are complexes of mono-functional proteins characterized by their iterative use of a single set of distinct enzymes to construct polyketide chains which are then cyclized to produce small molecules with aromatic ring systems. The third type belongs to the chalcone and stilbene synthase family of enzymes from plants and unlike the Type I and II systems, contain one protein, a single domain and active site to carry out the three essential mechanisms of chain initiation, elongation and cyclization (Salomon et al. 2004).

NRPS system of Streptomyces sp. strain Aer003

An interesting type of NRPS system was detected and identified in *Streptomyces* sp. strain Aer003 and was found to be widely distributed in different sponge samples, an ascidian as well as from seawater as supported by phylogenetic analysis of their adenylation domains. These results also indicate that the peptide encoded by this biosynthetic cluster is of actinobacterial origin and that this could be produced by actinomycetes associated with the invertebrate samples that are presumably not of symbiont nature since the same NRPS system was found in seawater. Notably, the overlap between PCR-based, culture-independent identification of a widely distributed seawater and sponge-associated bacteria and successful isolation of a corresponding strain is extremely rare. So far, this is the first report of a widely distributed NRPS system present in a cultivated actinomycete strain as well as in different sponge species.

Since an entire biosynthetic cluster could not be constructed from the sequence data of the two cosmids, prediction of the peptide product is therefore not possible. The only speculations that can be drawn from the individual modules are the amino acids that are incorporated into the peptide chain. In most NRPS systems, the organization of the modules maps in a 1:1 manner to the amino acid sequence of the peptide products following the co-linearity principle and therefore allowing the possibility of assigning a specific amino acid activation function to each module (Challis et al. 2000). From the partial cluster flanked by the NRPS regions in cosmid 1/C13, 4-hydroxy-phenylglycine,

aspartic acid and an unknown modified amino acid constitute the peptide product, without any modification after release of the product. On the other hand, the peptide product of the 2/E12 NRPS cluster consists also of 4-hydroxy-phenylglycine and an unknown modified amino acid. Additionally, the thioester-bound amino acid to 4'-phosphopantetheine co-factor in the thiolation domain is converted from the L- into the D-configuration as evidenced by the presence of the additional epimerization domain in both modules found in this cosmid. Whether the peptide has additional amino acids incorporated still remains to be elucidated by finding the preceding modules that make up the entire cluster. It also remains unclear whether the peptide is structurally related to enduracidin as previously shown by phylogenetic analysis of the A domains with that of *Streptomyces fungicidus.* It can be speculated further that the cosmids are part of different NRPS gene clusters and that these probably encode a large peptide molecule.

In a previous study, the crude ethyl acetate extract obtained from *Streptomyces* sp. strain Aer003 exhibited antibacterial activity against *Staphylococcus aureus* (Scheuermayer 2006). It is therefore interesting to pursue the chemical analysis of the metabolite to identify the compound responsible for the biological activity. The NRPS gene cluster identified in this particular strain provides a valuable hint that the bioactive compound could possibly be the same peptide encoded by the said cluster. Identification of the entire biosynthetic cluster is beyond the scope of this Ph.D. study but nevertheless warrants further investigation. Primers flanking the ends of the two NRPS-bearing cosmids can be designed to screen the genomic library to find overlapping cosmids to assemble the rest of the NRPS cluster. Prediction of the chemical structure and biosynthetic pathway of the encoded peptide will therefore be possible having the sequence of a complete biosynthetic cluster and the isolation of such peptide from the strain will be a worthwhile task.

PKS system of Saccharopolyspora cebuensis SPE 10-1^{T}

The second actinomycete strain where the biosynthetic gene cluster was investigated in detail is *Saccharopolyspora cebuensis* SPE 10-1. A type I PKS system was detected in this isolate. Furthermore, the strain has also been found to produce the macrolactam polyketides, cebulactams A1 and A2 (Pimentel-Elardo et al. 2008). Since the entire PKS cluster was not completely sequenced, the ASMPKS (Analysis System for Modular Polyketide Synthesis) (Tae et al. 2007) online tool (http://gate.smallsoft.co.kr:8008/~hstae/asmpks/index.html) was used to predict the PKS domain organization and biosynthetic pathway for these polyketides and then compared with identified PKS genes in the sequenced cosmids. As is typical of all ansamycins, the

