Chemical and Taxonomic Investigation of Indonesian Soil-dwelling Bacteria

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Declaration

I, Saefuddin Aziz declare that this thesis is an original report of my research, has been written by myself and has not been submitted for any previous degree. The experimental work is almost entirely my own work; the collaborative contributions have been indicated clearly and acknowledged. Due references have been provided on all supporting literatures and resources.

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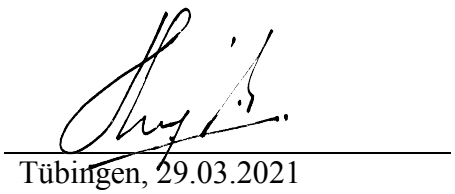

Tübingen, 29.03.2021

Zusammenfassung

Ziel des Projekts war es, taxonomisch neuartige bakterielle Stämme aus dem Biodiversitäts-Brennpunkt Indonesien zu isolieren und hieraus neue chemische Verbindungen zu isolieren. Zu diesem Zweck wurden 25 Stämme gesammelt und auf antimikrobielle Eigenschaften hin untersucht. Daraus resultierend wurden zunächst fünf und im späteren Verlauf dann nur noch ausschließlich die beiden Stämme *Streptomyces* sp. SW4 und *Pseudomonas aeruginosa* SW5 priorisiert.

Während der Stamm SW5 nur bekannte Naturstoffe produzierte und eine bereits bekannte Spezies darstellte, erwies sich der Stamm SW4 im Rahmen von polyphasischen taxonomischen Untersuchungen als völlig neuartige Spezies. Um die Ergebnisse zu untermauern, wurde das komplette Genom des Stamms sequenziert und mit den nächsten verwandten Typ-Stämmen verglichen. Diese bioinformatischen Experimente konnten die initialen Ergebnisse bestätigen.

Berücksichtigt man, dass Biodiversität üblicherweise direkt proportional zur chemischen Diversität und Novität ist, wurde nachfolgend erforscht, in wie weit der Stamm befähigt ist, Sekundärmetaboliten zu biosynthetisieren. Neben den 17 Biosynthesegenclustern, die im Genom sichtbar sind, stach ein Biosynthesegencluster heraus, welches für Pristinamycin-artige Verbindungen kodierte und daher eingehender untersucht wurden. Es konnte gezeigt werden, dass es trotz unterschiedlicher Größe, vollständig funktional war. Des Weiteren konnte gezeigt werden, dass die Produktion durch das Hormon γ-Butyrolakton reguliert war. Eine Molekulare Netzwerkanalyse förderte zudem zutage, dass der Stamm ein neues Derivat in sehr geringen Mengen produzierte.

Summary

The aim of the study was the discovery of new chemical entities and taxonomic bacterial strains from the biodiversity hotspot Indonesia. For this purpose, 25 strains were collected and pre-screened using an antimicrobial bioassay panel. From these investigations, first five and later on two strains were prioritized: *Pseudomonas aeruginosa* SW5 and *Streptomyces* sp. SW4. While strain SW5 produced only known chemistry and represented a known species, SW4 proved, within the frame of polyphasic taxonomic experiments, to be a completely new species. To support the taxonomic investigations, its genome was sequenced and compared with closely related type strains, which confirmed the initial results. Taking into account that biodiversity will be translated into chemical diversity and novelty, the potential of strain SW4, to biosynthesize secondary metabolites, was also investigated. Beside the 17 biosynthetic gene clusters which are present in the genome of the strain, a pristinamycin-like gene cluster was outstanding and further analyzed. It could be shown that it was functional, despite being different concerning the size. Furthermore, it could be shown that the production is regulated by γ-butyrolactone and a molecular network analysis unveiled that a new derivative is produced in minor amounts.

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Table of Contents

Table of figures

List of tables

Abbreviations and Units

°C	degrees Celsius
1D	one dimensional
2D	two dimensional
A (domain)	adenylation domain
ACP	Acyl Carrier Protein
AMP	adenosine monophosphate
AT	acetyltransferase
conc.	concentration
C (domain)	condensation domain
Da	Dalton
DAD	Diode Array Detector
DCM	dichloromethane
DMSO	dimethyl sulphoxide
ESI ionisation	electrospray
g	gram
HCl	hydrochloric acid
HR	(MS) high resolution
HSQC	Heteronuclear Single Quantum Coherence
Hz	Hert
LR	(MS) low resolution
m/z	mass to charge ratio
MeOD	deuterated methanol
MeOH	methanol

mg	milligram
MHz	Megahertz
min	minutes
ml	millilitre
MS	Mass Spectroscopy
MS/MS	Tandem Mass Spectrometry
NADPH	reduced nicotinamide adenine dinucleotide phosphate
nm	nanometre
NMR	Nuclear Magnetic Resonance
NRPS	Non-Ribosomal Peptide Synthetase
PCP	Peptidyl Carrier Protein
pH	potential of hydrogen
PKS	Polyketide Synthase
ppm	parts per million
qd	(NMR) quadruplet of doublets
RP	Reversed Phases
SAM	S-adenosyl methionine
TOCSY	Total Correlation Spectroscopy
UV	ultraviolet (spectroscopy)
VLC	Vacuum Liquid Chromatography
α	rotation (°)
δ	NMR chemical shift (ppm)
Δ	difference
λ	wavelength
μ	micro (10^{-6})

I. Introduction

1.1. Natural product discovery

Natural products play a vital role in the development of pharmaceutical drugs. They are organic compounds build by the primary or secondary metabolism of living organisms.[1] However, many scientists refer to natural products generally only for secondary metabolites which are not directly involved in essential physiological process, such as cell growth, development and division.[2] Secondary metabolites offer several advantages, including better protection or a defense system, rivalling with other living organisms, and intercommunication with other species.[3] They may also serve as differentiation effectors, sexual hormones, and many of them, act as antibiotics.[4]

Natural resources are widely acknowledged as untapped sources of chemical metabolites with extraordinaire features, highly morphed structures and biological functions. Since thousands of years, natural products have been used for a great variety of applications in the fields of human health care and well-being such as food, medication and farming. [5-7] Many natural products come from plants. Studies of the earliest known records of natural products, like the 4000-year-old Sumerian clay tablet reports about hundreds of natural products from plants as remedies for various illnesses, including the juice of poppy seed, *Papaver somniferum,* the oil of *Cedrus* species (cedar), the resin of *Commiphora myrrha* (myrrh) and, many of which are still in use today.[8] Papyri from 2900 BC have likewise documented various Egyptian plant-derived medicines, [9] and the Chinese Materia Medica and the written accounts of the Indian Ayurvedic medicine can be traced back for more than 2000 years. [10]

Natural products are also found in microorganisms. The discovery of penicillin by Alexander Fleming in 1928 by accident from a fungal metabolite that shows antimicrobial activity is mainly based on the previous studies of the microbial agent causing infectious disease conducted by Louis Pasteur, Joseph Lister and Robert Koch.[11, 12] In 1939, René Dubos discovered tyrothricin, which contains as active agent two distinct polypeptides tyrocidine and gramicidin from *Bacillus brevis,* which contained as active agents the two distinct polypeptides tyrocidine and gramicidin and which were found to be is useful in killing Gram-positive bacteria.[12, 13] Since then,

gramicidin has become the first natural antibiotic product to be widely used and clinically tested. Selman Waksman and Albert Schatz also isolated a chemical compound from *Streptomyces griseus* which is active against *Mycobacterium tuberculosis*. This compound was then named streptomycin.[14] The discovery of streptomycin and further antibiotics changed the medical practice. Operations could be performed at lower risk, and many life-threatening diseases, like tuberculosis, could be treated.[15]

The "Golden Age of Antimicrobial Discovery" is the time-frame between the 1950s and the 1970s, when nearly all precious antibiotics were found, such as chloramphenicol, cephalosporin, vancomycin, and carbapenems.[16] From the 1970s to the 1990s, more or less only analogues of formerly known metabolites were added. It is also noted that there was an innovation gap during the period between 1962 and 2000, which means that there were no new classes of antibiotics discovered after 1987 (Fig. 1).[17]

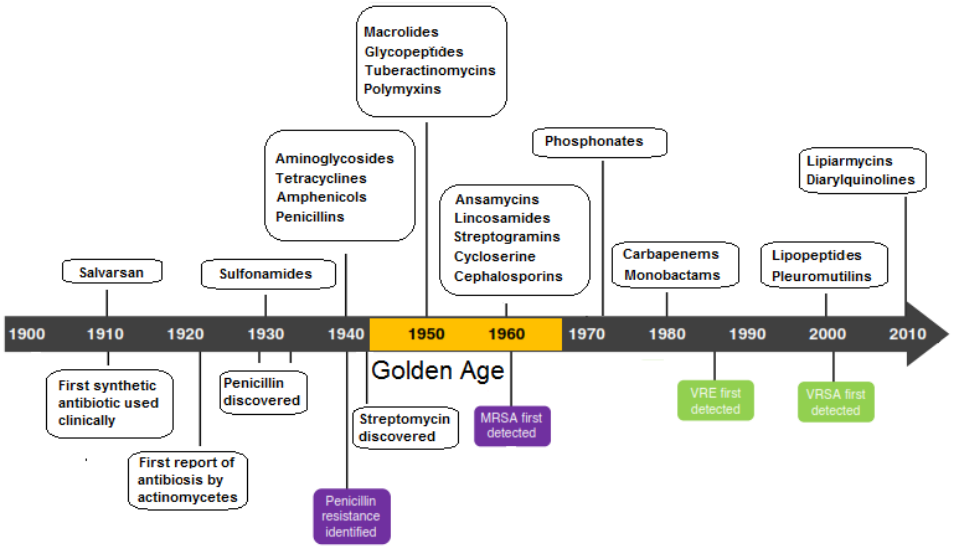


Figure 1. Timeline of introduced antibiotics. Adopted from Hutchings *et al.*[18]

The use of antibiotics is not only crucial in the pharmaceutical industry but also for agriculture. The modernization of antibiotic production has led to a striking decline in the price of antibiotics which has led to a tremendous and sometimes irrational use of antibiotics. Factory farms improperly administer antibiotics to prevent infections and promote growth in food-producing farm animals which over time created resistances and rendered the antibiotics ineffective to humans. The emergence of antibiotic-resistant pathogens and their spread throughout the biosphere can be observed and

involves an uninterrupted process of natural selection from human use of antibiotics due to dosing, overuse or abuse of antibiotics. [19]

The increasing occurrence of bacterial infections that cannot be treated sufficiently with existing antimicrobial therapies poses a substantial problem to public health until now. Problems caused by the emergence of antibiotic-resistant pathogens influence the effectiveness or efficiency of health care systems and have also social consequences. [20, 21] According to a report in 2019, each year, it is estimated that 35,000 people die in the US due to antibiotic-resistant infections. Also, in the European Union, there are 25,000 additional deaths,[22] and in Thailand, it is estimated that 19,000 people die each year as a victim of the infection of multi-drug resistant bacteria.[22] A total of 700,000 deaths occur annually worldwide caused by infections of multi-drug resistant bacteria, and this number could rise to 10 million by 2050 depending on the evolution of resistance patterns and the discovery of effective antibiotics (Fig. 2).[22] A most recent report by the World Bank (2017) highlights as one of their key findings that AMR leads to loss of global gross domestic product (GDP) of 1.1 - 3.8% per year (additional 1.2 trillion USD), and that consequently 28.3 million people are lost to extreme poorness. Antibiotic-resistant pathogens will also have an impact on reducing the global gross domestic product by 60 to 100 trillion US $, or about 2 to 3.5% by the year 2050.[23]

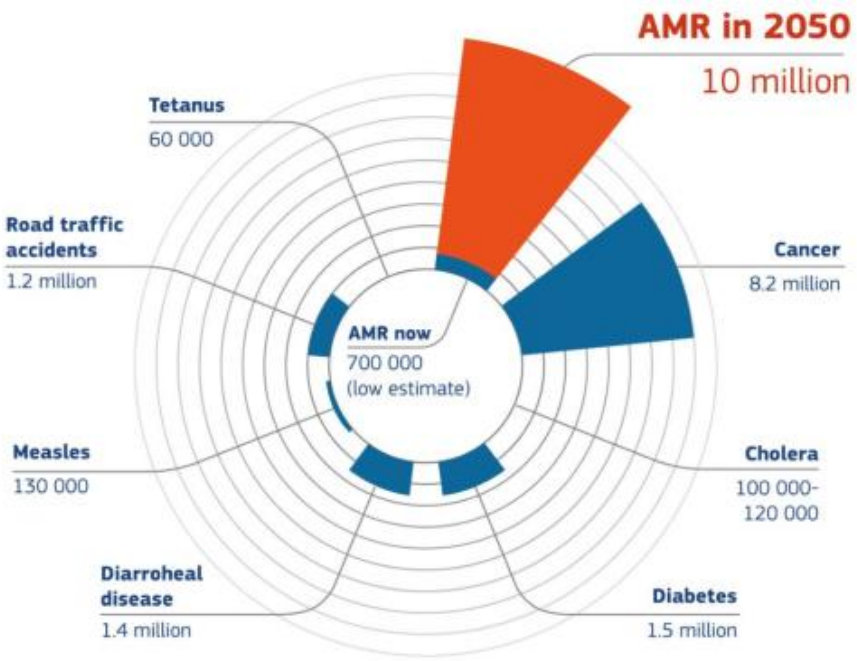


Figure 2. Worldwide death attributed to AMR every year, compared to other major causes of death. The blue region represents the number of deaths as of 2014 whereas the brown one highlights projected deaths by 2050. Adapted from O'Neil[24]

The necessity for new antibiotics continues to increase due to the rapid emergence of multiple antibiotic-resistant pathogens rousing deadly diseases.[25] Along with the rational use of existing antimicrobial drugs, new strategies are needed to discover effective novel antibiotics. Novel strategies for drug discovery have been carried out in recent years. These include modifying cultivation conditions, genome mining, and the application of a biodiversity-based method.[26, 27]

Concerning the biodiversity-based method, finding new antibiotics can be done by looking for new species of bacteria, e.g. from the talented taxon of actinomycetes. Unusual strains of actinomycetes produce diverse compounds that still have to be elucidated. The possibility to isolate new actinomycetes is still given because many environmental niches till now remain unexplored such as the endosymbiotic environment, the desert, and the deep sea.[27] Many new actinomycetes have been isolated from marine substrates such as sponges and tunicates. They produce several novel metabolites with antibiotic and antitumor properties, such as salinosporamides, sporolides, terpenoid chloro-dihydroquinones, marinomycins and rifamycins.[28] These "rare actinomycetes" are the genera from actinomycetes besides *Streptomyces* such as *Actinomadura*, *Actinoplanes*, *Micromonospora*, *Nocardia*, *Saccharopolyspora* and *Streptosporangium.*[29]

Indonesia represents one of the underexplored countries regarding the biodiversity of Actinobacteria. As an archipelago of more than 17,000 islands, Indonesia is recognized as one of the biodiversity hotspots of the world with a high level of endemic species and microorganisms (Fig. 3). It is estimated that Indonesia exhibits more than 25% of the world's microorganisms living in the various ecosystems of the country. Therefore, isolation of Actinobacteria from Indonesian samples bears the chance to obtain bacteria that are unique in terms of taxonomy and the ability to produce novel bioactive compounds.[27, 30]

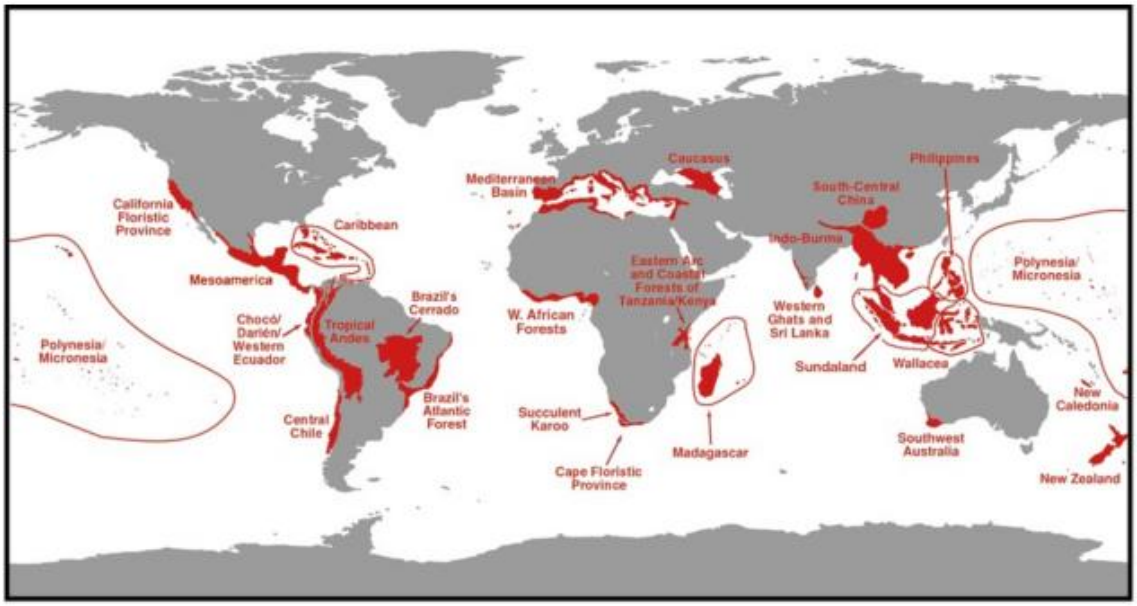


Figure 3. The hotspots area in the world. Most of the Indonesian area belong to the biodiversity hotspot area.[31]