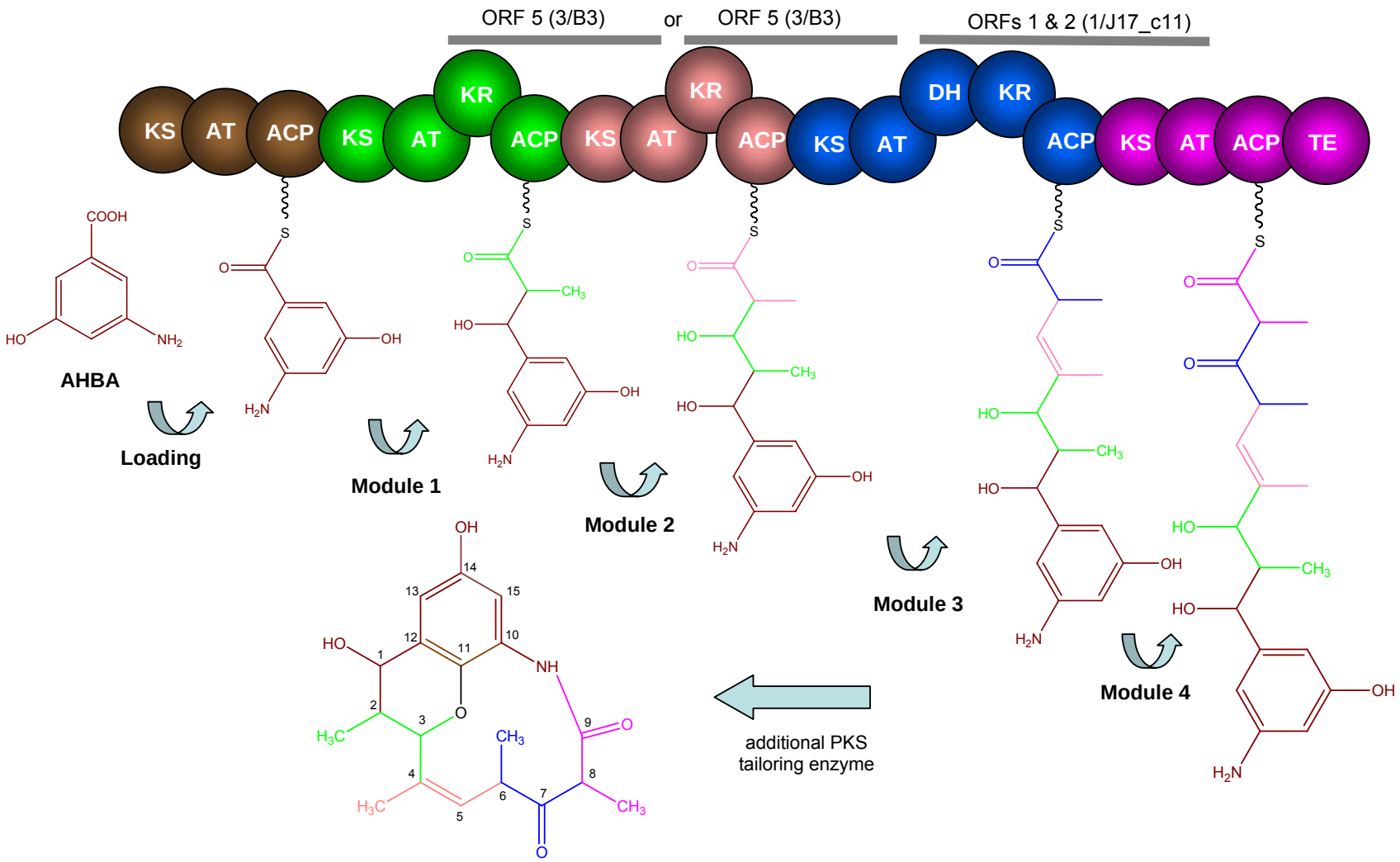


Fig. 4.5 Proposed PKS assembly and pathway for cebulactam biosynthesis. AHBA, 3-amino-5-hydroxybenzoic acid; KS, ketosynthase; AT, acyltransferase; ACP, acyl carrier protein; KR, ketoreductase; DH, dehydratase, TE, thioesterase. Lines above the domain architecture indicate where some of these domains have been identified in the open reading frames (ORF) of the sequenced cosmids (3/B3 and 1/J17_c11).

carbon skeleton of cebulactams from *S. cebuensis* strain SPE 10-1^T is assembled from the 3-amino-5-hydroxybenzoic acid (AHBA) starter unit (Kim et al. 1998) followed by successive condensation of two-carbon building blocks in four chain-elongation steps each utilizing methylmalonyl extender units (Fig. 4.5). Each module loads one methylmalonyl unit into the growing ketide chain. The involvement of this particular extender unit is further confirmed by the identification of two methylmalonate-specific AT domains in the sequenced cosmids. In this proposed pathway, the activated form methylmalonyl-CoA is added to the following carbon skeletons: C2 (module 1), C4 (module 2), C6 (module 3) and C8 (module 4). Modification of the ketide chain appears to involve the reduction of the β-keto function (modules 1-3) by the auxiliary KR domain as well as dehydratization of the β-hydroxyl group (module 3) by the DH domain in the polyketide assembly. The involvement of these domains is confirmed by their presence in the sequenced cosmids such as ORF 5 (3/B3) bearing KR-ACP-KS-AT either flanking modules 1 and 2 or modules 2. Furthermore, ORFs 1 and 2 (1/J17_c11) bearing DH-KR-ACP-KS-AT can be found in modules 3 and 4 (Fig. 4.5). Final product release is made possible by the TE or thiosterase domain. As with all ansamycins, the C-terminus of the assembled polyketide eventually forms an amide linkage to the amino group of the AHBA moiety to close the macrolactam ring (Kim et al. 1998). An additional PKS tailoring maybe involved in the oxidation of the aromatic system and cyclization as well as an isomerase enzyme to account for the production of the two constitutionally identical cebulactam polyketides by *Saccharopolyspora cebuensis*. Although not all predicted modules are found in the sequenced cosmids, these domains displayed close homologies with biosynthetic gene clusters such as the archetype ansamycin, rifamycin produced by *Amycolatopsis mediterranei* (August et al. 1998) and structurally striking similarities with the benzoquinone ansamycins geldanamycin (Rascher et al. 2003) and herbimycin (Rascher et al. 2005) both produced by *Streptomyces hygroscopicus*. Only one set of AHBA synthesis genes was found in the cosmids indicating that there is a single PKS cluster involved in the synthesis of the cebulactams. The same set of AHBA synthesis genes can also be found clustered together in the rifamycin biosynthetic cluster (August et al. 1998).

Identification of the whole biosynthetic cluster encoding for the cebulactams is indeed worthwhile pursuing. Additional primers can be designed to find cosmid clones that harbor overlapping regions with the current cosmid sequences to complete the missing domains in the different modules. With the complete PKS gene cluster, the proposed PKS assembly as well as the cebulactam biosynthetic pathway described here can thus be

confirmed. Furthermore, gene disruption studies can be carried out to prove the identity of the cluster and its involvement in the biosynthesis of cebulactams or other polyketides, if any, that this organism may be capable of producing.

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Chapter 5
General discussion and outlook

5.1 Secondary metabolite recovery by cultivation

Marine actinomycetes are abundant in various ocean sediments (Grein and Meyers 1958; Mincer et al. 2002; Fiedler et al. 2005; Jensen et al. 2005; Maldonado et al. 2005; Bredholdt et al. 2007), with an estimated >13,000 different actinobacterial taxonomic units, a great proportion of which are predicted to represent novel species and genera (Stach and Bull 2005). Actinomycetes, or members of the phylum *Actinobacteria* have also been derived from marine sponges using cultivation-dependent and cultivation-independent approaches (Webster and Hill 2001; Webster et al. 2001; Hentschel et al. 2002; Kim et al. 2005; Montalvo et al. 2005; Zhang et al. 2006; Jiang et al. 2007; Xin et al. 2008). The strains cultivated in this Ph.D. study have not been isolated from sponges before. Their absence in the sponge-derived 16S rRNA gene libraries suggests that the strains are probably not sponge symbionts but rather transient bacteria that were incidentally present during the collection of the sponge samples. It is also highly likely that these strains are present in the surrounding seawater or sediments and that there is a given probability of re-isolating the same strains.

The isolation strategy used in this study has yielded six diverse actinomycete genera comparable to those isolated from previous cultivation-dependent studies (Webster et al. 2001; Montalvo et al. 2005; Zhang et al. 2006; Jiang et al. 2007). Several known actinomycete species were cultivated, the majority of which belonged to the ubiquitous genus *Streptomyces*. Interestingly, two novel actinomycete taxa were isolated, namely *Saccharopolyspora cebuensis* (Pimentel-Elardo et al. 2008c), the first obligate marine strain of this genus and *Streptomyces axinellae* (Pimentel-Elardo et al. 2008b). There are however, other cultivation media and pretreatments that can be employed aside from those used in this Ph.D. study to isolate taxonomically novel and rare actinomycetes. For example, the use of low-nutrient media (Santavy and Colwell 1990; Olson et al. 2000) can be used to selectively isolate for oligotrophic bacteria that have the competitive advantage to reproduce even with the minimal concentrations of organic substances in the medium (Kuznetsov et al. 1979). The use of humic acid-vitamin agar medium facilitates the efficient recovery of actinomycetes and promotes sporulation while restricting the growth of non-filamentous bacterial colonies. Humic acids are heterogenous cross-linked polymers that are generally resistant to biological decomposition and are utilized by actinomycetes as sources of carbon and nitrogen in the

medium (Hayakawa 2008). Furthermore, the use of pretreatments such as UV irradiation and high frequency radiations have been shown to effectively stimulate the germination and outgrowth of spores of rare actinomycete genera (Bredholdt et al. 2007). The chemotactic method using xylose and γ-collidine has also been proven to be effective for isolating motile and novel actinomycetes (Takahashi and Omura 2003; Hayakawa 2008). The cultivation media can also be supplemented with other antibiotics such as novobiocin, rifampicin and chlortetracycline and gellan gum can be used instead of agar as solidifying agent to select for specific actinomycete genera (Takahashi and Omura 2003).