Previous investigations suggested that the entire biosynthetic potential of a typical organism is not expressed under a single set of conditions. Changing the cultivation conditions such as nutrient composition, incubation temperature, speed of agitation and the rate of aeration can alter the whole physiology of a microorganism and can affect the production pattern of secondary metabolites significantly. This method is known as the One Strain-Many Compounds (OSMAC) approach. The technique is suggested to stimulate the expression of cryptic (silent) metabolic pathways in microbial strains that can enlarge the variety of compounds they produce. Additional approaches such as co-cultivation with other microorganisms and the addition of chemical inducers have also been used to augment the number of secondary metabolites. However, for this method it is difficult to establish general standards for all the strains.[27, 32-34]

Retrieving information from an organism based genome sequence or genome mining can detect and analyse the biosynthetic gene clusters encoding secondary metabolites of a certain organism. Due to genome mining, corresponding chemical entities of organisms can be predicted. Genome mining is highly dependent on the available bioinformatics tools and computational technology. Biosynthetic gene clusters are physical groups of all the genes that encode the enzymes that are required for the metabolic pathways for the specific production of secondary metabolites such as

antibiotics, anticancer, insecticides, and immunosuppressants. The determination of the biosynthetic gene cluster can be achieved by checking the homology with renowned secondary metabolite gene clusters. This homology is obtained by the classification of previously recognized biosynthetic gene clusters of encoded compounds relating to the chemical structure, conserved domains, and biosynthetic pathways. The discovery of novel metabolic pathways is being enabled by the increasing easiness of high-quality genome sequencing, coupled with the development of powerful computational technology to identify metabolic gene clusters. By using whole-genome sequencing, it is finally understood that most fungi and bacteria can generate more biochemical compounds than they usually produce under the standard cultivation conditions. The information derived from biosynthetic gene clusters can be applied to different methods like more targeted drug discovery techniques and enabling the heterologous expression in the best expression host.[27, 30, 35, 36]

1.2. Actinobacteria

Actinomycetes are aerobic, gram-positive bacteria that are capable of forming spores and generate substrate and aerial mycelium during their growth.[37] They represent one of the major taxonomic groups among the 18 main lineages of the bacterial domain.[38] They are mostly characterized by having a high guanine and cytosine content within their genomes (>55%).[39] They generate about two-thirds of all clinically used antibiotics and further therapeutic compounds such as immunosuppressive, antitumor, antialgal, antimalarial, and antihelmintic agents.[40, 41]

Actinobacteria have been identified as one of the essential groups of soil population. They participate in the decomposition, and mineralization cycles of complex mixtures of polymers in dead plants, animals and fungal material with the production of extracellular enzymes, such as cellulase, chitinase, and lignin peroxidase.[42] They also can be found as plant symbionts, nitrogen-fixing commensals, and gastrointestinal tract inhabitants. However, a small numbers of Actinobacteria are recognized as pathogens, such as *Mycobacterium tuberculosis* that causes tuberculosis (TB) in humans, *Nocardia* spp., *Tropheryma* spp. and *Corynebacterium* spp.[38, 39]

Many Actinobacteria produce, like fungi, a mycelium. Because of this, they are considered as the transitional organisms between fungi, and bacteria. The term actinomycetes is originated from the Greek words “aktis or aktin” (ray) and “mukēs” (fungi). The difference between actinomycetes and fungi is that actinomycetes have no nucleus and contain peptidoglycan in their cell wall. Most of them are saprophytic and soil-dwelling bacteria that spend most of their life cycles as semi-dormant spores, particularly under the condition when nutrients are scarce. Some of them can also be found in aquatic ecosystems. Actinomycetes mainly grow better at neutral pH, and the temperature for their optimal growth is between 25-30°C.[38]

The phylum Actinobacteria has six classes which include *Actinobacteria, Acidimicrobidiia, Coriobacteria, Nitriliruptoria, Rubrobacteria*, and *Thermoleophilia*. The class Actinobacteria contains 16 orders and 62 families. The majority of these families of the phylum (around 74%) belong to the class of Actinobacteria (https://www.ncbi.nlm.nih.gov/taxonomy).[38] The determination of Actinobacteria at genus and species levels are primarily based on characteristics of morphology and chemotaxonomy. However, with the recent development of molecular studies, some species have recently been reclassified since they were inadequately positioned in particular taxonomic groups.[38] Nowadays, the hierarchic classification structure for the taxonomic levels of Actinobacteria is proposed based on a wide array of chemotaxonomic, morphological, physiological properties and genotypic methods. For genotypic taxonomy, the determination of Actinobacteria can be carried out based on the 16S rRNA sequence analysis, DNA-DNA hybridization, multilocus sequence analysis (MLSA), and comparison of whole-genome sequencing.[38, 43]

1.3. The genus *Streptomyces*

Streptomyces is a type genus of the sole member of the family Streptomycetaceae. Belonging to the Actinobacteria phylum and the Actinomycetales order within the classes Actinobacteria, *Streptomyces* can be easily recognized by their distinctive earthy smell generated by the secretion of the secondary metabolite, geosmin. They are effectively adapted to survive in soil by forming a substrate mycelium that can help to get the nutrition. They represent 40% of the soil bacteria and can secrete various enzymes to digest insoluble organic materials

and can produce numerous secondary metabolites for competing for other soil organisms and communication between others.[44]

Streptomycetes are well known of producing complex secondary metabolites. It is estimated, that over two-thirds of the therapeutic substances today, such as antibacterial, antifungal, antiparasitic drugs, and immunosuppressants are originating from *Streptomyces* spp. *Streptomyces* represent one of the largest taxonomic groups of Actinomycetes in terms of number and variety of identified species. [45, 46] Currently, *Streptomyces* encompass nearly 576 species with a growing number every year.

The life cycle of *Streptomyces* bacteria consists of three growing phases: vegetative hyphae, aerial hyphae and spores (Fig. 4). Starting with the germination of spores, *Streptomyces* develop into a long branched network of filamentous and multinucleotidal vegetative hyphae to penetrate the medium for getting nutrients. The cell division during vegetative growth creates cross-walls that separate the hyphae into interconnected compartments. Under environmentally unfavourable conditions, such as nutrient shortage, *Streptomyces* start to develop aerial hyphae, non-branching hyphae extend into the air away from the vegetative hyphae. Aerial hyphae continue to develop into spores through synchronous cell division and cell maturation. This developmental stage usually correlates with the start of secondary metabolite production, such as antibiotics. However, many industrial companies accomplished liquid cultivation platforms for secondary metabolite production from *Streptomyces*. Under these conditions, usually *Streptomyces* strains do not sporulate.[38, 47, 48]

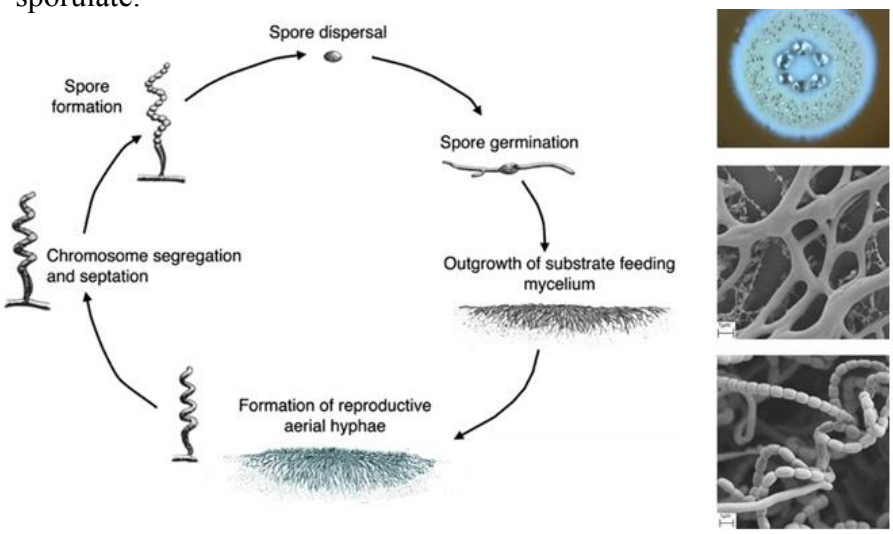


Figure 4. *Streptomyces* life cyle. Adapted from Seipke *et al.*[47]

Streptomyces strains can produce various antibiotics with different characters and functions. Those antibiotics generally fall under five mechanisms of action against the bacterial cell. The most common mechanism involves the inhibition of cell wall synthesis, while the second-largest class is targeting the protein synthesis. Another mechanism is mediated by altering the enzymes involved in nucleic acid metabolism and reparation. The fourth mechanism consists of the alteration of the cell membrane, and the final mechanism is by exerting antimetabolite activity. Some examples of antibiotics produced by *Streptomyces* species are chloramphenicol from *S. venezuelae*, novobiocin from *S. niveus*, daptomycin from *S. roseosporus*, platensimicin from *S. platensis*, and streptomycin from *S. griseus* (Fig. 5). Streptomycin and chloramphenicol inhibit the initiation of protein synthesis in bacteria. Streptomycin binds to the small subunit of ribosomal bacteria especially to 16S rRNA, while chloramphenicol binds to the large subunit of ribosomal bacteria especially to 23S rRNA. Novobiocin can disrupt the synthesis of nucleic acid by inhibiting the DNA gyrase, while daptomycin inhibits cell evelope synthesis by interfering with fluid membrane microdomains.[49] Platensimicin mediates its antibacterial activity by inhibiting the fatty acid production.[50, 51]

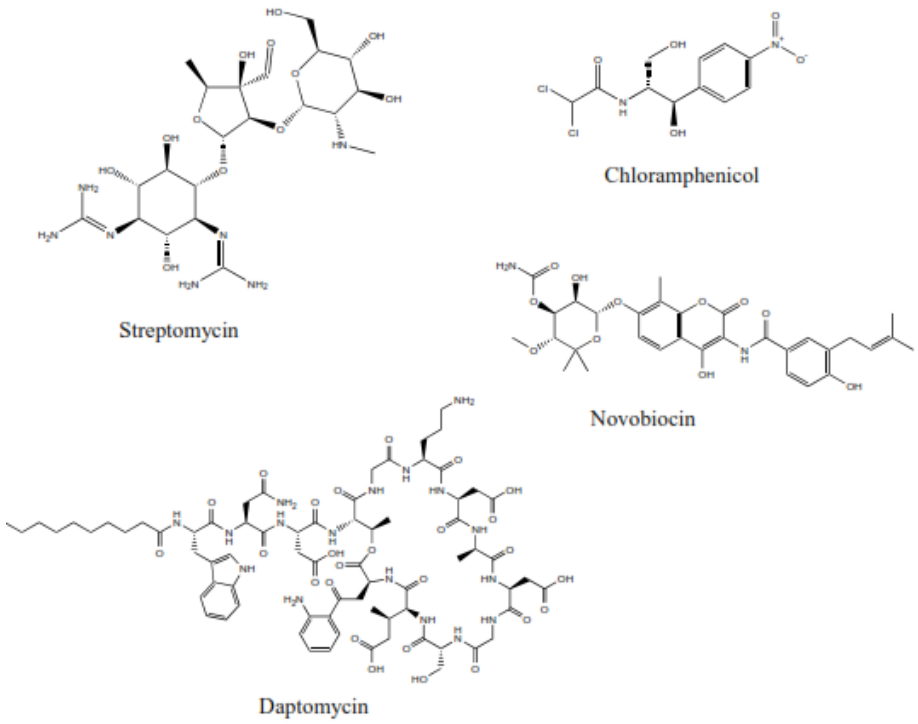


Figure 5. Some antibiotic structurs produced by *Streptomyces* species

Table 1. Important antibiotics produced by *Streptomyces* species

Isolate	Antibiotic	Class of molecule	Function	Reference
Streptomyces sp. TÜ 6075	Arylomycin	Lipopeptides	Inhibition of type I signal peptidase	[52]
S. capreolus	Capreomycin	Peptides	Inhibition of protein synthesis	[53]
S. clavuligerus	Cephalosporins	ß-Lactams	Inhibition of cell wall synthesis	[54]
S. venezuelae	Chloramphenicol	Chloramphenicols	Inhibition of protein synthesis	[55]
S. garyphalus	Cycloserine	Analog of Dalanine	Inhibition of cell wall synthesis	[56]
S. roseosporus	Daptomycin	Lipopeptides	Destruction of the membrane potential	[57]
S. fradiae	Fosfomycin	Fosfomycin	Inhibition of cell wall synthesis	[58]
S. fradiae	Neomycin	Aminoglycosides	Inhibition of protein synthesis	[59]
S. kanamyceticus	Kanamycin	Aminoglycosides	Inhibition of protein synthesis	[60]
S. lincolnensis	Lincomycin	Lincosamides	Inhibition of protein synthesis	[61]
S. niveus	Novobiocin	Aminocoumarins	Inhibition of DNA gyrase	[62]
S. antibioticus	Oleandomycin	Macrolides	Inhibition of protein synthesis	[63]
S. platensis	Platensimycin	Platensimycin	Inhibition of fatty acid production	[64]
S. pristinaespiralis	Pristinamycin	Streptogramins	Inhibition of protein synthesis	[65]
S. ribosidificus	Ribostamycin	Aminoglycosides	Inhibition of protein synthesis	[66]
S. ambofaciens	Spiramycin	Macrolides	Inhibition of protein synthesis	[67]
S. griseus	Streptomycin	Aminoglycosides	Inhibition of protein	[68]

			synthesis	
S. aureofaciens	Tetracycline	Tetracyclines	Inhibition of protein synthesis	[69]
Streptomyces sp. ICBB8309	Actiphenol	Angucyclinones	Inhibition of DNA synthesis	[70]
S. vinaceus	Viomycin	Peptides	Inhibition of protein synthesis	[71]
S. virginiae	Virginiamycin	Streptogramins	Inhibition of protein synthesis	[72]

1.4. Polyphasic taxonomy of Actinobacteria

Microbial taxonomy is essential for studying organisms by which microorganisms show similarities to the members of the same group. Therefore, they are separated from other groups of microorganisms that have different characteristics. Taxonomy comprises three main parts, i.e. classification, nomenclature, and identification. Classification is the arrangement of organisms into groups (taxa). Nomenclature refers to the assignment of names to taxonomic groups. The term nomenclature includes giving the label of the units determined in classification. Identification refers to the determination of the particular taxon to which a specific isolate belongs. Taxonomic information helps to understand and organize the biodiversity and also to find out similarity among organisms from different niches. Taxonomy in the field of microorganisms has an essential function in supporting the accurate identification of strains from different species.[73]

Bacteria were at first classified in the 19th century solely based on simple phenotypic markers such as their shape of colonies and cells, gram stain, presence of endospores and motility. This system changed by incorporating additional properties, i.e., physiological and biochemical parameters. In the period between1960-1980, further characterization such as numerical taxonomy and DNA–DNA hybridization, were applied in the classification of bacteria and archaea. Later, since 1980, the emergence of DNA amplification and sequencing techniques provided the complete genetic information of a single strain and represented a significant progress in bacterial classification and identification. Nowadays, genetic characters such as 16S rRNA sequence analysis, multilocus sequence analysis and whole-genome analysis are commonly used in the taxonomy study of procaryotes.[74]

To establish a reliable taxonomy of microorganisms, the integration of phenotypic data and genotypic data is needed. This approach is called polyphasic taxonomy.[40] Phenotypic information is derived from the main physical characteristics such as morphological, physiological and chemotaxonomical properties. Meanwhile, genotypic data are obtained from the nucleic acids (DNA and RNA) in the cells by analysis of 16S rRNA gene sequencing, GC content and DNA–DNA hybridization (DDH).[73, 75, 76] Some other methods, recently used for genomic taxonomy studies include ribotyping and whole-genome analysis.[77, 78] Phylogeny shows biological entities that are connected through common ancestry. There can be species, genus or higher-level taxonomic groups given. The phylogenetic tree is used for understanding not only the relationships among taxa but also their hypothetical related ancestors. Currently, most phylogenetic trees are constructed from molecular data corresponding to DNA or amino acid sequences.[79]

1.5. Phenotypic analysis

Various morphological properties can be found in Actinobacteria. Classic identification of actinomycetes is well-developed in the appearance of the radial mycelium. Based on the difference between morphology and function, mycelia can be divided into substrate mycelium and aerial mycelium. Some actinobacteria can form complicated structures, such as spores, spore chains, sporangia and sporangiospores. The various shapes include irregular rods (*Propionibacterium*), rod-coccoid (*Arthrobacter*) or coccoid (*Micrococcus*). The various forms of mycelia include fragmental mycelia (*Nocardia*), and permanent and highly differentiated branched mycelia (*Kitasatospora, Frankia* and *Streptomyces*). The members of the genus *Rhodococcus* form elongated filaments on the substrate without producing true mycelia, while the members of the genus *Corynebacterium* do not produce mycelia at all. There is also a group that can break up its substrat hyphae into flagellated motile elements (*Oerskovia*). Some members of *Rhodococcus* and *Mycobacterium* do not usually form aerial hyphae.[38, 79-83]

Some Actinobacteria have long been known to produce pigments depending on the strain, the used medium and the age of the culture. These may be red, green, yellow, orange, purple, brown, blue, grey or black. Melanins are polymers with various structures that usually appear dark from brown to black and are derived from a

heterogenic polymer of phenolic and indolic molecules. They have a similar character to humic soil substances. They are not necessary for growth and development, but they can help the host for the defense and competition against other organisms. The presence of melanins can be used in taxonomic studies of Actinobacteria.[84]

According to the Shirling and Gottlieb (1966) methods for *Streptomyces* isolation and identification, the essential observations should concern the cultivation of cultures on various media from International Streptomyces Project (ISP) such as yeast extract – malt extract agar (ISP2), inorganic salts – starch agar (ISP4), oatmeal agar (ISP3) and glycerol – asparagine agar (ISP5). The specific growth and morphology features of *Streptomyces* species can be observed when cultures become mature with rising spore mass. Then the aerial spore mass colour, the substrate mycelium and diffusible pigment colour production can be judged. The initial grouping of actinomycetes could be completed by comparison of morphological and physiological properties based on their colour alignment.[79, 85]