The cultivation of *Saccharopolyspora cebuensis* and *Streptomyces axinellae*, producing cebulactams (Pimentel-Elardo et al. 2008a) and tetromycin derivatives, respectively further underlines the potential of taxonomically novel actinomycetes to produce new secondary metabolites. This can be explained by the premise that novel species will most probably contain unique compounds and that the evolution of novel secondary metabolites acts as the driving force for bacterial speciation (Czaran et al. 2002; Bull and Stach 2007). It is a given fact that actinomycetes are known to produce bioactive secondary metabolites, particularly antibiotics. The genus *Streptomyces* produces 80% of currently characterized actinomycete natural products with a predicted diversity exceeding 100,000 (Watve et al. 2001) and with other smaller contributions from genera such as *Saccharopolyspora, Amycolatopsis, Micromonospora* and *Actinoplanes* (Challis and Hopwood 2003). Secondary metabolites are non-essential for the growth of the producing organism and the metabolic pathways are only activated during particular stages of growth or during periods of stress caused by nutritional limitation or microbial attack (Mann 1987). Furthermore, the biosynthetic pathways for secondary metabolite production are usually activated in the late logarithmic to the stationary phase of the growth period, after cell division and biomass accumulation have ceased (Yarbrough et al. 1993). For example, *Streptomyces* antibiotics are typically produced in small amounts at the transition phase when the growth of the vegetative mycelium is slowing as a result of nutrient exhaustion while the aerial mycelium starts to develop at the expense of the nutrients released by the breakdown of the vegetative hyphae (Miguelez et al. 2000; Challis and Hopwood 2003). These antibiotics have been proposed to defend the food source from competing microorganisms that are attracted to the amino acids, sugars and other small molecules arising from the degraded vegetative mycelium (Shi and Zusman 1993; Challis and Hopwood 2003).

Given the metabolic diversity of actinomycetes, it is highly probable that the strains cultivated in this Ph.D. study are capable of producing several additional compounds. The use of a starch-based M1 medium (Mincer et al. 2002) has been shown to be effective for cultivating marine actinomycetes and promote secondary metabolite production in this study as well as in other previous studies (Mincer et al. 2002; Soria-Mercado et al. 2005; Kwon et al. 2006). Nevertheless, the culture media and other growth conditions can also be manipulated not only to cultivate novel taxa such as the alternative methods previously mentioned, but also to fully exploit the metabolic potential of actinomycetes. Strain optimization for secondary metabolite production was not the major aim of this study, yet this endeavor is worthwhile to pursue. In fact, it has been shown that a single strain has the potential to produce various compounds when grown under different culture conditions. The so-called OSMAC ('one strain-many compounds') approach has yielded more than 100 compounds belonging to more than 25 different structural classes from only six different microorganisms (Bode et al. 2002). Metabolite diversity and production are functions of the biosynthetic capabilities of the organism and thus, fermentation parameters can be manipulated to encourage the production of diverse secondary metabolites (Yarbrough et al. 1993). Previous studies have also shown that even small changes in the cultivation conditions can completely shift the metabolic profile of various microorganisms, including actinomycetes (Bode et al. 2002). The organism should be first grown in a seed medium that has been optimized to promote cell growth and once the organism has grown, these can be transferred to multiple production media, varying for example in carbon and nitrogen sources and other cultivation parameters (i.e. $CaCO_3$ or Al_2O_3 and pH alteration) to promote the production of different metabolites. However, this approach poses practical limitations particularly when handling numerous strains. For example, a limited number of growth conditions can only be applied to each strain. Therefore, there is a need for a high throughput screening that allows the exhaustive analyses of the effects of fermentation on secondary metabolite production of numerous strains. The application of HPLC for the selection of production media for actinomycetes based on their metabolite profiles provides a useful tool for identifying growth conditions that support the synthesis of secondary metabolites (Tormo et al. 2003). Tormo and co-workers (2003) have shown that each strain can be grown in a panel of ten media or different culture conditions in a small-volume format and the metabolite profiles of each extract can be analyzed by high pressure liquid chromatography coupled with photodiode array detection (HPLC-DAD) in a semi-automated fashion. The media or growth conditions with the most diverse and least overlapping chemical profiles can then be selected and scaled-up to the appropriate volumes required for secondary metabolite isolation. The use of mass spectrometry (i.e. direct infusion electrospray) can be used to

complement this method to guide the development of improved conditions for expression of secondary metabolites from actinomycetes (Higgs et al. 2001; Zahn et al. 2001). Efficient dereplication protocols including access to current natural products databases should also be designed to minimize re-isolation of previously identified compounds (VanMiddlesworth and Cannell 1998). Another consideration is finding the optimal method to capture the secondary metabolites for bioactivity screening. The metabolites of interest may include intracellular molecules and/or those that are secreted into the fermentation media. In this Ph.D. study, both types of metabolites were extracted by lysis of the cells with methanol to release intracellular components followed by organic solvent partitioning of the whole broth or in the case of solid cultures, direct extraction of the mycelial mass with ethyl acetate. Methanol has been shown to dissolve most natural products at the same time enhancing their release from the cellular matrix or cell surface by permeabilizing the physical barrier of the cell walls (Cannell 1998) and the bulk of the insoluble material was then removed by filtration. There are however, alternative methods to capture and concentrate as many diverse metabolites as possible with the general consideration that the method should not interfere with succeeding bioassays. One alternative is filtration of the broth supernatant through a high molecular weight exclusion filter with subsequent freeze-drying of the filtrate. Another method involves mixing of the whole broth with polystyrene resin followed by washing with water and eluting the bound materials using methanol or acetone (Yarbrough et al. 1993; Fiedler et al. 2005; Soria-Mercado et al. 2005; Kwon et al. 2006). The latter method has been utilized in this Ph.D. study to isolate the cebulactams from the strain *S. cebuensis* (data not shown). The use of Amberlite XAD-7 adsorbent resin (Sigma) and elution with methanol produced low yields of the compounds as compared to solvent partitioning with ethyl acetate which proved to be a more effective extraction method. Therefore, each strategy has its own advantages as well as limitations and metabolite properties vary among strains. Factors such as efficiency in optimum isolation of the metabolites, assay compatibility and cost should thus be considered.