Some phenotypic characteristics of actinobacteria, including physiological and biochemical properties, are of primary importance in taxonomy studies for a genus or species description. Those studies include data about the growth at different temperatures, pH values, salt concentrations, atmospheric conditions (aerobic/anaerobic), growth in the presence of various substances such as antimicrobial agents and data on the presence or activity of various enzymes.[39, 86-89]

In the classification of Actinobacteria, the chemotaxonomic properties correlate with the distribution of specific chemicals of the cell envelope such as amino acids, sugar, polar lipids, menaquinones, and fatty acids.[90-92] Some genera contain 2, 6-diaminopimelic acid (DAP) in their cell-wall structure, which has isomers, i.e. meso-DAP and LL-DAP. Meso-DAP can be found in members of the *Mycobacterium*, *Nocardia* and *Pseudonocardia*.[93] The genera of *Streptomyces*, *Intrasporangium,* and *Sporichthya* are examples, which possess only LL-DAP in their cell-wall. Hydroxy-diaminopimelic acid (OH-DAP) which is a derivative of DAP, that is detected in *Micromonospora* strains. For whole-cell sugar patterns, *Mycobacterium*, *Nocardia* and *Pseudonocardia* species have galactose and arabinose as their whole sugar pattern. *Microbispora* and *Streptosporangium* strains contain madurose, whereas the members of *Micromonospora, Actinoplanes*, and *Dactylosporangium* contain xylose

and arabinose in their cell-wall. Meanwhile, *Streptomyces* species have no characteristic sugar patterns.[79, 94, 95]

1.6. Genomic and phylogenetic analysis

The traditional identification of bacteria based on phenotypic characteristics is generally not as accurate as the identification based on genotypic methods. In the 1980s, a new standard for identifying bacteria other than the classical approach was developed. This new standard is based on the comparison of the bacterial 16S rRNA gene sequence. Sequences of 16S rRNA of bacteria and archaea are the part of the DNA which is now most commonly used for taxonomic classification. This sequence is highly conserved and can be compared among all bacteria. Phylogenetic relationships of bacteria and indeed, all life forms, could be determined by comparing a stable part of the genetic code. The 16S rRNA sequence is used as the backbone for the classification of bacteria because it is ubiquitous, functionally stable, highly conserved and poorly subject to horizontal gene transfer. The 16S rRNA sequence provides the basis for identification and allows the investigation of the evolution and phylogentic studies between species of bacteria. The 16S rRNA gene sequence is about 1,550 bp long and is composed of variable and conserved regions. Universal primers are usually chosen as a complementary to the conserved regions at the beginning of the gene and at the 540-bp region or at the end of the whole 16S rRNA gene sequence, and the sequence of the variable region in between is used for comparative taxonomy. The gene is large enough, with sufficient interspecific polymorphisms of the 16S rRNA gene, to provide specific and statistically valid measurements. Sometimes, sequencing of the entire 1,500-bp region is necessary to distinguish between particular taxa or strains. However, 500 to 1,500 bp are quite common lengths for sequencing and comparing sequences in databases that can have various lengths. Sequencing of the entire 16S rRNA sequence is also required in case, a new species is described. Besides the 16S rRNA gene, other candidates for this genetic area in bacteria include the genes that code for 5S rRNA, 23S rRNA and the spaces between these genes.[79, 96, 97]

In the recent decade, comparative analysis of the 16S rRNA sequence has been used to study prokaryote phylogeny. The tested bacterial isolate indicated a distinct genus when the similarity was below 95%, whereas the cut-off identity values for novel species is below 98.7%.[98] The 16S rRNA gene sequence analysis is reproducible, and the database of 16S rRNA gene sequence of all published bacterial name is available in Genbank (www.ncbi.nih.nlm.gov). To depict the novelty of the tested bacterial strains, a phylogenetic tree is build based on the closest related type species from the database.[99-101] In the case of *Streptomyces* species, previous studies found that, despite they were representing novel *Streptomyces* species, they still showed a 99.1-99.9% similarity value at the 16S rRNA level.[102-104]

Furthermore, the ratio of guanine and cytosine within the total number of nucleotides in the genome is one of the taxonomic markers that can be employed for the discrimination of microorganisms. It is also considered as one of the primary characteristics of cellular DNA, related with the codon usage of mRNA, the amino acid composition of proteins, auxotrophy for specific bases, and other features are of overall biological importance.[75, 105] Based on the percentage of GC-content, bacterial groups could be classified into one of three categories: GC-rich (Deinococcus-Thermus: 64.4%, Actinobacteria: 62.1%), GC-intermediate (Proteobacteria: 56.4%, Euryarchaeota: 53.7%, Crenarchaeota: 49.7%), and GC-poor (Bacteroidetes: 46.0%, Firmicutes: 43.1%, Spirochaetes: 40.6%, Chlamydiae: 40.3%). The class of Actinobacteria encompasses mostly Gram-positive bacteria with a high GC content (>55 mol% in genomic DNA). Some members of the genus *Corynebacterium*, however, have a GC content lower than 55% and even *Gardnerella* strains have a GC content which is less than 45%, therefore representing the exemption from the rule.[106]

DNA–DNA hybridization generally refers to a molecular biology technique that measures the degree of genetic similarity between certain pools of DNA sequences. It is usually used to determine the genetic distance between two organisms. This has been extensively used in phylogeny and taxonomy. This method is still known as the 'gold standard' principle for species delineation of prokaryotes. If the DDH value of the tested bacterial strain is lower than 70%, it indicates a different species.[107] However, DDH analysis has some shortage of reproducibility because the cut-off values for all prokaryote genera are not the same. The measurement of DDH values is

laborious, requires special facilities and exhibit a high experimental error. These reasons explain why it is still challenging to create a comparative database from DDH.[99, 108]

Recent technological progress in the area of genome sequencing calls for bioinformatics methods to replace the wet-lab DDH by in-silico genome-to-genome comparison. The latter process is also known as digital DNA-DNA hybridization (dDDH). Algorithms to efficiently determine high-scoring segment pairs or maximally unique matches perform well as a basis of inferring intergenomic distances. In-silico methods for the comparison of genome sequences can be used to replace DDH. Digitally derived genome-to-genome distances show a better correlation with 16S rRNA gene sequence distances than DDH values.[107, 109]

Multilocus sequence typing (MLST) schemes analyse nucleotide sequence data from several conserved housekeeping genes to find out a combination of alleles known as a sequence type. For each housekeeping gene, a different sequence is given for a species. The degree of similarity in the sequence type among isolates correlate to their evolutionary distance. Multilocus sequence analysis has been used for the delineation of strains, samples or isolates of several bacterial species including Actinobacteria. Nowadays, at least 50 housekeeping genes, are specifically applied in taxonomy studies of several bacterial species.[110] However, it was shown that an MLST scheme based on 5 housekeeping genes gave a good resolution in the taxonomy studies in *Mycobacterium*, *Streptomyces* and *Kitasatospora*. These five house-keeping genes are *rpoB* (RNA polymerase, beta subunit), *trpB* (tryptophan synthase, beta subunit), *atpD* (ATP synthase F1, beta subunit), *gyrB* (DNA gyrase, B subunit), and *recA* (recombinase A). The sequences derived from the selected housekeeping genes can be used for generating phylogenetic trees. The reproducibility of the MLST method is very high and can be comparable to DDH. The distance of 0.007 derived from five gene-based MLST analysis corelates to a DDH value of 70%, which means that this value could be employed as the species cut-off for identifying novel species in the genus *Streptomyces*.[111, 112]

Complete genome sequences of all type strains are critical for the future of microbial systematics. The incorporation of genomics into the taxonomy and systematics of the microorganism, coupled with computational advances, will boost the credibility

of conclusion in taxonomy studies. Whole-genome data has also been used in Actinobacteria taxonomy by aligning and comparing between two whole-genome sequences from closely related species. The measurement to show the similarity between two tested genomes sequence is called an overall genome relatedness index (OGRI). The most common algorithm used for the taxonomic studies is the average nucleotide identity (ANI). The ANI value can be used to determine a novel species of bacteria if the score of the ANI value is below 95-96%. This score is equivalent to 70% DDH.[98, 113, 114]

1.7. Isolation and dereplication of natural products from Actinobacteria

Identification of bioactive molecules from natural sources requires highly integrated interdisciplinary approaches, recent technological advances, time and human resources. The crucial part is the effort to concentrate only on the discovery of novel bioactive compounds. This effort requires the identification of known substances, which is called dereplication. Several procedures of dereplication were developed in the past twenty years to enhance the success of natural product screening programs.[115, 116] Researchers have been using multifaceted approaches by merging different areas of knowledge to improve the efficiency of drug discovery program. Multidisciplinary dereplication processes will result in an enhancement of the number of natural product discoveries and approved drugs in the imminent future.[117]

Dereplication can be used as an untargeted strategy for the rapid identification of the major compounds in a sample. This strategy is usually combined with biological assays and fractionation procedures. In other cases, dereplication is fully integrated with metabolomic studies to profile all of the chemical metabolites of natural extract collections, both for untargeted identification or targeted identification of predetermined compounds. Dereplication is also used for taxonomic identification of microbial strains by comparing their gene sequences.[118, 119]

The dereplication strategies in natural product research include some methods, e.g. bioactivity-guided assays in combination with LC-MS (liquid chromatography-mass spectrometry) techniques and MS libraries or databases, also nuclear magnetic resonance (NMR) spectroscopy. Gene sequence analysis of certain genes, e.g. 16S rRNA gene or whole genomic sequence has also been used as a modern dereplication tool.[120]

Isolation of natural products deriving from Actinobacteria may be carried out following the bioassay-guided fractionation strategies. This strategy leads the discovery of major bioactive compounds since decades. Using a combination of solvent-solvent partitioning and chromatography techniques, the fractions from a culture of an Actinobacterial extract can be isolated and tested for antibacterial, antifungal, anticancer or antiviral activity.[121] The effectiveness of fractions against specific organisms is important to identify their spectrum of activity and can be conducted either by using a filter paper disk diffusion test[122] or a serial microdilution test in a 96-well plate.[123] The determination of the chemical formula of an isolated compound is employed by LCMS (liquid chromatography-mass spectrometry) and confirmed by high-resolution mass spectrometry (HR-MS) to provide accurate mass measurements.[124] Finally, NMR is used to determine the molecular structure of the compound.[125]

1.8. Molecular networking of Metabolomes

The metabolome is the final manifestation of biochemical pathways. Temporal and spatial changes in the metabolite composition reflect the outcome (phenotype) of interactions at the genomic, transcriptomic and proteomic level. Molecular networking is becoming more and more popular into the metabolomic community to organize tandem mass spectrometry (MS/MS) data. The aim of untargeted metabolomics is the global profiling of small molecule biomarkers that are characteristic for a particular physiological state. A major requirement is to quickly pinpoint and identify those compounds.[126, 127]

One of open source sofware is Global Natural Products Social Molecular Networking (GNPS; http://gnps.ucsd.edu). GNPS is an open-access knowledge base for community-wide organization and sharing of raw, processed or identified MS/MS spectrometry data. In GNPS, crowdsourced curation of freely available community-wide reference MS libraries will underpin improved annotations. Data-driven social-networking should facilitate identification of spectra and foster collaborations through continuous reanalysis of deposited data.[128] This platform creates databases which are available to every scientist to caption and find readily new chemistry and to solve the problem to perform dereplication.

Even though this approach allows the treatment and comparison of large data sets, several drawbacks related to the MS-Cluster tool routinely used on the GNPS platform limit its potential. MS-Cluster cannot distinguish between chromatographicaly well-resolved isomers as retention times are not taken into account. Annotation with predicted chemical formulas is also not implemented and semi-quantification is only based on the number of MS/MS scans. The software Mzmine introduces here a data-preprocessing workflow including the preliminary data treatment to clear one of the major drawbacks previously mentioned above.[129] Before sending data to the GNPS, metabolic profiles of the extracts were analyzed by ultra-performance liquid chromatography (UPLC), coupled with electrospray ionization high-resolution mass spectrometry (ESI-HRMS-MS). Mass spectrometry-based molecular networking was employed to aid in metabolite annotation and for the visual investigation of known metabolites and their analogues.[130]

1.9. Previous work

In order to find novel antibiotic chemical entities with novel modes of action, Indonesian microbial samples were collected, purified and screened for production of novel antimicrobial compounds.[2,3] Consequently, more than 124 soil isolates have been collected and screened for bioactivity towards both gram negative (*Eschericia coli*, *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Vibrio* sp.) and gram positive bacteria (*Staphylococcus carnosus*, *Streptococcus pneumoniae*, *Micrococcus luteus*, *Bacillus subtilis*). This preliminary screening revealed five isolates with confirmed antimicrobial activity. Further characterization of these isolates by 16S rRNA analyses was used to determine their species. Based on their 16S rRNA sequences, three isolates belong to the phylum Actinomycetes (99%) *(Streptomyces* sp. SW4, *Actinomadura* sp. PT1, *Promicromonospora* sp. SW3), one isolate belongs to the genus *Bacillus* (99%) (*Bacillus licheniformis* SB3) and the remaining isolate to the genus *Pseudomonas* (99%) (*Pseudomonas aeruginosa* SW5).

This study was also conducted within the frame of the project NabaUnAkt (New Antibiotics from unknown Aktinomycetes) isolation of new unique natural compounds from unknown actinomycetes under the umbrella of the Biodiversity and Health cooperative from the DAAD. This project is in turn a collaboration project between Indonesian institutes and German Universities. The ultimate goal of this

project is to isolate new antimicrobial compounds from unknown Indonesian soil actinomycetes and investigate their medical potential.

1.10. Aim of the study

Taking into account that biodiversity will be translated into chemical diversity and novelty, the potential of the five prescreened Indonesian bacteria shall be investigated. For prioritized samples, the chemical analyses shall be complemented by genome sequencing analyses. In case one sample represents a new species, a taxonomic classification process will be initiated to document the given microbial biodiversity of Indonesia.

II. Materials and Methods

2.1 Materials

Table 2. List of chemicals and media used in this study

Chemicals	Manufacturer
Acetonitrile	J.T. Baker
Agar Bacteriology grade	AppliChem
API 50 CH strip	bioMérieux SA
Bacto TM yeast extract	Difco
Copper(II) sulfate pentahydrate pure Ph. Eur., USP	AppliChem
Dichloromethane	VWR
Ethyl acetate	VWR
Ethanol	Sigma Aldrich
Glutamine, L-	Sigma Aldrich
Glycerol 86-88%	Sigma Aldrich
Gamma butyrolactone	Sigma Aldrich
Manganese(II)-chloride monohydrate	Roth
Methanol HPLC grade	VWR
Methanol LC-MS grade	VWR
Sodium dihydrogen phosphate heptahydrate	Sigma Aldrich
peqGreen	Peqlab
Polygoprep 50-60 C18 RP silica gel	Macherey-Nagel
Potassium, di- ; hydrogen phosphate	Roth
Sodium hydroxide	Sigma Aldrich
10mM dNTP-mix (2.5 mM each)	BioLine
1 kb DNA Ladder	BioLine
Lysozyme	AppliChem
Proteinase K	Bioscience
Taq-polymerase	Roche
RNAse A (100g/mL)	Qiagen, Hilden, Germany
Rifampicin	Sigma Aldrich

Table 3. List of instruments used in this study

Instruments	Manufacturer
Autoclave (Systec VX-150)	Systec
Centrifuge (5424 R)	Eppendorf
Centrifuge (Heraeus Multifuge 4KR)	ThermoScientific
Clean bench (Safe 2020)	ThermoScientific
Gel Doc Imaging System XR+	Bio-Rad
Gel electrophoresis apparatus	VWR
HPLC system	Waters
Incubation shaker Multitron Pro	Infors
LC (1100 Series; coupled to MS)	Agilent
Mass spectrometer (MS; QTRAP 3200)	AB Sciex
Electroporator (MicroPulser™)	Bio-Rad
Microscope (Leica DM750)	Leica Microsystems
pH meter (FiveEasy)	Mettler Toledo
Pipettes (Discovery Comfort: 1000 µl; 100 µl; 10 µl; 2 µl)	HTL Lab Solutions
Scale (B22002)	Oxford

Special accuracy weighing scale (BP210D)	Sartorius
Thermo Cycler peqSTAR 96x Universal	VWR
Thermo Mixer	VWR
UV/vis spectral photometer	Eppendorf
Vortex-Genie 2	Scientific Industries
Water purification system (Elga^{TM}Purelab^{TM}Flex)	Bioscience
Taq-polymerase	Roche
RNAse A (100g/mL)	Qiagen, Hilden, Germany

2.1.1. Media

The following list shows the media used in this study. Unless otherwise stated, the media were prepared with distilled water up to a final volume of 1 liter and later autoclaved for 20 min at 121°C (2 bar).