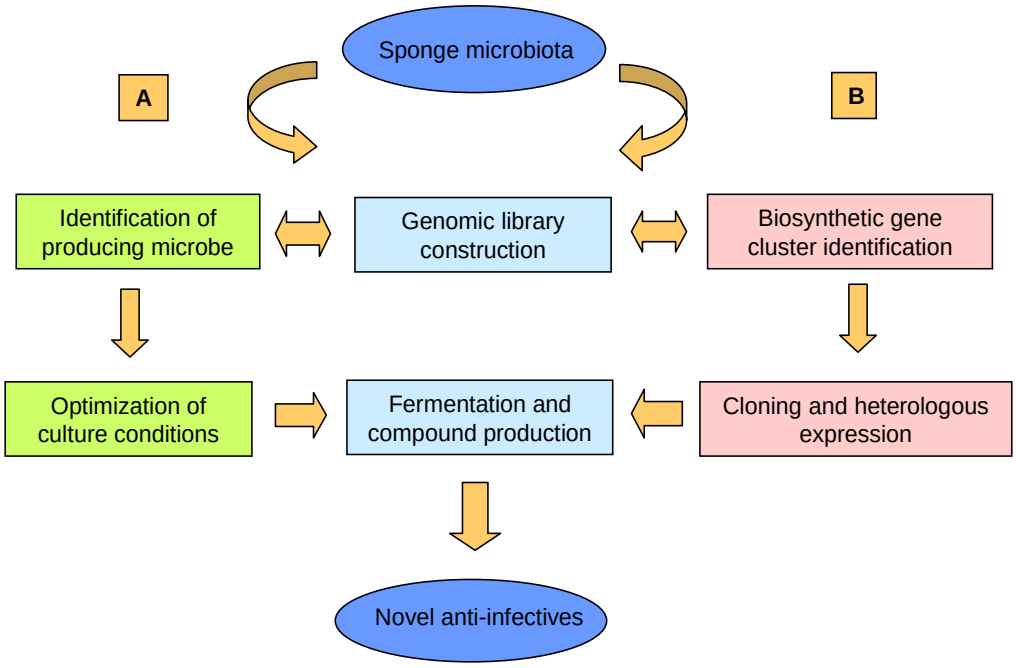

Fig. 5.1 Culture-dependent (A) and culture-independent (B) strategies to access the biosynthetic potential of sponge-associated microorganisms

5.2 Secondary metabolite recovery by cultivation-independent techniques

Genome sequencing of actinomycetes and other microorganisms has revealed several biosynthetic enzymes unrelated to known metabolites, indicating that natural products are still underexplored (Van Lanen and Shen 2006). Actinomycetes producing NRPS and PKS-derived compounds have multiple biosynthetic gene clusters, consisting of an average of 12 clusters per genome (Farent and Zazopoulos 2005; Bull and Stach 2007). Furthermore, actinomycetes with large genomes devote 5-10% of their coding capacity to secondary metabolism (Bentley et al. 2002; Ikeda et al. 2003; Oliynyk et al. 2007; Udwary et al. 2007; Baltz 2008). The field of genomics has also paved the way for the discovery of orphan biosynthetic gene clusters that are phenotypically silent but capable of activation by fermentation and/or genetic manipulation (Bull and Stach 2007). For instance, when 60 actinomycete species were screened by traditional fermentation procedures, these were found to produce 65 natural products which is in contrast to the results obtained from genome scanning of the same species revealing ~700 natural product biosynthetic gene clusters including those found by fermentation (Farent and Zazopoulos 2005).

It is therefore not surprising that NRPS and PKS systems were detected in all of the actinomycete strains cultivated in this Ph.D. study. In particular, an interesting NRPS system found in *Streptomyces* sp. strain Aer003 was also detected in several sponge samples, in an ascidian and in seawater revealing the wide distribution of such system. These results also indicate that the NRPS system is of actinobacterial origin, thus pointing out the possible involvement of actinomycetes in the secondary metabolism of these marine invertebrates. Sequencing results of the NRPS-bearing cosmids from the genomic library of the strain indicated the involvement of different gene clusters encoding for a large peptide molecule. However, identification of the entire biosynthetic cluster as well as prediction of the encoded peptide were beyond the scope of this PhD study but nevertheless warrants further investigation. On the other hand, the polyketide gene cluster of *Saccharopolyspora cebuensis* strain SPE 10-1^T was investigated. Sequencing results of the PKS-bearing cosmids from the genomic library of this strain pointed out the involvement of 3-amino-5-hydroxybenzoic acid (AHBA) as the starter unit of the polyketide. This is further supported by the isolation of the novel polyketides, cebulactam A1 and cebulactam A2 (Pimentel-Elardo et al. 2008a) produced by the strain. Moreover, prediction of the cebulactam biosynthetic pathway also revealed the involvement of AHBA synthase in its initiation module followed by the successive condensation in a series of chain-elongation steps each utilizing methylmalonyl extender units. The involvement of auxiliary domains and other PKS tailoring enzymes is also predicted to be responsible for the novelty of the compound structures. Biosynthetic feeding studies or gene disruption work should be carried out to provide actual insights into the polyketide biosynthetic pathway and eventually prove the identity of the gene cluster present in this novel actinomycete strain.

There are different strategies involved in developing an integrated biosynthetic study that can be used to fully exploit the secondary metabolite potential of sponge-associated microorganisms (Fig. 5.1). Using the cultivation-independent approach, gene cluster sequences can be probed from metagenomic libraries constructed for example, from the microbial consortia of marine sponges. The cluster sequences can be compared with those found in online databases to identify related genes and their source organisms. The compound-specific genes combined with 16S rRNA probes can be used to identify and localize the source of the cluster (Salomon et al. 2004). The identification of the likely microbial producer can direct efforts to better replicate appropriate conditions to obtain the producing microorganism in culture using the cultivation-dependent approach. Moreover, identification of the gene cluster will provide valuable information about the secondary metabolite. A major step in identifying specific gene clusters is predicting the

biosynthetic pathway of the encoded secondary metabolites. This is crucial for the identification of specific key enzymes that can be used as molecular probes to screen genomic libraries (Salomon et al. 2004). Furthermore, heterologous expression of the gene clusters in suitable hosts for compound production poses a major hurdle. The implementation of a genetically similar host strain should maximize the potential for productive transcription, translation and finally, metabolite production (Fortman and Sherman 2005). Heterologous expression has been achieved from entire biosynthetic clusters of actinomycetes (Martinez et al. 2004) and circumvents the need for numerous fermentations to obtain the compound of interest. More importantly, this valuable technique offers a sustainable supply of important bioactive metabolites.

In summary, this Ph.D. study has clearly shown that diverse actinomycete genera are associated with marine sponges, highlighting the cultivation of two new actinomycete species. Although their functions in sponges are not known, it is evident that these actinomycetes play an important role in secondary metabolite production. The strains produced diverse chemical structures including new (cebulactams A1 and A2, tetromycin derivatives) and known (valinomycin, staurosporine, cycloisoleucylprolyl, butenolide) compounds with interesting anti-infective properties, underscoring the potential of the novel actinomycete taxa to produce novel compounds. Lastly, the presence of biosynthetic gene clusters identified in this study substantiates the biosynthetic potential of actinomycetes to produce exploitable natural products and hopefully provide a sustainable supply of anti-infective compounds.

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ANNEX

I. Abbreviations and Acronyms

δ_C	chemical shift (ppm), ^{13}C NMR
δ_H	chemical shift (ppm), ^{1}H NMR
°C	degree Celsius
ASW	artificial seawater
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
bp	base pair
br (NMR)	broad
ca.	approximately
^{13}C NMR	correlation spectroscopy
COSY	carbon nuclear magnetic resonance
d (NMR)	doublet
dd (NMR)	double doublet
dq (NMR)	double quartet
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EtOH	ethanol
EtOAc	ethyl acetate
g	gram
h	hour
H_2O_d	distilled water
H_2O_{dd}	double distilled water
HMBC	heteronuclear multiple bond correlation
^{1}H NMR	proton nuclear magnetic resonance
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
Hz	hertz
IC_{50}	concentration required for 50% inhibition
J	coupling constant
kb	kilobase
L	liter
M	molar
m (NMR)	multiplet
MHz	megaherz

MeOH	methanol
mg	milligram
MIC	minimum inhibitory concentration
min	minute
ml	milliliter
mM	millimolar
MS	mass spectrometry
mult	multiplicity
NMR	nuclear magnetic resonance
NRPS	nonribosomal peptide synthetase
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PKS	polyketide synthase
ppm	parts per million
RFLP	restriction fragment length polymorphism
rDNA	ribosomal DNA
rRNA	ribosomal RNA
RNA	ribonucleic acid
rpm	revolutions per minute
R_t	retention time
RT	room temperature
s	second
s (NMR)	singlet
sp.	species
strain^T	type strain
t (NMR)	triplet
TFA	trifluoroacetic acid
U	enzyme units
µg	microgram
µl	microliter
µM	micromolar
V	volt
w/v	weight per volume
vol/vol	volume per volume

II. Buffers, Solutions and Media

The following were sterilized by autoclaving at 121°C for 30 min. Antibiotics and other solutions, when specified are added to the media at hand warm temperature following sterilization. Media containing agar were aseptically poured into sterile disposable Petri dishes and allowed to solidify at room temperature.

A. Buffers and Solutions

1. Artificial seawater

NaCl		234.70 g
Na_2SO_4		39.20 g
$MgCl_2.6H_2O$		106.40 g
$CaCl_2$		11.00 g
$NaHCO_3$		1.92 g
KCl		6.64 g
KBr		0.96 g
H_3BO_3		0.26 g
$SrCl_2$		0.24 g
NaF		0.03 g
H_2O_{dd}	ad	10.00 L

2. EDTA (ethylenediamine tetraacetic acid) (0.5 M, pH 8.0)

$Na_2EDTA.2H_2O$		93.05 g
NaOH (10 M)	ca.	25.00 ml
H_2O_{dd}	ad	500 ml

The pH of the solution was adjusted to 8.0 with NaOH since EDTA does not go dissolve until the pH reaches 7.0. Water was added to make a final volume of 500 ml.

3. Gel-loading buffer (5x)

Bromphenol blue		25.0 mg
Xylene cyanol		25.0 mg
Ficoll (type 400)		1.5 g
H_2O_{dd}	ad	10.0 ml

4. IPTG (Isopropyl-β-D-thiogalactopyranoside) (1 M)

IPTG		2.38 g
H_2O_{dd}	ad	10.00 ml

5. $MgSO_4$ (1 M)

$MgSO_4.7H_2O$		24.6 g
H_2O_{dd}	ad	100.0 ml

6. NaCl (5 M)

NaCl		292.2 g
H_2O_{dd}	ad	100.0 ml

7. Phage dilution buffer

NaCl (1 M)		1.0 ml
$MgCl_2$ (1 M)		0.1 ml
Tris-Cl (1 M, pH 8.3)		0.1 ml
H_2O_{dd}	ad	10.0 ml

8. Plasmid mini-prep buffers

Buffer P1

Tris-Cl (1 M, pH 7.5)		5.0 ml
EDTA (0.5 M, pH 8.0)		2.0 ml
RNAse A (10 mg/ml)		1.0 ml
H_2O_{dd}	ad	100.0 ml

Buffer P2

NaOH (2 M)		5.0 ml
SDS (10%)		10.0 ml
H_2O_{dd}	ad	100.0 ml

Buffer P3

Potassium acetate		29.4 g
Glacial acetic acid	ca.	11.5 ml
H_2O_{dd}	ad	100.0 ml

The pH of the solution was adjusted to pH to 5.5 using glacial acetic acid and water was added to make a final volume of 100 ml.

9. SDS (sodium dodecyl sulfate) solution (10% w/v)

SDS		10.0 g
H_2O_{dd}	ad	100.0 ml

SDS was first dissolved in 80 ml water, with warming at 50°C to accelerate dissolution. The pH of the solution was adjusted to 7.0 by adding several drops of concentrated HCl and water was added to make a final volume of 100 ml.

10. SET buffer

NaCl (1 M)		750 µl
EDTA (0.5 M, pH 8.0)		500 µl
Tris-Cl (1M, pH 7.5)		200 µl
H_2O_{dd}	ad	10.0 ml

11. Sodium acetate (3 M)

Sodium acetate.$3H_2O$		40.8 g
H_2O_{dd}	ad	100.0 ml

12. TAE buffer (5x)

Tris base		242.00 g
Sodium acetate.$3H_2O$		136.12 g
$Na_2EDTA.2H_2O$		19.00 g
H_2O_{dd}	ad	1.00 L

Dissolve the above reagents in 700 ml water and adjust the pH to 7.2 with acetic acid. Add water to make a final volume of 1 L.

13. TE buffer

Tris-Cl (2 M, pH 7.4)		0.5 ml
EDTA (0.5 M, pH 8.0)		20.0 µl
H_2O_{dd}	ad	100.0 ml

14. Tris-Cl (2 M, pH 7.4)

Tris		242.2 g
H_2O_{dd}	ad	1.0 L

The pH of the solution was adjusted to 7.4 by adding about 140 ml concentrated HCl and water was added to make a final volume of 1 L.

15. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (5% w/v)

X-gal		0.5 g
Dimethylformamide	ad	10.0 ml

B. Media

1. Czapek medium

$NaNO_3$	3.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
KCl	0.5 g

	$FeSO_4.7H_2O$		10.0 mg
	Saccharose		30.0 g
	Agar		18.0 g
	H_2O_d	ad	1.0 L

2. ISP 2

	Yeast extract		4.0 g
	Malt extract		10.0 g
	Glucose		4.0 g
	Agar		18.0 g
	H_2O_d	ad	1.0 L

3. ISP 3

	Oatmeal		20.0 g
	Agar		18.0 g
	H_2O_d	ad	1.0 L

4. ISP 4

	Soluble starch		10.0 g
	K_2HPO_4		1.0 g
	$MgSO_4$		1.0 g
	NaCl		1.0 g
	$(NH_4)_2SO_4$		2.0 g
	$CaCO_3$		2.0 g
	$FeSO_4.7H_2O$		1.0 mg
	$MnCl_2.4H_2O$		1.0 mg
	$ZnSO_4.7H_2O$		1.0 mg
	Agar		18.0 g
	H_2O_d	ad	1.0 L

5. ISP 5

	Glycerol		10.0 g
	Asparagine		1.0 g
	K_2HPO_4		1.0 g
	Trace element solution		1.0 ml
	H_2O_d	ad	1.0 L

6. ISP 6

	Peptone		15.0 g
	Proteose peptone		5.0 g
	K_2HPO_4		1.0 g

Yeast extract		1.0 g
Ferric ammonium citrate		0.5 g
Sodium thiosulfate		0.08 g
Agar		18.0 g
H_2O_d	ad	1.0 L

7. ISP 7

L-tyrosine		1.0 g
Agar		18.0 g
H_2O_d	ad	1.0 L

8. LB (Luria-Bertani) agar

Peptone		10.0 g
Yeast extract		5.0 g
NaCl		5.0 g
Agar		18.0 g
H_2O_d	ad	1.0 L

9. LB/amp

LB agar	1.0 L
Ampicillin (100 mg/ml)	1.0 ml

Filter-sterilized ampicillin (100 μg/ml) was added to LB agar after autoclaving.

10. LB/amp/IPTG/X-gal

LB agar	1.0 L
Ampicillin (100 mg/ml)	1.0 ml
IPTG (1 M)	0.4 ml
X-gal (5%)	1.6 ml

Filter-sterilized ampicillin (100 μg/ml), IPTG (0.5 mM) and X-gal (80 μg/ml) were added to LB agar after autoclaving.

11. M1

Soluble starch		10.0 g
Yeast extract		4.0 g
Peptone		2.0 g
Agar		18.0 g
Artificial seawater	ad	1.0 L

12. M2

Glycerin (100%)		6.0 ml
Arginine		1.0 g
K_2HPO_4		1.0 g
$MgSO_4$		0.5 g
Agar		18.0 g
Artificial seawater	ad	1.0 L

13. M7

Peptone		2.0 g
Asparagine		0.1 g
Sodium propionate		4.0 g
K_2HPO_4		1.0 g
$MgSO_4$		0.1 g
$FeSO_4$		0.001 g
Glycerol		5.0 g
NaCl		20.0 g
Agar		18.0 g
Artificial seawater	ad	1.0 L

14. MS

Mannitol		20.0 g
Soybean flour		20.0 g
Agar		18.0 g
H_2O_d	ad	1.0 L

15. NaSt21Cx

Solution A

K_2HPO_4		1.0 g
Agar		18.0 g
Artificial seawater		750.0 ml

Solution B

KNO_3		1.0 g
$MgSO_4$		1.0 g
$CaCl_2.2H_2O$		1.0 g
$FeCl_3$		0.2 g
$MnSO_4.7H_2O$		0.1 g
Artificial seawater		250.0 ml

Solutions A and B were autoclaved separately and subsequently combined. Trace element solution (1 ml) was added to the final solution.

Trace element solution

$ZnSO_4.7H_2O$		0.1 g
$FeSO_4.7H_2O$		0.1 g
$MnCl_2.4H_2O$		0.1 g
$CaCl_2.6H_2O$		0.1 g
NaCl		0.1 g
Distilled H_2O	ad	1.0 L

16. SOC medium

Tryptone		20.0 g
Yeast extract		5.0 g
NaCl (1 M)		10.0 ml
KCl (1 M)		2.5 ml
$MgCl_2$ (1 M)		10.0 ml
$MgSO_4$ (1 M)		10.0 ml
Glucose (2 M)		10.0 ml
H_2O_d	ad	1.0 L

Tryptone (2%), yeast extract (0.5%), NaCl (10 mM) and KCl (10 mM) were added to 970 ml distilled water prior to autoclaving. The following filter-sterilized solutions: $MgCl_2$ (10 mM), $MgSO_4$ (10 mM) and glucose (20 mM) were subsequently added to the medium after autoclaving.

17. YPD medium

Yeast extract		10.0 g
Peptone		20.0 g
Glucose		20.0 g
H_2O_d	ad	1.0 L

18. Zobell medium

Yeast extract		1.0 g
Peptone		5.0 g
Artificial seawater		750.0 ml
H_2O_d	ad	1.0 L

III. Chemicals

Chemical name	Manufacturer
Acetic acid	AppliChem
Acetonitrile	Sigma
Agar, granulated	Difco
Agarose, ultrapure	Gibco
Ammonium sulfate [$(NH_4)_2SO_4$]	AppliChem
Ampicillin	AppliChem
Arginine	Sigma
Asparagine	Sigma
Boric acid (H_3BO_3)	AppliChem
5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)	Sigma
Bromphenol blue	Merck
Calcium carbonate ($CaCO_3$)	AppliChem
Calcium chloride ($CaCl_2$)	AppliChem
Chloramphenicol	Sigma
Chloroform	Roth
Cycloheximide	Sigma
Diaion HP-20ss	Mitsubishi
Dipotassium hydrogen phosphate (K_2HPO_4)	Roth
Dimethylformamide	AppliChem
Dimethylsulfoxide	Sigma
Ethanol absolute (EtOH)	Merck
Ethanol denatured (EtOH)	Roth
Ethidium bromide (1% solution)	Roth
Ethyl acetate	Roth
Ethylenediamine tetraacetic acid dihydrate ($Na_2EDTA.2H_2O$)	Serva
Ferric ammonium citrate	AppliChem
Ferrous sulfate heptahydrate ($FeSO_4 .7H_2O$)	Fluka
Gentamicin	Sigma
Glucose	AppliChem
Glutaraldehyde	Sigma
Glycerin/ Glycerol	Roth
Hydrochloric acid (HCl)	AppliChem
Isopropanol	Roth
Isopropyl-β-D-1-thiogalactopyranoside (IPTG)	Sigma
Kanamycin	Merck
Lincomycin	Sigma

Chemical name	Manufacturer
Malt extract	AppliChem
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	AppliChem
Manganous chloride tetrahydrate ($MnCl_2.\ 4H_2O$)	AppliChem
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	AppliChem
Mannitol	AppliChem
Methanol	Sigma, Roth
Nalidixic acid	Sigma
Nystatin	Sigma
Oatmeal	Gesitz Haferflockenfabrik
Osmium tetroxide	Sigma
Oxacillin	Sigma
Penicillin	Sigma
Peptone	Roth
Phenol	AppliChem
Phytagel	Sigma
Potassium acetate	AppliChem
Potassium bromide (KBr)	AppliChem
Potassium chloride (KCl)	Fluka
Potassium nitrate (KNO_3)	AppliChem
Propylene oxide	Roth
Rifampin	Sigma
Saccharose	Roth
Sodium acetate trihydrate	AppliChem
Sodium bicarbonate ($NaHCO_3$)	Merck
Sodium chloride (NaCl)	Roth
Sodium dodecyl sulfate (SDS)	AppliChem
Sodium fluoride (NaF)	Fluka
Sodium hydroxide (NaOH)	AppliChem
Sodium nitrate ($NaNO_3$)	Merck
Sodium propionate ($C_3H_5NaO_2$)	AppliChem
Sodium sulfate (Na_2SO_4)	Merck
Sodium thiosulfate ($Na_2S_2O_3$)	AppliChem
Soybean flour	Neuform
Starch	Roth
Streptomycin	Sigma
Strontium chloride ($SrCl_2$)	Fluka
Tetracycline	Sigma
Trifluoroacetic acid (TFA)	Sigma