Table 4. List of media and ingredients used in this study

Medium	Ingredients	Amount/l	Final pH (at 25^{o}C)
ISP-1/Tryptone–yeast extract	Pancreatic digest of casein	5 g	7.0±0.1
	Yeast extract	3 g	
	Agar	20g	
	Deionized water	1000 ml	
ISP-2/GYM	Yeast extract	4 g	7.2±0.1
	Malt extract	10 g	
	Dextrose	4 g	
	Agar	20 g	
	Deionized water	1000 ml	
ISP-3/Oatmeal agar	Oat Meal	20 g	7.3±0.1
	Ferric sulphate heptahydrate	1 mg	
	Manganese chloride tetrahydrate	1 mg	
	Zinc sulphate heptahydrate	1 mg	
	Agar	20g	
	Deionized water	1000 ml	
ISP-4/Inorganic salts starch	Soluble Starch	10 g	7.2±0.1
	Dipotassium phosphate	1 g	
	Magnesium sulfate USP	1 g	
	Sodium chloride	1 g	
	Ammonium Sulfate	2 g	
	Calcium carbonate	2 g	
	Ferrous sulfate	1 mg	
	Manganous chloride	1 mg	
	Zinc Sulfate	1 mg	
	Agar	20 g	
	Deionized water	1000 ml	
ISP-5/Glycerol asparagine	L-Asparagine	1 g	7.4±0.1
	Dipotassium phosphate	1 g	
	Glycerol	10 g	
	*Trace salt solution (ml)	1 ml	
	Agar	20 g	
	Deionized water	1000 ml	
	*Trace salt solution contains:		
	Ferrous sulphate heptahydrate	1 mg	

	Manganese chloride tetrahydrate	1 mg	
	Zinc sulphate heptahydrate	1 mg	
ISP-6/Peptone yeast extract iron Agar	Peptone	15 g	6.7±0.1
	Protease peptone	5 g	
	Yeast extract	1 g	
	Ferric ammonium citrate	0.5 g	
	Dipotassium hydrogen phosphate	1 g	
	Sodium thiosulphate	1 g	
	Agar	20 g	
	Deionized water	1000 ml	
ISP-7/Tyrosine agar	L-Asparagine	1 g	7.3±0.1
	L-Tyrosine	0.5 g	
	Dipotassium phosphate	0.5 g	
	Magnesium sulphate heptahydrate	0.5 g	
	Sodium chloride	0.5 g	
	*Trace salt solution (ml)	1 ml	
	Agar	20 g	
	Deionized water	1000 ml	
	*Trace salt solution contains:		
	Ferrous sulphate heptahydrate	1.360 mg	
	Copper chloride dihydrate	0.027 mg	
	Cobalt chloride heptahydrate	0.04 mg	
	Sodium molibdat dihydrat	0.025 mg	
	Zink chloride	0.020 mg	
	Boric acid	2.850 mg	
	Manganese chloride tetrahydrat	1.8 mg	
	Sodium tartrate	1,77 mg	
SCA/Starch casein agar	Soluble starch	10 g	7.0±0.1
	Casein	0.3 g	
	Potassium nitrat	2 g	
	Magnesium sulphate heptahydrate	0.05 g	
	Dipotassium phosphate	2 g	
	Sodium chloride	2 g	
	Calcium carbonate	0.02 g	
	Ferrous sulphate heptahydrate	0.01 g	
	Agar	20 g	
	Deionized water	1000 ml	
SNA/Starch nitrate agar	Soluble starch	20 g	7.0±0.1
	Potassium nitrate	1 g	
	Magnesium sulphate heptahydrate	0.5 g	
	Dipotassium phosphate	0.5 g	
	Sodium chloride	0.5 g	
	Ferrous sulphate heptahydrate	0.01 g	
	Agar	20 g	
	Deionized water	1000 ml	
MEA/Malt extract agar	Malt extract	30 g	5.4±0.2
	Peptone	5 g	
	Agar	20 g	
	Deionized water	1000 ml	
TSA/Tryptic soy agar	Pancreatic digest of casein	17 g	7.3±0.1

	Papaic digest of Soybean	3 g	
	Dextrose	2.5 g	
	Sodium chloride	5 g	
	Dipotassium phosphate	2.5 g	
	Agar	20 g	
	Deionized water	1000 ml	
NA/Nutrient agar	Lab-Lemco' Powder	1 g	7.4±0.1
	Yeast extract	2 g	
	Peptone	5 g	
	Sodium chloride	5 g	
	Agar	20 g	
	Deionized water	1000 ml	
YMA/Yeast mannitol agar	Yeast extract	1 g	6.8±0.1
	Mannitol	10 g	
	Dipotassium phosphate	0.5 g	
	Magnesium sulfate USP	0.2 g	
	Sodium chloride	0.1 g	
	Agar	20 g	
	Deionized water	1000 ml	
MSA/Mannitol salt agar	Protease peptoe	10 g	7.4±0.1
	D-Mannitol	10 g	
	Beef extract	1 g	
	Sodium chloride	75 g	
	Agar	20 g	
	Deionized water	1000 ml	
Grauze medium	Soluble Starch	20 g	7.4±0.1
	Potassium nitrat	1 g	
	Dipotassium phosphate	0.5 g	
	Magnesium sulfate USP	0.5 g	
	Sodium chloride	0.5 g	
	Ferrous sulfate	10 mg	
	Agar	20 g	
	Deionized water	1000 ml	
HT7T7 medium	Soluble Starch	10 g	7.4±0.1
	NZ amine	2 g	
	Beef powder	1 g	
	Yest extract	1 g	
	*Trace salt solution (ml)	1 ml	
	Deionized water	1000 ml	
	*Trace salt solution contains:	1000 ml	
	Calcium chloride dihydrate	11 g	
	Ferrous sulphate heptahydrate	7 g	
	Manganese chloride tetrahydrat	2 g	
	Zink sulphate heptahydrate	2 g	
	Copper sulphate heptahydrate	0.4 g	
	Cobalt chloride heptahydrate	0.4 g	
	Sodium EDTA dihydrate	45 g	
API 50 CHB/E medium	Ammonium sulfate	2 g	7.4±0.4
	Yeast extract	0.5 g	
	Tryptone (bovine/porcine origin)	1 g	
	Disodium phosphate	3.22 g	
	Monopotassium phosphate	0.12 g	
	Phenol red 0.17 g		
	*Trace salt solution (ml)	1 ml	
	Deionized water	1000 ml	

	*Trace salt solution contains:	1000 ml	
	Calcium chloride dihydrate	11 g	
	Ferrous sulphate heptahydrate	7 g	
	Manganese chloride tetrahydrat	2 g	
	Zink sulphate heptahydrate	2 g	
	Copper sulphate heptahydrate	0,4 g	
	Cobalt chloride heptahydrate	0.4 g	
	Sodium EDTA dihydrate	45 g	
Müller Hinton agar	Beef infusion solids	2 g	7.4±0.4
	Starch	1.5 g	
	Casein hydrolysate	17.5 g	
	Agar	20 g	
	Deionized water	1000 ml	

Table 5. Solution of antibiotics

Antibiotic	**Concentration in media (µg/ml)**	**Solvent**
Rifampicin	50	H_2O

Table 6. Buffer for DNA gel electrophoresis

Buffer/Solution	**Composition**	**Final concentration**	**Preparation**
50X TAE	Tris Base	2 M	pH 8.0
	EDTA	0.05 M	
	Acetic acid glacial	57.1 ml/l	
Loading Buffer	Glycerol	30% (w/v)	
	Bromophenol blue	0.25% (w/v)	
Ethidium bromide solution	Ethidium bromide	1 µg/ml	

Table 7. PCR Primers

27F	5-AGAGTTTGATCCTGGCTCAG-3	16S rRNA amplification and sequencing
1492R	5-TACGGCTACCTTGTTACGACTT-3	

Table 8. Bacterial strains used in this study

Strain	**Description/origin**
Streptomyces sp. SW4 DSM 110508	This study
Streptomyces leeuwenhoekii DSM 42122	DSMZ
Streptomyces indiaensis DSM 43803	DSMZ
Streptomyces lomondensis DSM 41428	DSMZ
Streptomyces glomeratus DSM 41457	DSMZ
Streptomyces purpurascens DSM 40310	DSMZ
Streptomyces rameus DSM 41685	DSMZ
Streptomyces resistomycificus DSM 40133	DSMZ
Streptomyces iakyrus DSM 40482	DSMZ
Streptomyces laurentii / S spinoverrucosus DSM 41648	DSMZ
Streptomyces niger DSM 43049	DSMZ
Streptomyces muensis DSM 103493	DSMZ
Streptomyces thermodiastaticus DSM 40573	DSMZ

Streptomyces pristinaspiralis	Wohlleben group, University of Tübingen
Pseudomonas sp. SW5	This study

Table 9. HPLC Columns

Column name	Manufacturer
Luna C18 omega polar, 4.6 x 250mm, 5 µm	Phenomenex
Aeris C18, 4.6 x 250mm, 3.6 µm	Phenomenex

2.2 Methods

2.2.1. Description of sampling sites

Sampling site was the jungle of Nusakambangan island. Sample were collected from the soil and beach of that island (Lat.7° 44' 0'' S and Long. 108° 55' 0'' E).

2.2.2. Isolation of soil actinobacteria

The 15 cm size samples were collected in sterile plastic bags during August 2015. Five grams of soil were air-dried at room temperature and then suspended in 50 ml of sterile sample buffer in 100 ml Erlenmeyer flasks by stirring with a magnetic bar for 30 min. These 10^{-2} diluted aliquots were serially diluted to 10^{-6}. Then 100 µl of dilutions 10^{-3} – 10^{-6} were spread (two replicates at each dilution) onto plates containing sterile starch casein agar (SCA) medium. The medium was supplemented with rifampicin 5 mg/l to prevent bacterial and fungal growth. The plates were incubated at 28±2°C for 7 to 14 days. Then, the isolated strains were further purified by the streak plate technique in the same media and stored at 4°C for further investigation.

2.2.3. Preliminary characterization of actinobacterial isolates

2.2.3.1. Colony morphology

The colony morphology of the purified actinobacterial isolates on SCA medium was recorded regarding the colour of aerial spores, size and nature of the colonies, colour on the reverse side and diffusible pigmentation.

2.2.3.2. Light microscopy

Purified actinobacterial culture plates were prepared and 3 to 4 sterile coverslips were inserted at an angle of 45°. Then the plates were incubated at 30°C for 4-8 days. The coverslips were removed at a 2-3 day interval and observed under the high power magnification. The structure and arrangement of conidiospores and arthospores on aerial and substrate mycelia were observed and determined with the help of Bergey's Manual of Determinative Bacteriology. All the actinobacterial isolates were identified to their generic level based on their morphological properties.

2.2.3.3. Scanning Electron Microscopy

Scanning electron microscopy carried out in collaboration with the Biology Centre CAS, Institute of Soil Biology, České Budějovice, Czech republic. The potential *Streptomyces* were grown in suspension culture and harvested by centrifugation at 10,000 rpm for 10 min. The remaining cell pellet was washed repeatedly three times in phosphate buffer (0.1 M and pH 7.2-7.4). The cells were fixed in fixative glutaraldehyde for 3-6 h at room temperature. The cell pellets were washed repeatedly for three times with phosphate buffer for a 20 min. interval at 4°C. The samples were serially dehydrated through a graded serial of acetone. Then, the samples were transferred to a stub and observed under the scanning electron microscope.

2.2.3.4. Gram staining

Thin smears of the *Streptomyces* isolates were made on a clean glass slide and heat fixed. Then the smear was stained by crystal violet for 1 min. and subsequently washed with water, followed by staining with Gram's iodine. After 1 min., the slide was washed and decolourized with 95% ethyl alcohol. After decolourization, the smear was counterstained with safranin for 1 min. Then, the slide was washed, air dried and examined microscopically.

2.2.3.5. Antagonistic screening

The antimicrobial activity test of the actinobacterial isolates against bacteria was using the cross streak plate method. The single streak of the actinobacteria was made on the surface of the nutrient agar medium and incubated at 30°C. After observing a good ribbon-like growth of the actinobacteria at the center of the plates, the pathogens were streaked at right and left angles to the original streak of actinobacteria and then

incubated at 37 °C for 24 h. Inhibition zones of pathogens were formed towards the actinobacteria that were measured and recorded in millimeter scale. Based on the presence and absence of inhibition zones, the antimicrobial compound producing actinobacteria were selected. Further, the isolates producing inhibition zones were considered as antibiotic producer and selected for secondary screening.

2.2.4. Secondary screening

The selected actinobacteria with antimicrobial activity in preliminary screening were further subjected to a secondary screening by a paper disc diffusion assay. All the active actinobacterial isolates selected by primary screening were inoculated into flasks containing 50 ml SNA and incubated in an orbital shaker (at 200 rpm) at 30 °C for 6 days. After incubation, the broth cultures were centrifuged at 10,000 rpm for 10 min. Supernatant was collected aseptically and their antimicrobial properties were tested against test bacteria. Paper disc diffusion assays weremade on Müller Hinton agar with fresh test bacterial lawn cultures (*B. subtilis, S. aureus, S. pneumoniae, E. coli, S. typhi* and *S. pneumoniae*). Each paper disc was loaded with 10 µl of actinobacterial extract. All plates were incubated at appropriate incubation conditions and examined for inhibition zones. The diameter of inhibition zones was measured and recorded in millimeter.

2.2.5. Isolation of chromosomal DNA

Streptomyces bacteria were grown up to the late exponential phase in starch casein broth at 30°C, then the cells were harvested and washed twice with Tris EDTA buffer or 10.3% sucrose prior to DNA preparation. Chromosomal DNA was isolated by resuspending (0.5-1.0 g) the cells with 5 ml lysis buffer (25 mM Tris: EDTA, pH 8.0; 10-15 µg lysozyme and 50 µg/ml RNAse) and then incubated for 30-80 min at 37°C, followed by the addition of 500 µl of a 5 M NaCl solution. The suspension was agitated on a vortex mixer until the cell suspension became translucent. Cells were lysed by the addition of 1.2 ml of 10% SDS. The lysates were incubated for 15-30 min at 65°C. After addition of 2.4 ml of 5 M potassium acetate, the solution was mixed and left on ice for 20 min. The precipitate was removed by centrifugation for 30 min at 6,000 rpm and the volume of the supernatant was adjusted to 8 ml. The DNA was recovered by precipitation with 2 volumes of isopropanol. The precipitate was dissolved in 700 µl/g of

50 mM Tris/10 mM EDTA (pH 8.0) and the aqueous phase was transferred to a 1.5 ml microcentrifuge tube. Subsequently, 75 µl 3 M sodium acetate and 500 µl isopropanol were added and the solution was then centrifuged for 30 sec. to 2 min. The precipitate was washed with 70% cold ethanol, dried and dissolved in 100 µl TE buffer (10 mM Tris/1 mM EDTA, pH 8.0).

2.2.6. PCR amplification of 16S rRNA

A mixture of sterile distilled deionized water 49 µl, upstream primer 27 F (100 pmol) 10 µl, downstream primer 1492R (100 pmols) 10 µl, 10× PCR buffer 10 µl, 25 mM MgCl 8 µl, dNTP mix 6 µl, *Streptomyces* template DNA (50 ng), 5 µl and *Taq* DNA polymerase (3U/ µl), 2 µl in a 0.5 ml micro centrifuge tube was taken. The total 100 µl mixture in a tube was gently spun for 10 sec. and allowed to settle the contents. The samples were kept in an Eppendorf PCR thermal cycler. The amplification was carried out in the following manner: 35 cycles, denaturation for 60 sec. at 92 °C, primer annealing for 60 sec. at 54 °C and polymerization for 90 sec. at 72 °C, finally the tubes were ensured complete polymerization at 72 °C for 15 min.

2.2.7. Agarose gel electrophoresis of DNA

Gel electrophoresis with 1% (w/v) agarose was used to separate DNA fragments. The employed buffer was 1×TAE buffer. 10 µl of PCR products with 2 µl of loading dye were mixed and analyzed by electrophoresis at 120 Volts for 45 min. After running the gels, they were stained with the fluorescent peq Green, detected under the UV light at 312 nm and photographed using an Eagle Eye II System (Stratagene, Heidelberg, Germany). The visible gel band were compared to a commercial 1kb DNA ladder.

2.2.8. 16S rRNA sequencing

The PCR products were purified by using a Microcon PCR centrifugal filter device (Millipore Corp. Bedford, Mass.) and sequenced using by the Eurofins Genomics GmbH. (Ebersberg, Germany).

2.2.9. Nucleotide sequence accession

The 16S rDNA sequences of the one potential streptomyces *Streptomyces* sp. SW4 have been deposited in a genbank http://www.ncbi.nlm.nih.gov/genebank and the sequence accessions numbers were received as MH517391.

2.2.10. Phylogenetic analysis

The reference sequences required for comparison were down loaded from the EMBL database using the site http://www.ncbi.nlm.nih.gov/genebank. All the sequences were aligned using the multiple sequence alignment program CLUSTAL W. The aligned sequences were then checked for gaps manually and arranged in a block of 250 bp in each row and saved as format in software MEGA 5. The pair wise evolutionary distances were computed using the Kimura 2- parameter model. To obtain the confidence values the original data set was resampled 1,000 times by using the Boot strap program of PHYLOGENY and subjected to bootstrap analysis. The bootstrapped data set was used directly for constructing the corresponding phylogenetic tree using the MEGA program or used for calculating the multiple distance matrix. The multiple distance matrix obtained was then used to construct a phylogenetic tree using the Neighbour-Joining method. All these analysis were performed using the MEGA X software.

2.2.11. Whole genome sequencing and analysis

Streptomyces sp. SW4 was isolated from a Nusakambangan Island in Central Java-Indonesia (DSM 110508 = BCCM/LMG 31269). The isolate was maintained on GYM agar at 30°C. Long term preservation was performed with 30% (w/v) glycerol and then stored at -80°C. The whole genome sequence analysis of *Streptomyces sp*. SW4 was conducted by the facility of Macrogene lnc. (Geumcheon-gu, Seol, Korea) and the draft genome was deposited at DDBJ/EMBL/Genbank under the accession no. QKWM00000000.

2.2.12. Gene prediction and annotation

Multiple bioinformatics software tools were used for the gene prediction and annotation. Gene functions were annotated with Prokka (v1.12b) (http://www.vicbioinformatics.com/software.prokka.shtml) and also using Prokaryotic Genome Annotation Pipeline (PGAP) http://www.ncbi.nlm.nih.gov/genome/annotation_prok/). Whole genome sequence of *Streptomyces leeuwenhoekii* DSM 42122, *Streptomyces lomondensis* DSM 41428, *Streptomyces glomeratus* DSM 41457,

Streptomyces purpurascens DSM 40310, and *Streptomyces thermodiastaticus* DSM 40573 were obtained from whole genome sequencing center of the DSMZ.