Chemical name	Manufacturer
Tris (hydroxymethyl) aminomethane hydrochloride	Sigma
Tryptone	Roth
Tyrosine	Sigma
Uranyl acetate	Sigma
Vancomycin	Sigma
Xylene cyanol	AppliChem
Yeast extract	Gibco
Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$)	Sigma

IV. Computer Programs

Software	Application	Reference
Align	sequence alignment and editing	Hepperle 2002
AntiMarin database	marine natural products database	University of Canterbury, New Zealand
ARB	phylogenetic tree construction	http://www.arb-home.de/
ASMPKS	analysis and prediction of modular PKS systems	http://gate.smallsoft.co.kr:8008/~hstae/asmpks/index.html
BLAST	sequence comparison/alignment	http://www.ncbi.nlm.nih.gov/BLAST/
ChemDraw Ultra 8.0	Chem Office 2004	Chem Office 2004
ClustalX	alignment of nucleotide and amino acid sequences	http://www-igbmc.u- strasbg.fr/BioInfo/ClustalX/Top.html
Dictionary of Natural Products	online database of natural products	http://www.chemnetbase.com
Multi Analyst 1.1	documentation of agarose gel	BioRad
NRPSpredictor	NRPS adenylation analysis	http://www.tigr.org/jravel/nrps/
UpSol NMR Prediction	prediction of NMR shifts	Pretsch et al. 2000
Phylip	phylogenetic tree construction	http://evolution.genetics.washington.edu/phylip/getme.html
PKS analysis tool	prediction of PKS domains	http://www.nii.res.in/searchall.html
Primer3	designing of PCR primers	http://frodo.wi.mit.edu/
SciFinder Scholar	natural products access tool	http://www.cas.org/scifinder/scholar/
Treeview	visualization of phylogenetic trees	http://taxonomy.zoology.gla.ac.uk/rod/treeview.html
Vector NTI Advance™ 10	ORF identification and sequence annotation	https://catalog.invitrogen.com/index.cfm?fuseaction=userGroup.downloadCenter

V. Enzymes and Kits

Name of Enzyme/ Kit	Manufacturer
ABI Prism™ Big Dye ™ terminator cycle sequencing ready reaction kit	Applied Biosystems
API kits	Biomerieux
Biolog SF-P2	Biolog
FastDNA® spin kit for soil	Q-Biogene
Lysozyme	Sigma
Proteinase K	Sigma
pGEM-Teasy vector system	Promega
pWEB™ cosmid cloning kit	Epicentre
QIAquick PCR purification kit	Qiagen
REDTaq® ReadyMix™ PCR reaction mix	Sigma
Restriction endonucleases and buffers	New England Biolabs
RNase	Roche
Taq DNA polymerase and buffer	Qiagen
T4 DNA ligase and buffer	New England Biolabs
Molecular weight markers	
GeneRuler™ 1kb DNA ladder	Fermentas
GeneRuler™ 100bp DNA ladder	Fermentas
FastRuler™ DNA Ladder, high range	Fermentas
GeneRuler™ DNA Ladder, hrRange	Fermentas

VI. Equipment and Supplies

Equipment/ Supplies	Manufacturer	Specifications
Autoclave	Fedegari	Tec 120, 9191E, FV 3.3
	H+P Labortechnik	Varioklav 500, 135S
Benchtop centrifuge	Hereaus Instruments	Biofuge Frasco
Distilling apparatus for H_2O_{dd}	GFL	Bi-Dest 2304
Disposable cuvette	Plastibrand	halbmikro 1,5 ml
Electroporator	EquiBio	Easyject PRIMA
Electroporation cuvette	EquiBio	EPC 102
ELISA plate reader	Thermo Electron	Multiskan Ascent
Filter disks	Becton Dickinson	-
Filter membranes	Millipore	Millex-GS 0.22 µm
Gel documentation	BioRad	Gel Doc 2000
Gel electrophoresis chamber	BioRad	-

Equipment/ Supplies	Manufacturer	Specifications
Heat block	Laboratory Devices	Digi-Block Jr.
HPLC	Agilent	Agilent 1100
HPLC columns	Phenomenex	RP18
Ice maker	Scotsman	AF-20
Incubator	Heraeus	Kelvitron®t
	Memmert	TV 40b
Magnetic stirrer	Labinco	L32
Micropipettes	Microlab	MicroOne 0,5-10 µl
	Microlab	MicroOne 2-20µl
	Microlab	MicroOne 20-200µl
	Microlab	MicroOne 100-1000µl
Microfuge tubes	Sarstedt	1,5 ml; 2,0 ml
	Abgene	0,5 ml Thermo tubes
	Abgene	0,2 ml Thermo Stripes
	Greiner	15 ml, 50 ml Bio-one CELLSTAR®
Microplates	Nalgene	Nunclon™
Microwave	AEG	Micromat
	Privileg	8020
NMR	Varian	Varian Inova 400, 500, 600 MHz
	Bruker	Avance 400 MHz
MS	Waters, Bruker	Micromass Q-TOF, MicroTOF
PCR cycler	Biometra	T3-Thermocycler
Petri dishes, round	Greiner	-
Petri dishes, square	Nalgene	Nunclon™
pH Meter	WTW	MultiLine P4, SenTix 41
Pin replicator	Nalgen Nunc International	384 pin replicator
Quartz cuvette	Hellma	Suprasil
Refrigerator	Privileg	Superöko
Rotary evaporator	Heidolph	Laborota 4010
Scanning electron microscope	Zeiss	DSM 962
Sequencer	ABI Prism	ABI 377XL
Spectrophotometer	Pharmacia Biotech	Ultraspec 3000
	PeqLab	NanoDrop ND1000

Equipment/ Supplies	Manufacturer	Specifications
Speedvac concentrator	Thermo Scientific	Savant
Shakers	Braun	Certomat U
	Edmund Bühler	SM-30
	Eppendorf	Rotationsmischer 3300
	Infors	HT
Transmission electron microscope	Zeiss	EM 10

VII. Microorganisms

Microorganism	Application
Staphylococcus aureus NCTC 8325	Bioactivity testing
Staphylococcus epidermidis RP62A	Bioactivity testing
Enterococcus faecalis JH212	Bioactivity testing
Enterococcus faecium 6413	Bioactivity testing
Escherichia coli 536	Cloning
Escherichia coli XL1-Blue	Bioactivity testing
Escherichia coli EPI100-T1^{R}	Cloning
Pseudomonas aeruginosa	Bioactivity testing
Yersinia pseudotuberculosis 252 01A	Bioactivity testing
Yersinia pestis KUMA	Bioactivity testing
Candida albicans 5314 (ATCC 90028)	Bioactivity testing
Trypanosoma brucei brucei 221	Bioactivity testing
Leishmania major	Bioactivity testing

VIII. Oligonucleotides

Oligonucleotide	Sequence 5' – 3'	Annealing (°C)	Reference	Specificity
27f 1492r	GAGTTTGATCCTGGCTCA TACGGCTACCTTGTTACGACTT	56	Lane 1991	bacterial 16S rRNA gene (universal)
540f 1100r	GGITGCACSTCIGGIMTSGAC CCGATSGCICCSAGIGAGTG	64	Wawrik et al. 2005	type II PKS
A7R A3	SASGTCVCCSGTSCGGTAS GCSTACSYSATSTACACSTCSGG	59	Ayuso and Genilloud 2003	NRPS adenylation domain
degAH-F2 degAH-R2	ATCATGCCSGTSCAYATGGCSGG CKRTGRTGSARCCASTKRCART	52	Rascher et al. 2003	AHBA synthase
K1 M6R	TSAAGTCSAACATCGGBCA CGCAGGTTSCSGTACCAGTA	55	Ayuso and Genilloud 2005	type I PKS
KS α KS β	TSGRCTACRTCAACGGSCACGG TACSAGTCSWTCGCCTGGTTC	58	Ayuso et al. 2005	type II PKS
KS 1 KS 2	TSGCSTGCTTCGAYGCSATC TGGAANCCGCCGAABCCGTC	58	Metsa-Ketela et al. 1999	type II PKS

Oligonucleotide	Sequence 5' – 3'	Annealing (°C)	Reference	Specificity
PKS III Fwd	TCGCTSCTSTCGAACGGCCTSTT	58	Cortes et al. 2002	type III PKS
PKS III Rev	CGGCGACGCSCTSTCGGC CTCSGCGGTGATSCCGGGSCCG AAGCCSGCGATSAGGC			
S-C-Act-0878-A-19	CCGTACTCCCCAGGCGGGG	60	Stach et al. 2003	*Actinobacteria* 16S rRNA gene
S-C-Act-0235-a-S-20	CGCGGCCTATCAGCTTGTTG			
sebNRPS-1	GGCAGGCTGGTTGACGTAG	59	Proksch 2008	NRPS adenylation domain
sebNRPS-2	GTGTGGTGGAGCTGGCTTT			
SP6	ATTTAGGTGACACTATAG	45	Promega	pGEM-T easy cloning vector
T7	GTAATACGACTCACTATAGGG			

Publications

Pimentel-Elardo S, Scheuermayer M, Kozitska S, Hentschel U (2008) *Streptomyces axinellae* sp. nov., a novel actinomycete isolated from the Mediterranean sponge, *Axinella polypoides* (Porifera). Int J Syst Evol Microbiol: In Press.

Pimentel-Elardo S, Gulder TAM, Hentschel U, Bringmann G (2008) Cebulactams A1 and A2, new macrolactams isolated from *Saccharopolyspora cebuensis*, the first obligate-marine strain of the genus *Saccharopolyspora*. Tetrahedron Lett 49: 6889-6892.

Pimentel-Elardo S, Tiro LP, Grozdanov L, Hentschel U (2008) *Saccharopolyspora cebuensis* sp. nov., a novel actinomycete isolated from a Philippine sponge (*Porifera*). Int J Syst Evol Microbiol 58: 628-632.

Sertan-de Guzman AA, Predicala RZ, Bernardo EB, Neilan BA, **Elardo SP**, Mangalindan GC, Tasdemir D, Ireland CM, Barraquio WL, Concepcion GP (2007) *Pseudovibrio denitrificans* strain Z143-1, a heptylprodigiosin-producing bacterium isolated from a Philippine tunicate. FEMS Microbiol Lett 277: 188-196.

Scheuermayer M, **Pimentel-Elardo S,** Fieseler L, Grozdanov L, Hentschel U (2006) Microorganisms of sponges: phylogenetic diversity and biotechnological potential. In: Proksch P, Müller W (eds) Frontiers in Marine Biotechnology. Horizon Bioscience, Norwich, pp 289-312.

Symposia, Workshops and Field Work

“Presenting Talks in Academic English”, workshop organized by the Graduate School of Life Sciences (Würzburg, June 12-13, 2008)

“Catalogue and Database Research”, workshop organized by the Graduate School of Life Sciences (Würzburg, May 15, 2008)

“Scientific Writing Course", workshop organized by the the Graduate School of Life Sciences (Würzburg, November 14-16, 2007)

“VIth US-Japan Symposium: Frontiers in Marine Natural Products Research”, organized by the University of Utah (Park City, Utah, July 1-6, 2007)

“3rd International Symposium”, organized by the International Graduate School (Würzburg, October 8, 2007).

“Young Investigator Symposium on Infection Biology”, organized by ZINF; Poster presentation (Würzburg, March 2-3, 2007).

Field work: sponge collection and cultivation of actinomycetes (Cebu City, Philippines, December 2006).

“New Trends in Infectious Disease Research”, symposium organized by the SFB 630 and SFB 544; Oral presentation (Heidelberg, November 2006).

“2nd International Symposium: Molecular Approaches for Novel Therapies”; organized by the International Graduate School (Würzburg, October 2006)

“Summer School on Sponges” organized by the Biotecmarin (Rovinj, Croatia, August 2006).

“1st International Symposium: Novel Agents against Infectious Diseases - an Interdisciplinary Approach”, organized by the SFB 630; Poster presentation (Würzburg, February 2006).

Curriculum Vitae

Personal Information

Name	Sheila Marie Pimentel Elardo
Date of Birth	July 16, 1973
Place of Birth	Cebu City, Philippines
Nationality	Filipino
Status	Married

Educational Attainment

Feb 2006 – present	Ph.D. dissertation “Novel anti-infective secondary metabolites and biosynthetic gene clusters from actinomycetes associated with marine sponges” Julius-Maximilians-University Würzburg, Germany Research supervisor: Prof. Ute Hentschel
2002	Master of Science in Microbiology (Thesis “Isolation of planctomycetes from *Aplysina* sponges”) University of the Philippines, Quezon City, Philippines Thesis advisors: Prof. Wilfredo Barraquio (Philippines), Prof. Ute Hentschel (Germany)
1994	Bachelor of Science in Pharmacy (Thesis “Anti-spasmodic activity of *Blumea balsamifera*”) University of the Philippines, Manila, Philippines Thesis advisor: Prof. Mildred B. Oliveros
1994	Pharmacy Licensure Examination: Passed (Rating: 88.58%)

Professional Experience

1999 - 2005	Instructor College of Pharmacy, University of San Carlos, Cebu, Philippines
1996 -1999	University Research Associate (Supervisor: Dr. Gisela Concepcion) Marine Science Institute, University of the Philippines, Quezon City
1994 -1996	Branch Pharmacist Marsman & Co., Inc., Cebu City, Philippines

Research Grant

2004 - 2006	International Foundation for Science Research Grant F/3615-1