2.2.13. Bioinformatic analysis - Identification of the biosynthetic gene cluster

For the identification of the putative biosynthetic gene clusters, the web-based software antiSMASH was applied. For phylogenetic analyses, alignments were made with the CLUSTALX (version 1.81) software available at http://www.ebi.ac.uk/clustalw/. Trees were inferred by Neighbour Joining in CLUSTALX using 10,000 bootstrap replicates. Multiple alignments were performed by using MEGAX. Phylogenetic trees were visualized with Draw tree, MEGAX.

2.2.14. Characterization of *Streptomyces* sp. (SW4)

2.2.15. Biochemical characterization

2.2.16. API 50 CH test (Utilization of carbon sources)

The ability of *Streptomyces* to digest several carbon sourses was tested by an API 50 CH system. All streptomyces were inoculated into flasks containing 50 ml GYM and incubated in an orbital shaker (at 200 rpm) at 30^{o}C for 4-8 days. After incubation, the pellet cells were harvested and dissolved in the API 50 CH medium until the turbidity was equivalent to 2 McFarland. The API 50 CH strip was loaded with 100 µl of the suspension and incubated at 30^{o}C for 4-14 days. After incubation, the change of medium color was recorded. A positive test corresponds to acidification revealed by the phenol red indicator contained in the medium changing from red to yellow.

2.2.17. Diffusible pigment production test

The *Streptomyces* cultures were inoculated into different kinds of agar media and incubated at 30°C for 14 days. The formation of color such as yellow-brown, blue, green, red, orange and grey or violet in the medium was subsequently recorded.

2.2.18. Catalase test

The culture suspensions of *Streptomyces* were placed on a clean glass slide, few drops of hydrogen peroxide were added to the culture suspension. The evolution of air bubbles from the suspension indicated a positive reaction.

2.2.19. Physiological characterization

2.2.20. Effect of pH

Medium ISP2 broth was prepared and sterilized. The pH of the broth was adjusted to 3, 4, 5, 6, 6.5, 7, 8, 8.5, 9 and 10 using 0.1 N HCl or NaOH. The *Streptomyces* cultures were inoculated into the test tubes containing broth and incubated at 30 °C for 7-14 days. The growth was recorded after incubation.

2.2.21. Effect of temperature

Medium ISP2 broth was prepared and sterilized. The *Streptomyces* cultures were inoculated into the broth. The tubes were incubated at 26, 28, 30, 37 and 50 °C for 7-14 days. After incubation, the growth was recorded.

2.2.22. Antibiotic sensitivity

The lawn cultures of *Streptomyces* were prepared in SCA medium. The selected antibiotic discs (streptomycin, gentamycin, vancomycin, tetracycline, ampicillin, penicillin and co-trimazole) were placed over the media. The plates were incubated at room temperature. After incubation, the zones of inhibition were recorded and determined the sensitivity patterns of *Streptomycetes*.

2.2.23. Effect of NaCl

The basal medium was prepared with NaCl 0, 2, 4, 6, and 8%. The medium was autoclaved and poured into sterile petri plates. Agar plates were streaked with *Streptomyces* and incubated 30 °C for 7-14 days. After incubation, the tolerance limits of *Streptomyces* to NaCl were recorded.

2.2.24. Production of antimicrobial compounds

Seed cultures of *Streptomyces* sp. SW4 was grown in 12.5 ml GYM in 50 ml Falcon tubes for 3 days at RT with shaking (220 rpm) at 30°C. The production medium (HT7T) was prepared at 50 ml in a 250 ml Erlenmeyer flask and sterilized. After sterilization, the media was inoculated with 2 ml of seed culture and incubated in rotary shaker (at 200 rpm) for seven days at 30°C.

Seed cultures of *P. aeruginosa* SW5 were grown in 12.5 ml Difco Davis Minimal Broth without dextrose (Becton Dickinson; Sparks, MD) containing 20 mM glycerol (DMBgly) in 50 ml Falcon tubes for 3 days at RT with shaking (110 rpm) employing a Gerhardt LS30 horizontal shaker. For cultivation on a large scale, 5000 ml Erlenmeyer flasks containing either 1 or 1.5 L DMBgly were inoculated with 3 ml of seed culture and incubated for 48 h at 23°C in an INFORS HT Multitron Pro orbital shaker operating at 120 rpm.

2.2.25. Extraction of antimicrobial compounds of actinobacteria

The potential isolate was inoculated into 100 ml of ISP2 medium and incubated at 30 °C in a shaker at 200 rpm for 6 days. The broth culture was filtered using Whatman No. 1 filter paper (11 µm). The organic solvent ethyl acetate was added to the filtrate in the ratio of 1:1 (v/v). The mixture was shaken vigorously for 5 min; supernatant was collected and stored at 4 °C. The antimicrobial activities of the solvent extracts were determined by a paper disc-diffusion assay. Paper disc-diffusion assays were made on Müller Hinton agar with fresh test bacterial lawn cultures and each disc was loaded with 10 µl of actinobacterial solvent extracts. All plates were incubated at appropriate incubation conditions and examined for inhibition zones. The diameter of the inhibition zones was measured and recorded in millimeter. Separate control wells were maintained for each solvent alone.

2.2.26. Separation and purification of antimicrobial compounds

The antimicrobial compounds of *Streptomyces* sp. SW4 were purified by silica gel TLC. Using a capillary tube, a row of spots of the treated fermented broth sample was applied 1.5 cm above from the bottom of TLC plate, and the spots were allowed to dry. The TLC plate was placed vertically in a TLC solvent chamber containing suitable solvents (DCM-Methanol (9:1)). When the solvents moved up to 80% of TLC, the plates were taken out and dried. The TLC plates were observed and analyzed under UV light.

2.2.27. HPLC profiling

Profiling of the obtained extract triplicates was achieved using an HPLC system composed of the Waters 1525 Binary Pump with a 7725i Rheodyne injection port; a Kromega Solvent Degasser, a Waters 996 Photodiode Array Detector, and an Aeris

peptide XB-C18 column. ACN (solvent A) and H_2O + 0.1% TFA (solvent B) were used for the gradient elution of the analytes at a steady flow rate of 0.7 ml/min, with an injection volume of 5 µl. A non-uniform gradient was employed: for the initial 15 min, 50% A, followed by 100% A for 10 min, 50% A for additional 10 min and lastly 50% A for the last 4 min. The detection wavelengths were 238, 280, and 336 nm.

2.2.28. Mass spectroscopy

HR-ESI-TOF MS data were recorded using a Bruker Daltonic maXis 4G instrument. LCMS and MS2 experiments were carried out employing either an AB SCIEX LC/MS system consisting of an Agilent 1100 system and a 3200 Q TRAP mass spectrometer or an Agilent 1100 LC MSD ion trap system equipped with autosampler, binary gradient pump and column thermostat (Agilent, Waldbronn, Germany). The mobile phase contained (A) 0.1% formic acid and (B) 0.1% formic acid in acetonitrile. The LC-run was performed in gradient elution mode. First, the mobile phase was kept for 2.5 min on 10% B, increasing the percentage of B in 10 min to 64% followed by isocratic conditions until min 15, increasing the percentage of B to 80% in 2.5 min keeping it for 2.5 min, followed by reequilibration at 10% B for 3 min. The MS was run in the information dependent acquisition-mode (IDA) for simultaneous identification by high resolution TOF and confirmation by MS/MS. Mass accuracy was kept below 0.5 ppm for MS and below 1.3 ppm for MS/MS by interlaced calibration and sample measurement.

2.2.29. Molecular networking and metabolites annotation

The features list of the three extraction solvents was exported from Metaboscape as two single MGF files for both of the positive and negative measurements. Both MGF files were uploaded separately to the GNPS online platform where two molecular networks were generated with the online workflow (GNPS 2.0). A molecular network was created with a cosine score above 0.65 and 0.7 for positive and negative modes, respectively. The minimum number of matched fragment ions was adjusted to 6. Further edges between two nodes were kept in the network if (and only if) each of the nodes appeared in each other's respective top 10 most similar nodes. The network spectra were searched against GNPS' spectral libraries using a minimum of 6 matched fragments for spectral matching. The program Cytoscape 3.5.1 was used for molecular network visualization.

Manual putative structure identification was achieved by submitting the preprocessed MS^2.mgf output file from Metaboscape to Sirius + CSI: FingerID 4.0.1 for the prediction of the elemental composition (C, H, N, O, S, P) and the molecular structure database search with *m*/*z* tolerance was set to 20 ppm using online Pubchem database and the DEREP-NP database which was manually integrated into the software.

Data visualization was performed by using a Bruker Daltonics Data Analysis 4.4, while Metaboscape 3.0 (Bruker Daltonics) was used for the molecular features selection. Raw data files were imported into the program MetaboScape 3.0 for the entire data treatment and pre-processing. The T-ReX 3D (Time aligned Region Complete eXtraction) algorithm was used for the retention time alignment. It automatically detects and combines isotopes, adducts, and fragments intrinsic to the same compound into one feature. All detected features were displayed as a bucket table with their t_R, measured *m*/*z*, molecular weight, detected ions, and their intensity in each sample. The Bucket table was created with intensity threshold $10e^3$ and $10e^4$ for negative and positive ionization modes, respectively. The retention time range was set from 1 to 35 min and the mass range from 140 to 1800 *m*/*z*.

2.2.30. NMR spectral analysis

The pure compounds were characterized by ^1H and ^{13}C NMR spectroscopic experiments recorded on a Bruker Avance 400 MHz NMR spectrometer with $CDCl_3$ as solvent.

III. Result and Discussion

3.1. Results

In total, 110 isolates were obtained from soil of Nusakambangan island, Central Java-Indonesia. In the primary screening five isolates possessed antibacterial activity. Among 5 isolates, 4 isolates had activity against Gram positive bacteria and 1 isolate showed activity against Gram negative bacteria. (Tabel 10). Out of 5, three isolates were Actinobacteria. The strains were affiliated with the genera *Streptomyces* (SW4), *Promicromonospora* (SW3) and *Actinomadura* (PT1). Other two strains were affiliated with the genera *Bacillus* (SB3) and *Pseudomonas* (SW5). All of the strains were obtained using SNA medium. *Streptomyces* (SW4) represents possibly a novel species according to their 16S rRNA gene sequence similarities.

Table 10. Selected isolate from soil of Nusakambangan island, Indonesia.

Isolate code	Length (nt)	Related organism	Similarity	Antibacterial activity
PT1-A	1350	*Actinomadura sp* A01103 (KU382662.1)	100%	Bs, Ml
PT1-B	1347	*Actinomadura sp* A01103 (KU382662.1)	100%	Bs, Ec
SB3	1430	*Bacillus licheniformis* L5 (KU179324.1)	99%	Bs, Ec, Sp, Ml, Pf, Sc
SW3	1347	*Promicromonospora flava* old-3-4s-2-2 (AB562469.1)	99%	-
SW4	1516	*Streptomyces leeuwenhoekii* sleC34. (LN831790.1)	98,78%	Ml, Sp
SW5	1350	*Pseudomonas aeruginosa* R7-584-1 (JQ659909.1)	99%	Bs, Ec, Sp, Sc

Bs: *Bacillus subtilis*; Ec: *Escherichia coli*; Sp: *Streptococcus pneumoniae*; Ml: *Micrococcus luteus*; Pf: *Pseudomonas fluorescens*; Sc: *Staphylococcus carnosus*

3.2. Taxonomic and Chemical Investigations of Strain SW5

isolate SW5 was able to inhibit the growth of *Bacillus subtilis* as a gram positive bacteria and *Escherichia coli* as a gram negative bacteria. In order to find out that the correct name of microorganism, a 16S rRNA analysis was performed prior to PCR product sequencing of the gene 16S rRNA from SW5. Therefore, a series of taxonomic analyses were performed to characterize the strain and to assign it to the correct taxonomic class.

3.1.1. Taxononomic Characterization of SW5

The phylogenetic position of SW5 was determined by comparison of several 16S rRNA gene sequences. The 16S rRNA gene sequence (1350 bp) was obtained both from the 16S rRNA PCR sequence using the Illumina method. The 16S rRNA gene sequence then compared to the GenBank database using the NCBI-BLAST platform. Based on sequence similarity, strains of the genus *Pseudomonas* appeared to be the closest relatives. Therefore, further analyses were performed employing the EzTaxon-e server (http://www.ezbiocloud.net) and MEGA X. For reliable conclusions five different treeing methods were applied: neighbour-joining, maximum-likelihood, minimum evolution, maximum-parsimony and UPGMA. The result of this analysis is visualized in Figure 6. All three analyses confirmed with the highest bootstrap value that the strain SW5 should be positioned within the genus *Pseudomonas*, with *Pseudomonas aeruginosa* JCM5962^T as its closest relative. SW5 and *Pseudomonas aeruginosa* JCM5962^T share 100% 16S rRNA sequence similarity, followed by *Pseudomonas otitidis* MCC10330^T (98.7%), *Pseudomonas alcaligenes* NBRC14159^T (97.6%) and *Pseudomonas songnensis* NAE-ST5-5^T (96.8%). This locates SW5 in between *Pseudomonas aeruginosa* JCM5962^T and *Pseudomonas otitidis* MCC10330^T.

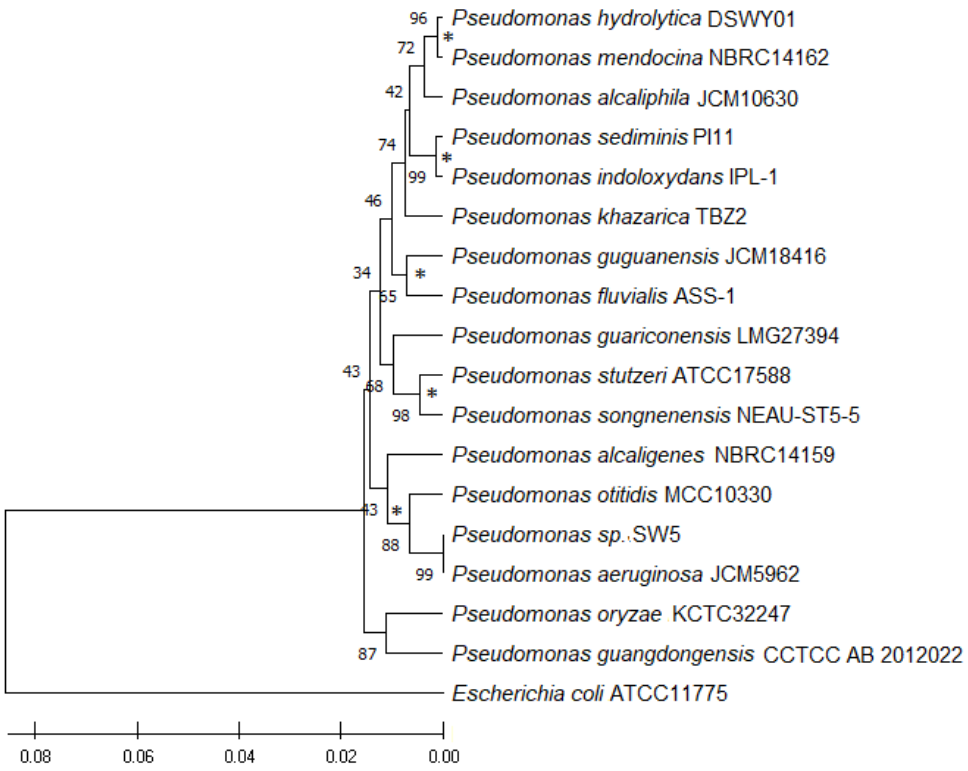


Figure 6. Neighbour-joining tree based on 16S rRNA gene sequences (1350 positions in the final dataset) showing relationships between strain SW5 and the type strains of closely related *Pseudomonas* species. The evolutionary distances were determined using the Kimura 2 parameter method. Asterisks indicate branches of the tree that were also found using the maximum-likelihood, minimum evolution, UPGMA and maximum parsimony tree-making algorithms. Numbers at the nodes are percentage bootstrap values based on a neighbour-joining analysis of 10,000 replicates. Bar 0.005 substitutions per nucleotide position. *Escherichia coli* ATCC11775 ^{T} was used as outgroup to root the tree.

3.1.2. Phenotypic and morphologic characteristics

The colony of strain SW5 is filamentous shape in SNA medium, transparent and thin, easy to scratch from the plate. Based on light microscopy, carried out in collaboration with Yvonne Mast from Biotechnology Department of the University of Tüebingen, strain SW5 is small single rod-shaped cell and shows a directed movement. (Fig. 7).

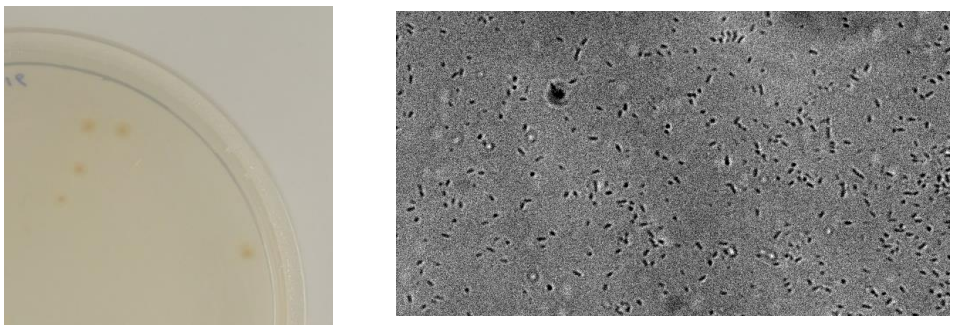


Figure 7. Morphology of *Pseudomonas aeruginosa* SW5 on SNA agar (left) and light microscopic picture of *Pseudomonas aeruginosa* SW5

3.1.3. Cultural characterization

Cultural characteristics of the isolate SW5 were studied with five different culture media. The colony of the isolate *Pseudomonas aeruginosa* SW5 looks transparent to yellowish forms raised either in irregular rough or in circular smooth, produce odor like grape and slimy. SW5 can grow at temperatures between 28^{o}C and 37^{o}C, but shows ideal growth at 30^{o}C. After 1-2 days under aerobic conditions at 30^{o}C, growth could be observed on DMB gly agar, ISP2 agar, ISP7 agar, TS agar, and SC agar. However no growth could be detected on MS agar. Furthermore, SW5 tolerates a pH range of 5.0 -9.0 and a salinity concentration of <4% (w/v) (Tabel 11).

Table 11. Cultural characteristics of isolate SW5.

Characteristic	Range	Optimum
Medium	DMBgly, ISP2, ISP7, TSB, SCA	DMBgly
Temperature (^{o}C)	28-37	30
Salinity (%)	0-4	0
pH	5-9	6

3.1.4. Biochemical characterization

Biochemical characteristics of the isolate SW5 were studied using API® 50CH. The utilization test for 49 carbon sources in API® 50CH shows that isolate SW5 uses glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D- galactose, D-mannose, D-melibiose, D-trehalose, gentiobiose, D-fucose, L-fucose, D-arabitol and potassium gluconate (Table 12). The SW5 isolate is similar with all strains in the species of *Pseudomonas aeruginosa* that not use lactose to growth and shows catalase positive.

Table 12. Utilization of carbon source of *Pseudomonas aeruginosa* SW5 using the API® 50CH test.

Caron source	Growth	Carbon source	Growth	Carbon source	Growth
Glycerol	+	D-mannitol	-	D-melezitose	-
Erythritol	-	D-sorbitol	-	D-raffinose	-
D-arabinose.	++	Methyl-alpha-D-mannopyranoside	-	Amidon (Starch)	-
L-arabinose	++	Methyl-alpha-D-glucopyranoside	-	Glycogen	-
D-ribose	++	N- Acetylglucosamine	-	Xylitol	-
D-xylose	++	Amigdalin	-	Gentiobiose	++
L-xylose	++	Arbutin	-	D-turanose	-
ADO,	-	Esculin ferric citrate	(+)	D-lyxose	-
Methyl-beta-D-xylopyranoside	-	Salicin	-	D-tagatose	-
D-glalactose	++	D-cellobiose	-	D-fucose	++
D-glucose	-	D-maltose	-	L-fucose	+
D-fructose	-	D-lactose	-	D-arabitol	+
D-mannose	++	D-melibiose	++	L-arabitol	-
L-sorbose	-	D-saccharose	-	Potassium gluconate	V
L-rhamnose	-	D-trehalose	+	Potassium 2-ketogluconate	-
Dulcitol	-	Inulin	-	Potassium 5-ketogluconate	-
Inositol	-				

++ very good growth; + good growth; (+) weakly growth; - no growth; V violet

3.1.5. Isolation of secondary metabolites from SW5

To isolate the potential secondary metabolites produced by SW5, the bacterium had to be cultivated in DMBgly medium. Pre-cultures were prepared in 50 ml DMBgly medium while the main cultures comprised 100 ml DMBgly broth per flask and were inoculated with 2 ml pre-culture. Cultures were incubated at 30°C for 2 days and extracted twice with ethylacetate. The dry crude extract was fractionated using HPLC (Fig. 8) and evaluated

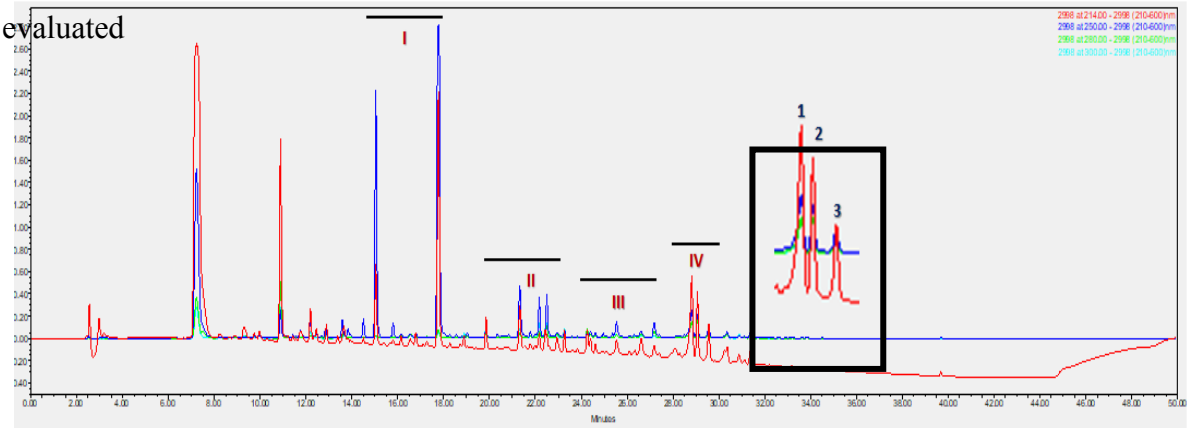


Figure 8. HPLC profiling and separation of the SW5 crude extract. The box represents a magnification of fraction IV.

In antimicrobial bioassays. Since solely fraction IV was highly active, this fraction was further purified by a second round of HPLC (Fig. 9).

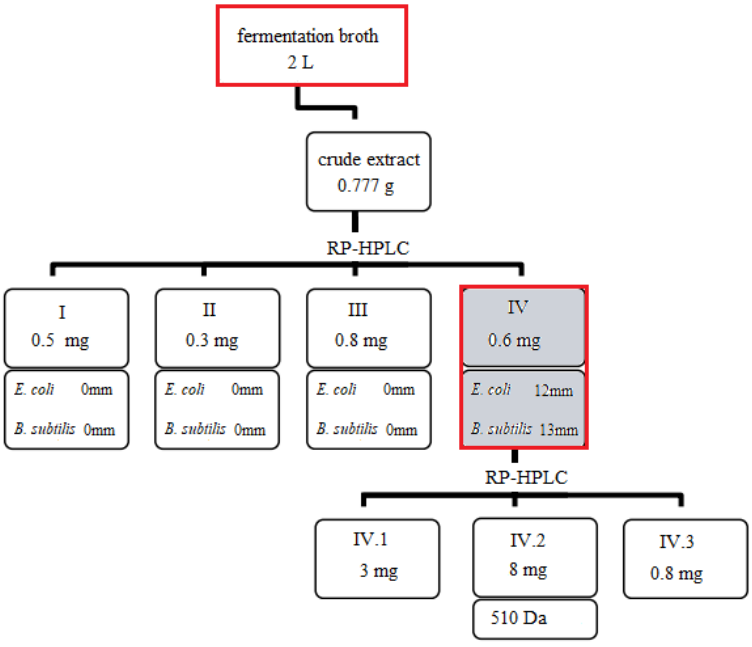
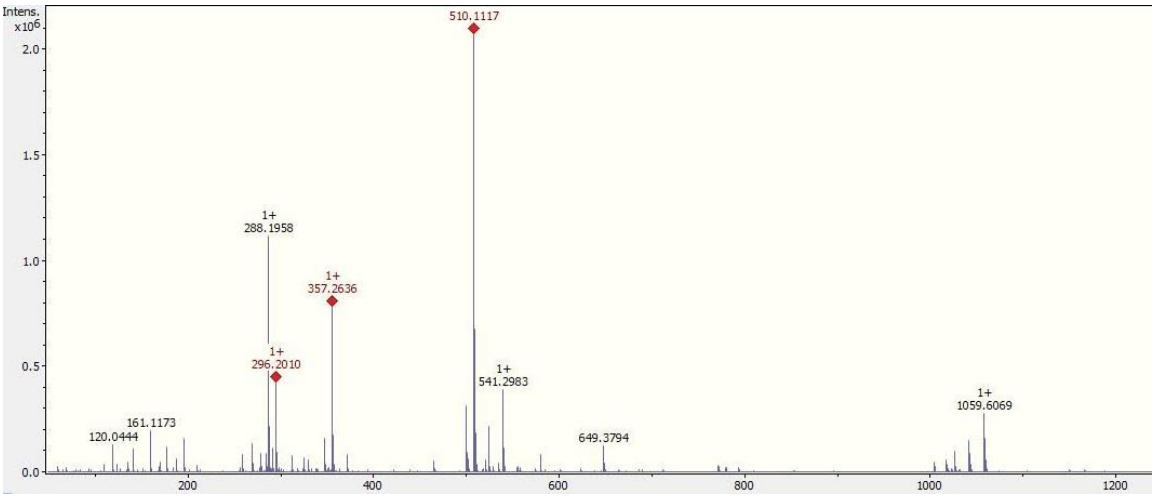


Figure 9. Fractination scheme of SW5. Red color indicates antibacterial activity

This effort resulted in the isolation of 3 putative pure compounds. Using database-based dereplication, compounds IV.1 and IV.3 represents 556 Da and 699 Da. Both of compound correlate with monorahmnolipids and dirhamnolipids.[131] However, fraction IV.2 contained a mass of 510 Da. (Fig. 10). Interestingly, based on the fragmentation pattern, this mass was not found in natural product databases.

A



B

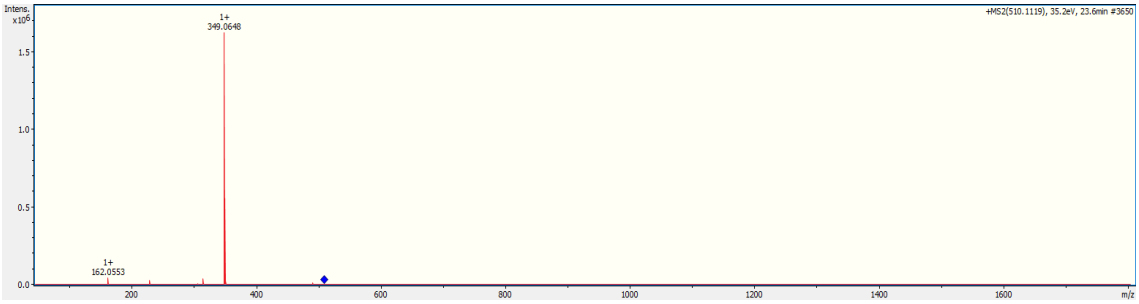


Figure 10. MS and MS/MS analysis of the SW5 crude extract. MS analysis of 510 Da (A), MS/MS Compound spectra of 510 Da (B).

Generating the prediction formula of 510 Da based on MS and MS/MS analysis from HRMS data of SW5 crude extract, the possible ion formula of the compound is $C_{33}H_{18}O_6$, (calcd. 510.1117, Δ-3.8 ppm) due to the lowest mass error (Fig. 10).

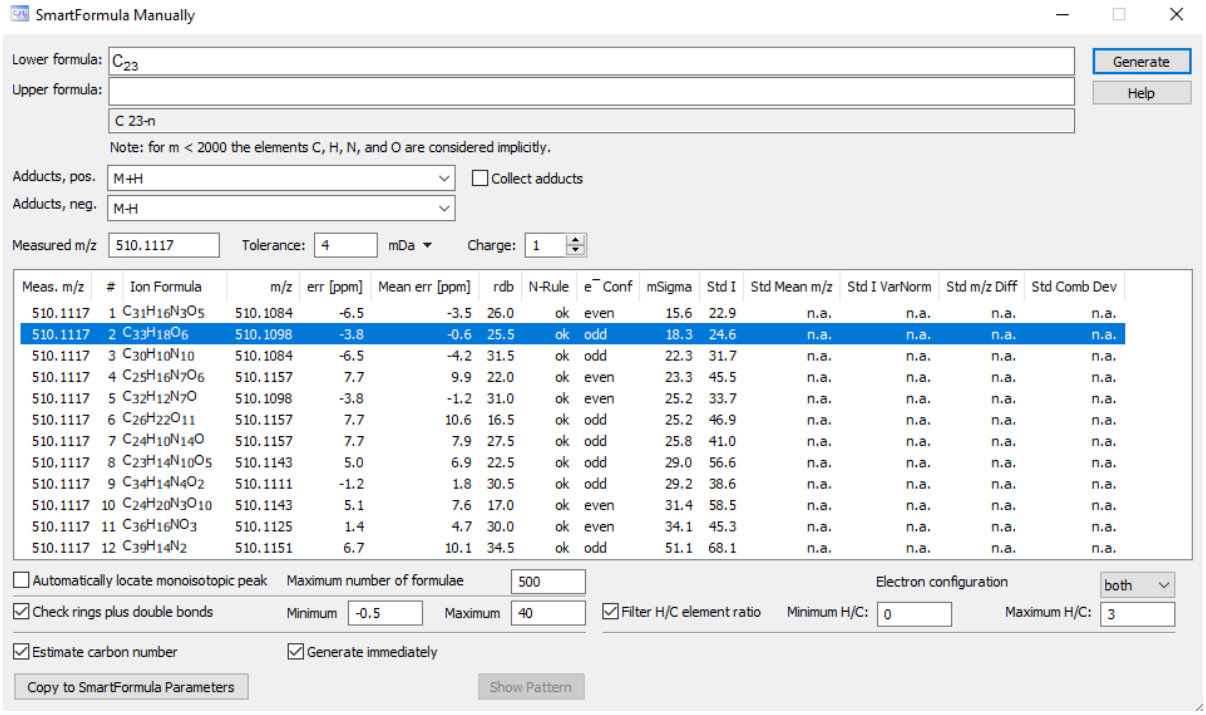


Figure 11. Formula prediction of 510.1 Da for compound fraction IV.2 of SW5 using HRMS data.

Subsequently, ^{1}H-NMR and ^{1}H-^{13}C-HSQC analyses were performed chracterize the compound class. The ^{1}H NMR spectrum of fraction IV.2 was indicative for a rhamnolipids by displaying anomeric H signals of sugar moieties (δ_H~ 5.5), CH-OH of sugar resonances (δ_H~ 3 - 4) and several resonances characteristic for a fatty acid moiety (δ_H~ 0.9 and 1.4) (Fig. 11).

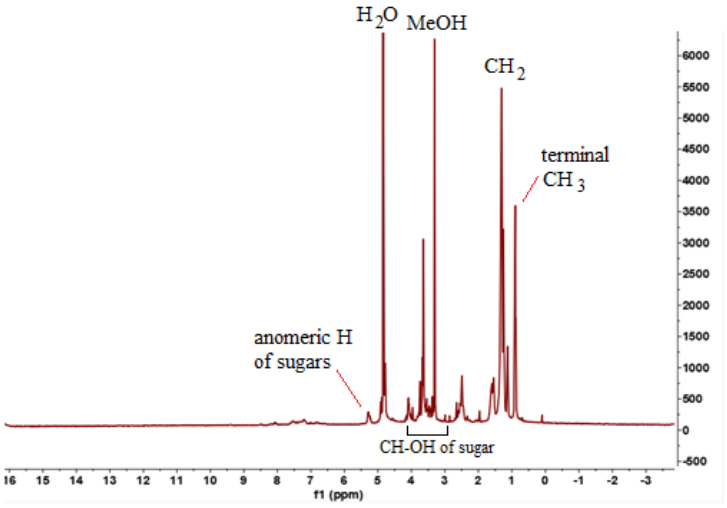


Figure 12. ^{1}H-NMR of compound IV.2.

Furthermore, NMR data from the ^{1}H-^{13}C 2D-HSQC spectrum exhibited six distinct spin systems (4.60–5.50 to 92.4–102.4 ppm) indicating the presence of sugar ring moieties and lipid sidechains, characteristic of rhamnolipids. (Fig.12), thereby confirming the first conclusion from the proton NMR analysis. Notably, it also revealed the presence of three anomeric protons at 5.27 ppm. Since tri-rhamnolipids are so far non/existant and would also not fit to the determined mass, it was concluded that fraction IV.2 does not a single pure compound, but rather a mixture of mono- and di/rhamnolipids. However, the mass of 510 could not be associated with these compounds and possibly a third compound with this mass co-eluted with the two rhamnolipids.

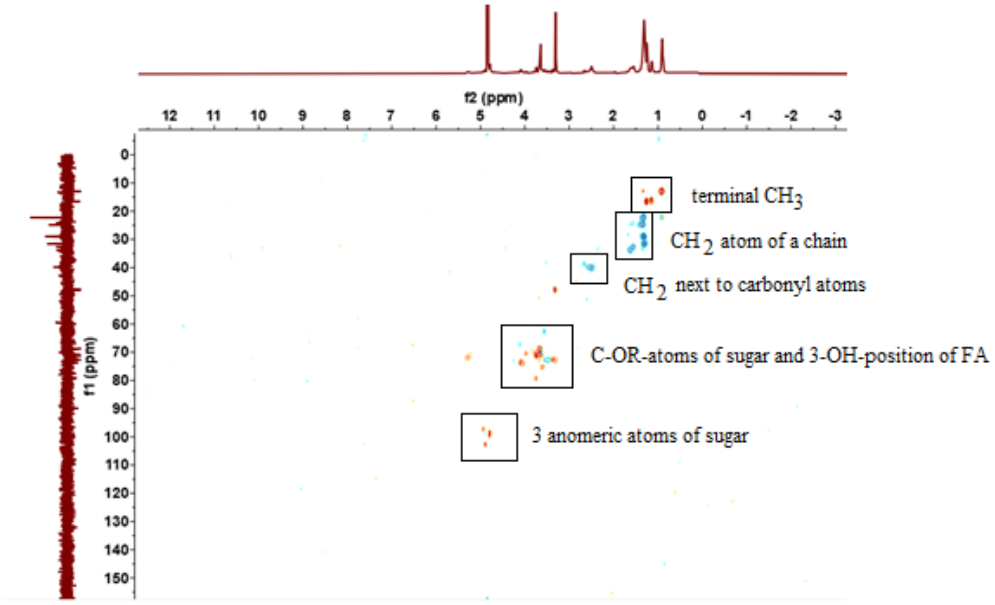


Figure 13. 400 MHz ^{1}H-^{13}C-HSQC-NMR spectrum fraction IV.2 of SW5.

3.3. Discussion and Outlook

SW5 was shown to be a potent isolate to produce antibacterial agents. The taxonomic classification of SW5 into *Pseudomonas aeruginosa* could be confirmed within these studies. The 16S rRNA of strain SW5 was identified to be highly similar (99%) to the one of the type species *P. aeruginosa* JCM5962^{T}. However, the strains differ in some phenotypic characteristics such as e.g. salinity preferences. Sachan and co-workers isolated *P. aeruginosa* JCM5962^{T} from soil of a sugarcane field in Uttar Pradesh-India.[132] However, identification of SW5 was only based on the 16S rRNA sequence.

Khan and co-workers found that oceanic *P. aeruginosa* strains better grew in the presence of a high concentration of NaCl rather then fresh water and clinical *P. aeruginosa* strains.[133] However, *P. aeruginosa* SW5 was able to grew in medium contain concentration of NaCl 4% (w/v). This finding is not surprising, taking into account that the sample SW5 was collected off an area which was located close to a beach.

The precence of rhamnolipids in the crude extract of the culture medium of *Pseudomonas aeruginosa* SW5 is line with literature reports. *Pseudomonas aeruginosa* are known to possess a rhamnolipid biosynthetic gene cluster and it actually represents a model strain to understand the genes that are critical for rhamnolipid biosynthesis.[134] Rhamnolipids are composed of either one or two rhamnose monosaccharides covalently bonded with up to two 3-(hydroxyalkanoyloxy)alkanoic acid (HAA) fatty acid tails ranging between 8 and 16 carbons in length.[135] The found rhamnolipids from fraction IV showed biaoactivity against *E.coli* and *B. subtilis* in the concentration up to 10 µg/ml. These findings are in good agreemnent with the reports stating that, above the critical micell concentration, rhamnolipids exhibit significant antibacterial activity.[136] Spectrum-wise rhamnolipids were reported to be active against *Listeria monocytogenes*, *Serratia marcescens, Enterobacter aerogenes, Klebsiella pneumoniae, Staphylococcus aureus, Staphylococcus epidermidis, Salmonella typhimurium, Escherichia coli* and *Bacillus subtilis*. [137-139] In addition, rhamnolipids are also able to affect the colony growth and biomass accumulation of plant pathogenic fungi, such as *Phytophthora infestans, Botrytis cinerea, Fusarium graminearum* and *Mucor* sp.[139, 140] Concerning the mode of action, it is hypothesized that rhamnolipids alter the cell membrane or envelop which in turn leads to enhanced membrane permeability and cell damage.[141]

Beside the antibacterial effect, rhamnolipids were assigned further different roles and tasks. Thus, taking into account its amphiphilic nature, rhamnolipids were reported to contribute to a naturally developed mechanism of bioremediation in the case of petroleum contamination. Other groups provided evidence that this compound class is involved in the enhancement of microbial motility or that they are responsible for the formation of biofilms.[142-146]

Production of rhamnolipids in *Pseudomonas* involves several steps. The precursors for rhamnolipid synthesis are the sugar (dTDP-L-rhamnose) and hydrophobic moieties such

as 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs). The sugar moiety can be synthesized from D-glucose, while the hydrophobic moiety can be synthesized through the fatty acid synthesis pathway, starting with two-carbon units. The three key enzymes for rhamnolipid biosynthesis, RhlA, RhlB and RhlC, are found almost exclusively in *Pseudomonas* sp. and *Burkholderia* sp., but have been successfully expressed in several non-pathogenic host bacteria to produce rhamnolipids in large scales.[147-149]

3.4. Chemical and Taxonomic Investigations the Strain SW4

The phylogenetic position of SW4 was determined by comparison of several 16S rRNA gene sequences. The 16S rRNA gene sequence (1516 bp) was obtained both from a 16S rRNA PCR sequencing experiment as well as from the whole genome sequence using the PacBio method. The 16S rRNA gene sequence was then compared to the GenBank database using the NCBI-BLAST platform. Based on sequence similarity, strains of the genus *Streptomyces* appeared to be the closest relatives. Therefore, EzBioCloud subjected every 16S rRNA sequence of *Streptomyces*-type strains to phylogenetic analysis, using MEGA X. For reliable conclusions four different treeing methods were applied: neighbour-joining, maximum-likelihood, minimum evolution and maximum-parsimony. The result of these analyses is visualized in Figure 13. All three analyses confirmed with the highest bootstrap value that strain SW4 should be positioned within the genus *Streptomyces*, with *Streptomyces spiralis* NBRC14215^T as its closest relative. SW4 and *Streptomyces spiralis* NBRC14215^T share 98.67% 16S rRNA sequence similarity, followed by *Streptomyces indiaensis* DSM 43803^T = NBRC 13964^T (99,30%), *Streptomyces leeuwenhoekii* DSM 42122^T = C34^T (98.75%) and *Streptomyces muensis* DSM 103493^T = MBRL 179^T (98.89%). This locates SW4 in between *Streptomyces spiralis* NBRC14215^T and *Streptomyces muensis* DSM 103493^T.

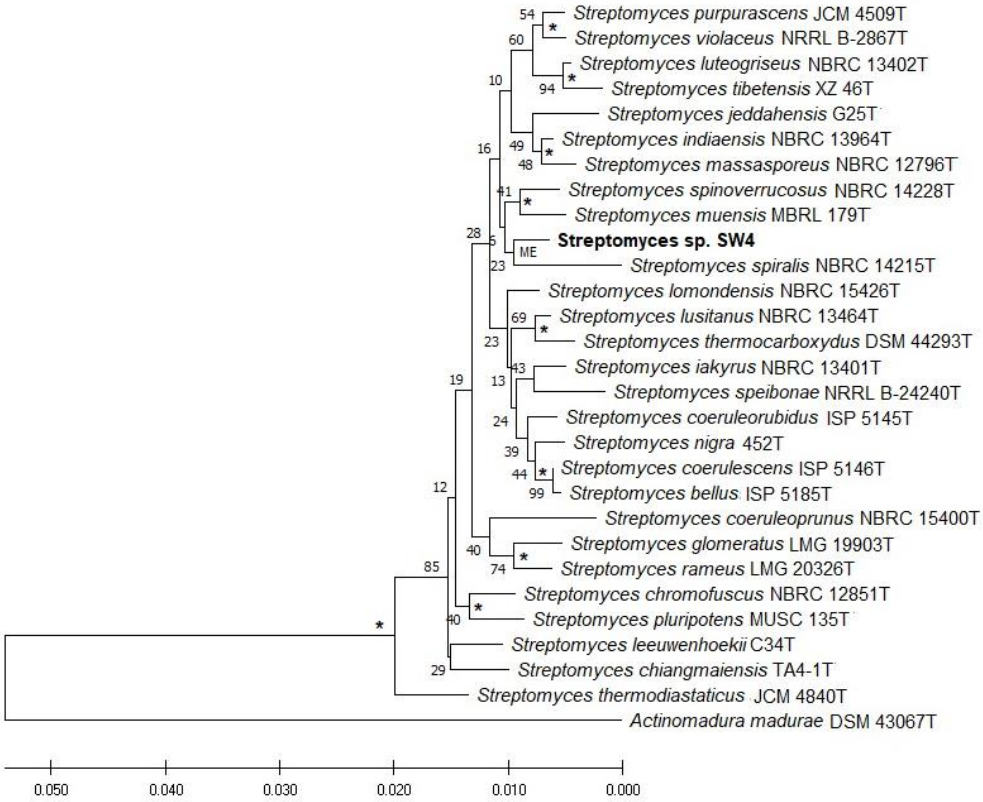


Figure 14. Neighbour-joining tree based on 16S rRNA gene sequences (1516 positions inthe final dataset) showing relationships between strain SW4 and the type strains of closely related *Streptomyces* species. The evolutionary distances were determined using the Kimura 2 parameter method[150]. Asterisks indicate branches of the tree that were also found using the maximum-likelihood, minimum evolution, and maximum parsimony tree-making algorithms. ME specify nodes that was also recovered using the minimum evolution. Numbers at the nodes are percentage bootstrap values based on a neighbour-joining analysis of 10,000 replicates. The bar indicates 0.01 substitutions per nucleotide position. *Actinomadura madurae* DSM 43067^T was used as outgroup to root the tree.

Next the *in silico* methods were applied. For genome to genome comparison, the software Type (strain) Genome Server (https://tygs.dsmz.de/) was used since it performs a comprehensive digital DNA-DNA hybridization (dDDH) comparison across the whole genome instead of random DNA portions. SW4 was investigated to establish whether it forms a distinct species within the genus *Streptomyces*. Unfortunately, from the 13 closely-related type strains of intersest, the corresponding genomic information was not available for all the strains. Therefore, only 9 closely-related type strains were included. Table 13 summarizes these results, and shows that SW4 shares less than 70% DNA sequence with closely-related type strains, thereby confirming its status as a novel species

Table 13. Classification of SW4 based on digital DNA-DNA sequence similarity

	Species	% dDDH												
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1	SW4 (QKWM00000000)	100												
2	*S. leeuwenhoekii* DSM 42122^T (LN831790)	33,8± 3	100											
3	*S. indiaensis* DSM 43803^T	-	-	100										
4	*S. lomondensis* DSM 41428^T (BMWC00000000.1)	33,9± 4	31,8± 3	-	100									
5	*S. glomeratus* DSM 41457^T	-	-	-		100								
6	*S. purpurascens* DSM 40310^T (BMUK00000000.1)	31,5± 6	29.9± 2	-	54,2± 13	-	100							
7	*S. rameus* DSM 41685^T	-	-	-	-	-	-	100						
8	*S. resistomycificus* DSM 40133^T (LMWZ00000000.1)	30,5± 2	-	-	38,1± 10	-	37,4± 10	-	100					
9	*S. iakyrus* DSM 40482^T (JNXI00000000.1)	32,7± 7	30,8± 3	-	51,0± 11	-	51,0± 4	-	36,4± 9	100				
10	*S. spinoverrucosus* DSM 41648^T (BJND00000000.1)	30.3± 4	29.0± 2	-	35.4± 7	-	33,2± 2	-	32,9± 4	30,3 ±4	100			
11	*S. niger* DSM 43049^T (JOFQ00000000.1)	19.8± 5	19.1± 5	-	19.2± 3		18.9± 4		18.4± 4	18.7± 4	18.4± 4	100		
12	*S. muensis* DSM 103493^T	-	-	-	-	-	-	-	-	-	-	-	100	
13	*S. thermodiastaticus* DSM 40573^T (BNBV00000000.1)	28.8± 1	27.9± 1	-	26.5± 1	-	24.9± 1	-	24.4± 1	25.0± 1	24.9± 1	18.9± 3	-	100

1: Strain SW4; 2: *S. leeuwenhoekii* DSM 42122^T; 3: *S. indiaensis* DSM 43803^T; 4: *S. lomondensis* DSM 41428^T; 5: *S. glomeratus* DSM 41457^T; 6: *S. purpurascens* DSM 40310^T; 7: *S. rameus* DSM 41685^T; 8: *S. resistomycificus* DSM 40133^T; 9: *S. iakyrus* DSM 40482^T; 10: *S. spinoverrucosus* DSM 41648^T; 11: *S. niger* DSM 43049^T; 12: *S. muensis* DSM 103493^T; 13: *S. thermodiastaticus* DSM 40573^T. All values are expressed in % sequence similarity ± differential between A x B and B x A mean value.

To complement the genotypic analysis, the G+C content was analyzed. This analysis was performed from *in silico* whole genome data sequencing from NCBI using an algorithm in Type Strain Genome Server (https://tygs.dsmz.de/).[151] The obtained G+C value of 73.32 mol% is specific for *Streptomyces* spp.

In order to support the classification of SW4 into the genus *Streptomyces*, the above mentioned findings need to be corroborated by phenotypic studies. Basic tests such as growth conditions and kinetics, Gram staining, cell and colony morphology were carried out in collaboraton with the DSMZ and the Biology Centre CAS, Institute of Soil Biology, České Budějovice, Czech republic.

3.1.6. Phenotypic and morphologic characteristics

Strain SW4 is a Gram-positive, non-motile bacterium. After one week of incubation, strain SW4 formed an aerial mycelium with brown spores on ISP2 agar (Fig. 14). Based

on the light and scanning electron microscopy carried out in collaboration with Biology Centre CAS, Institute of Soil Biology, České Budějovice, Czech republic, strain SW4 formed a straight to flexuous (rectiflexibiles) spore chain on an aerial mycelium with smooth surface. Single cells of SW4 represent a chain rod measuring 0.5 – 1.8 µm. The microscopical studies of this isolate undoubtedly placed these isolates under *Streptomyces* genera.

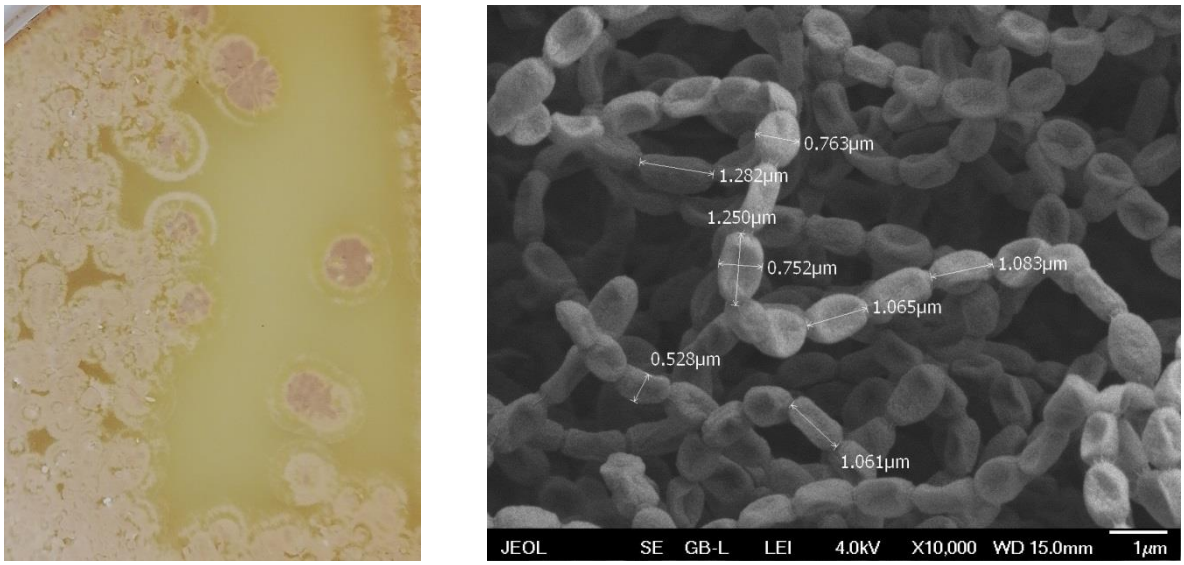


Figure 15. Morphology of *Streptomyces sp.* SW4 on ISP2 agar (left) and scanning electron micrographs of *Streptomyces sp.* SW4

3.4.1. Cultural characterization

3.4.3.1. Growth medium

Cultural characteristics of the isolates were studied with fourteen different culture media. The isolates *Streptomyces* sp. (SW4) produced grey, white and ash coloured spore mass and white, brown and yellowish on the reverse side in most of the media tested. SW4 produced diffusible pigments on ISP2 and MEA media (Table 14).

Table 14. Growth and characteristics of *Streptomyces sp*. SW4 cultivated on various agar media after incubation for 14 days at 30 ^{o}C.

Agar Medium	Growth	Substrate mycelium color	Aerial mycelium color	Soluble pigment
Yeast extract-malt extract (ISP 2)	Good	Purpish Pink	White	Yellow Green
Inorganic salts starch (ISP 4)	Good	White	White	None
Oatmeal (ISP 3)	Good	White	Yellow	None
Peptone–yeast extract-iron (ISP 6)	Good	Grey	Grey	None
Tryptone–yeast extract (ISP1)	Good	Buff	Buff	None
Tyrosine (ISP 7)	Good	White	Greenish Yellow	None
Glyserol-asparagine (ISP 5)	Good	Yellow Green	Yellow	None
Tryptic Soy Broth (TSB)	Good	White	White	None
Nutrient (NA)	Good	White	White	None
MEA	Good	Purpish Pink	Yellow Green	Yellow Green
YMA	Good	White	White	None
SCA	Good	White	White	None
Grauze's Medium No.1	Good	White	Yellow	None
MSA	Moderate	None	None	None

From the medium growth study, twelve related strains *S. leeuwenhoekii* DSM 42122^{T}, *S. indiaensis* DSM 43803^{T}, *S. lomondensis* DSM 41428^{T}, *S. glomeratus* DSM 41457^{T}, *S. purpurascens* DSM 40310^{T}, *S. rameus* DSM 41685^{T}, *S. resistomycificus* DSM 40133^{T}, *S. iakyrus* DSM 40482^{T}, *S. spinoverrucosus* DSM 41648^{T}, *S. niger* DSM 43049^{T}, *S. muensis* DSM 103493^{T} and *S. thermodiastaticus* DSM 40573^{T} were grown on the same media under aerobic condition following 14 days of growth at 30^{o}C. All strains grew well on all agar media. Only the medium MSA, which contained 10 mg/l NaCl, was not suitable for growing all *Streptomyces*. Color of the aerial mycelium, vegetative mycelium and diffusible pigment of strain SW4 shows wide variations across different mendia. The effect of the medium and time probably drives different biosynthetic gene cluster to produce their compouns.

3.4.3.2. Temperature condition

For the determination of temperature tolerance, SW4 was exposed to different temperatures (26, 28, 30, 37 and 50°C). The growth comparison of SW4 and 12 closely related type strains exposed to different temperatures was observed on ISP2 broth for 7 days.

Table 15. Growth comparison of *Streptomyces sp*. SW4 and closely related type strains, employing different temperatures.

Temperature	1	2	3	4	5	6	7	8	9	10	11	12	13
26°C	+	+	+	+	+	+	+	+	+	+	+	+	+
28°C	++	++	++	++	++	++	++	++	++	++	++	++	++
30°C	++	++	++	++	++	++	++	++	++	++	++	++	++
37°C	+	+	-	+	-	-	+	+	-	+	+	+	+
50°C	(+)	(+)	(+)	(+)	(+)	(+)	+	(+)	(+)	-	(+)	+	(+)
++ very good growth; + good growth; (+) weak growth; - no growth; 1: Strain SW4; 2: *S. leeuwenhoekii* DSM 42122^T; 3: *S. indiaensis* DSM 43803^T; 4: *S. lomondensis* DSM 41428^T; 5: *S. glomeratus* DSM 41457^T; 6: *S. purpurascens* DSM 40310^T; 7: *S. rameus* DSM 41685^T; 8: *S. resistomycificus* DSM 40133^T; 9: *S. iakyrus* DSM 40482^T; 10: *S. spinoverrucosus* DSM 41648^T; 11: *S. niger* DSM 43049^T; 12: *S. muensis* DSM 103493^T; 13: *S. thermodiastaticus* DSM 40573^T													

From the temperature growth study, it was found that strain SW4 grew at temperatures between 26-37°C but shows ideal growth at 28-30°C.

3.4.3.3. Salinity tolerance

Salinity tolerance was evaluated in ISP2 broth supplemented with 0-10% NaCl ((w/v), with 2% intervals). Growth kinetics were determined after 7 days of growth at 30°C by measuring OD600. SW4 grew very well in the presence of salt until a concentration of 2% was reached. The increasing concentration of salt drastically diminished cell growth.

Table 16. Growth comparison in the presence of sodium clorida of *Streptomyces sp. SW4* and closely related type strains.

Concentration	1	2	3	4	5	6	7	8	9	10	11	12	13
0%	++	++	++	++	++	++	++	++	++	++	++	++	++
2%	++	+	-	++	++	++	++	++	++	++	++	++	++
4%	+	-	-	+	+	+	+	+	+	+	+	-	+
6%	+	-	-	-	-	-	-	+	+	-	+	-	+
8%	(+)	-	-	-	-	-	-	(+)	+	-	-	-	-
10%	-	-	-	-	-	-	-	-	+	-	-	-	-
++ very good growth; + good growth; (+) weak growth; - no growth; 1: Strain SW4; 2: *S.*													

leeuwenhoekii DSM 42122^T; 3: *S. indiaensis* DSM 43803^T; 4: *S. lomondensis* DSM 41428^T; 5: *S. glomeratus* DSM 41457^T; 6: *S. purpurascens* DSM 40310^T; 7: *S. rameus* DSM 41685^T; 8: *S. resistomycificus* DSM 40133^T; 9: *S. iakyrus* DSM 40482^T; 10: *S. spinoverrucosus* DSM 41648^T; 11: *S. niger* DSM 43049^T; 12: *S. muensis* DSM 103493^T; 13: *S. thermodiastaticus* DSM 40573^T

3.4.3.4. pH tolerance

pH range for optimal growth was investigated at pH 3.0-10.0 (with 1 pH unit intervals) in ISP2 broth over 7 days. SW4 showed a high pH tolerance. Growth occurred between pH 5.0 and pH 9 with an optimum of pH 6.5

Table 17. Growth comparison in diffrent pH of *Streptomyces sp*. SW4 and closely related type strains.

pH	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
3	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-
5	+	-	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+
6,5	++	++	++	++	++	++	++	++	++	++	++	++	++
7	++	++	++	++	++	++	++	++	++	++	++	++	++
8	+	+	-	+	+	+	+	-	+	+	+	+	+
8,5	+	+	-	+	-	+	+	-	+	+	-	+	+
9	+	+	-	+	-	+	-	-	+	+	-	+	+
10	-	+	-	-	-	+	-	-	+	(+)	-	-	-

++ very good growth; + good growth; (+) weak growth; - no growth; 1: Strain SW4; 2: *S. leeuwenhoekii* DSM 42122^T; 3: *S. indiaensis* DSM 43803^T; 4: *S. lomondensis* DSM 41428^T; 5: *S. glomeratus* DSM 41457^T; 6: *S. purpurascens* DSM 40310^T; 7: *S. rameus* DSM 41685^T; 8: *S. resistomycificus* DSM 40133^T; 9: *S. iakyrus* DSM 40482^T; 10: *S. spinoverrucosus* DSM 41648^T; 11: *S. niger* DSM 43049^T; 12: *S. muensis* DSM 103493^T; 13: *S. thermodiastaticus* DSM 40573^T

3.4.2. Whole-cell fatty acid analysis

While *Streptomyces* characteristically make use of even-numbered straight-chain fatty acids, hydroxyl fatty acids and cyclopropane fatty acids. The genus *Streptomyces* mainly use odd-numbered, branched-chain saturated fatty acids. Determination of the cellular

Fatty Acid Methyl Ester (FAME) analysis of SW4 and 12 of its closely related type strains were performed at the Biology Centre CAS, Institute of Soil Biology, České Budějovice. Major cellular fatty acids (> 10%) for SW4 are anteiso-$C_{15:0}$ (22.61%), iso-$C_{15:0}$ (11.17%), anteiso-$C_{17:0}$ (16.17%) and iso-$C_{16:0}$ (14.49%). Overall, SW4 shows a cellular fatty acid profile similar to the twelve reference type strains, with the exception of e.g. iso-C18:1 family and $C_{18:2}$ (trans9,12) that showed noticeable differences, especially when SW4 is compared with *S. leeuwenhoekii* DSM 42122^T (Table 18). The latter shows an iso-C18:1 family value of below 1%, while SW4 has a value of 1.09%. For the unsaturated $C_{18:2}$ (trans9,12) variants, the values differ even more, with below 1% and 2,35%, respectively.

Table 18. Cellular fatty acid compositions (%) of *Streptomyces sp*. SW4 and closely related type strains.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13
Saturated													
$C_{14:0}$	-	-	1,61	-	-	1,33	-	1,06	1,28	1,60	-	-	-
$C_{15:0}$	1,22	-	1,37	1,11	-	1,63	-	1,25	1,38	1,38	-	1,11	1,73
$C_{16:0}$	8,09	6,60	13,28	5,20	5,13	17,52	3,10	13,99	26,15	9,56	3,36	3,35	4,28
$C_{17:0}$	-	-	-	-	-	-	-	-	-	-	-	-	-
$C_{18:0}$	1,72	-	1,67	-	2,90	1,22	1,15	-	2,12	1,46	-	-	-
Unsaturated													
$iC_{15:1}$	-	-	-	-	-	-	-	-	-	-	-	-	-
$C_{16:1}$ (cis11)	1,89	-	-	8,77	0,99	1,11	3,04	-	-	3,76	3,31	5,60	1,52
$C_{16:1}$ (cis9)	2,98	2,65	7,55	5,73	-	10,26	1,85	7,15	5,97	4,33	1,04	1,90	3,00
$C_{17:1}$ (cis7)	-	-	-	-	-	-	-	-	-	-	-	-	-
$C_{17:1}$ (cis9)	-	-	-	-	-	-	-	-	-	-	-	-	-
$aiC_{17:1}$	3,78	4,78	1,91	5,53	2,10	3,25	4,49	4,21	1,35	3,70	2,37	4,73	3,16
$C_{18:1}$ (family)	-	-	-	-	-	-	-	-	-	-	-	-	-
$C_{18:1}$ (family)	-	-	-	-	-	-	-	-	-	-	-	-	-
$C_{18:1}$ (family)	-	-	-	-	-	-	-	-	-	-	-	-	-
$C_{18:1}$ (family)	1,09	-	-	-	1,27	-	-	-	-	-	-	-	-
$C_{18:1}$ (family)	-	-	-	-	-	-	-	-	-	-	-	-	-
$cycC_{17:0}$ /$C_{17:1}$ (cis10)	3,00	2,23	2,68	3,36	-	5,20	1,76	2,51	2,81	4,18	3,48	2,74	1,92
$C_{18:2}$ (13,16ʊ)	-	-	-	-	-	-	-	-	-	-	-	-	-

$C_{18:2}$ (trans9,12)	2,35	-	1,07	-	1,17	-	-	-	-	-	-	-	-
$C_{18:1}$ (cis9)	-	-	-	-	-	-	-	-	-	-	-	-	-
Branched-chain													
$iC_{13:0}$	-	-	1,05	-	-	-	-	-	-	-	-	-	-
$iC_{14:0}$	1,32	-	1,91	3,47	1,12	-	3,54	-	-	2,26	5,54	4,24	2,54
$iC_{15:0}$	11,17	8,95	16,92	5,98	4,70	17,63	5,64	10,43	13,76	16,02	10,47	7,18	9,57
$iC_{16:0}$	14,49	6,99	5,51	26,18	11,33	4,92	33,53	5,66	6,64	15,96	21,58	25,76	11,74
$iC_{17:0}$	4,98	5,11	5,31	2,08	2,55	5,56	1,90	4,31	6,89	3,55	6,76	3,70	3,67
$aiC_{13:0}$	-	-	-	-	-	-	-	-	-	-	-	-	-
$aiC_{15:0}$	22,61	32,96	23,73	16,08	30,57	17,40	19,75	26,81	16,32	19,39	17,84	20,26	37,06
$aiC_{17:0}$	16,17	23,74	9,83	11,29	30,94	8,99	13,24	18,07	11,01	9,02	18,23	15,58	16,12
$C_{21:0}$ (methyl)	-	-	1,41	-	1,46	-	-	-	-	1,07	-	-	-
$cycC_{19:0}\omega$	-	-	-	-	-	-	-	-	-	-	-	-	-

1: Strain SW4; 2: *S. leeuwenhoekii* DSM 42122^T; 3: *S. indiaensis* DSM 43803^T; 4: *S. lomondensis* DSM 41428^T; 5: *S. glomeratus* DSM 41457^T; 6: *S. purpurascens* DSM 40310^T; 7: *S. rameus* DSM 41685^T; 8: *S. resistomycificus* DSM 40133^T; 9: *S. iakyrus* DSM 40482^T; 10: *S. spinoverrucosus* DSM 41648^T; 11: *S. niger* DSM 43049^T; 12: *S. muensis* DSM 103493^T; 13: *S. thermodiastaticus* DSM 40573^T. Major fatty acids (>10 %) are shown in bold type. Fatty acids that accounted for <1.0 % of the total in each strain are not shown.

3.4.3. Isoprenoid Quinones

Isoprenoid quinones or prenylquinones are isoprenoid compounds with a characteristic quinone structure and isoprenyl tail that are ubiquitous in almost all living organisms. There are four major prenylquinone classes: ubiquinone (UQ), menaquinone (MK), plastoquinone (PQ), and rhodoquinone (RQ). Side chains of isoprenoid quinones can also be used to differentiate between taxonomically distinct bacteria. The Identification Service of the DSMZ (Braunschweig, Germany) profiled SW4 for respiratory quinones. Hexaydrogenated and octahydrogenated menaquinones with nine isoprene units (MK-9(H_6) and MK-9(H_8)) were the predominant compounds identified in the genus *Streptomyces* and *Actinomadura*.[152-154] The total of MK-9 in cell wall of SW4 (4,95%) shows the lowest concentration compered with other closely related type strains. In the other hand, plastoquinone (PQ-6) is highly dominated in the cell wall of SW4 (92,64%), while other closely related type strains only produce plastoquinone (PQ-6) below 70% (Table. 19).

Table 19. Comparison of cell wall quinones (%) between *Streptomyces sp*. SW4 and closely related type strains

Cell wall quinones compound class	1	2	3	4	5	6	7	8	9	10	11	12	13
MK5	0,31	16,23	7,73	1,73	3,71	0,70	3,65	1,11	5,02	0,44	0,00	2,09	2,07
MK6	0,03	2,58	1,76	0,69	0,43	0,46	1,12	0,53	0,97	0,00	0,00	1,05	0,35
MK7	0,02	2,78	0,00	0,93	0,48	0,74	0,20	0,18	0,00	0,00	0,00	0,30	0,06
MK8	0,11	6,09	2,67	8,21	4,70	3,45	1,80	1,43	0,75	1,46	1,65	2,35	2,32
MK9	4,93	63,19	43,19	81,93	33,57	75,00	17,88	34,92	22,48	49,41	28,03	57,90	94,42
MK10	0,00	0,20	0,00	0,00	0,00	0,00	0,00	1,65	0,00	0,00	0,00	0,00	0,00
PQ4	0,17	1,81	3,97	0,12	1,74	0,29	0,25	0,78	3,18	0,23	0,26	0,17	0,07
PQ5	0,26	1,22	13,80	0,12	7,11	1,07	0,56	1,02	5,00	0,57	0,07	0,17	0,07
PQ6	92,64	0,28	0,00	0,08	30,58	14,16	58,41	53,42	49,85	42,88	68,19	32,49	0,01
PQ7	0,00	0,00	0,00	0,17	2,30	0,00	1,14	0,14	0,00	0,00	0,00	0,00	0,00
DMK4	0,29	1,14	1,91	0,14	5,29	0,65	0,59	1,24	6,72	0,33	0,13	0,46	0,15
DMK5	0,16	2,57	0,00	0,16	3,53	0,51	4,36	0,92	0,00	0,53	0,05	0,28	0,00
DMK8	0,00	0,00	0,00	0,54	0,00	0,71	0,00	0,18	0,00	0,10	0,21	0,85	0,04
DMMK8	0,00	0,73	0,00	2,14	0,21	0,00	0,39	0,07	0,00	0,00	0,25	0,00	0,00
UQ5	0,14	0,48	2,32	1,21	0,12	1,38	3,21	1,10	3,42	0,72	0,28	1,16	0,25
UQ6	0,93	0,68	22,66	1,83	6,23	0,88	6,44	1,30	2,62	3,34	0,89	0,73	0,20

MK, menaquinone; PQ, plastoquinone; DMK, demethylmenaquinone; UQ, ubiquinone; 1: Strain SW4; 2: *S. leeuwenhoekii* DSM 42122^T; 3: *S. indiaensis* DSM 43803^T; 4: *S. lomondensis* DSM 41428^T; 5: *S. glomeratus* DSM 41457^T; 6: *S. purpurascens* DSM 40310^T; 7: *S. rameus* DSM 41685^T; 8: *S. resistomycificus* DSM 40133^T; 9: *S. iakyrus* DSM 40482^T; 10: *S. spinoverrucosus* DSM 41648^T; 11: *S. niger* DSM 43049^T; 12: *S. muensis* DSM 103493^T; 13: *S. thermodiastaticus* DSM 40573^T.

Pastoquinone six or PQ-6 is 2,3-dimethyl-1,4-benzoquinone molecule with a side chain of six isoprenyl units. Plastoquinones is one of the electron acceptors majority in plant that accociated with Photosystem II in the process of photosynthesis.[154] The higest plastoquinone six concentration in the cell wall of the strain SW4 compared with other quinones, makes SW4 is very unique.

3.4.4. Biochemical Characterization

An API fingerprint was performed on SW4 and the twelve closely related type strains. The utilization of 49 different carbon sources by *Streptomyces* sp. SW4 was studied using the API® 50CH test. Among the 49 carbon sources, the isolate utilized glycerol, D-ribose, D-xylose, D-glucose, L-rhamnose, N-acetylglocosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-trehalose, D-raffinose, starch, glycogen, D-fucose, potassium gluconate (Table 20).

Table 20. Utilization of carbon sources of *Streptomyces* sp. SW4 and closely related type strains.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
API 50CH													
Glycerol	(+)	+	(+)	(+)	(+)	+	++	(+)	-	-	(+)	(+)	-
Erythritol	-	-	-	-	-	-	-	-	-	-	(+)	-	-
D-arabinose.	-	-	-	(+)	-	+	-	+	-	(+)	-	+	-
L-arabinose	-	-	(+)	-	-	(+)	++	+	-	(+)	(+)	(+)	+
D-ribose	(+)	+	-	-	-	(+)	(+)	+	-	(+)	++	++	-
D-xylose	-	-	-	-	-	(+)	(+)	(+)	-	-	(+)	(+)	-
L-xylose	-	-	-	-	-	-	-	-	-	-	-	-	-
ADO	+	-	-	-	-	-	++	-	-	-	++	-	-
Methyl-beta-D-xylopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-
D- glalactose	-	-	-	-	-	-	(+)	(+)	-	-	+	-	-
D-glucose	++	+	-	(+)	(+)	++	++	++	-	-	++	+	-
D-fructose	-	+	+	+	-	++	(+)	++	-	-	++	++	-
D-mannose	-	-	-	-	-	(+)	-	-	-	-	+	-	-
L-sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-
L-rhamnose	(+)	-	-	-	-	+	(+)	++	-	-	(+)	+	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	(+)	(+)	(+)	-	-	(+)	(+)	-
D-mannitol	-	-	-	-	-	(+)	(+)	(+)	-	-	++	(+)	-
D-sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl-alpha-D-mannopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl-alpha-D-glucopyranoside	-	-	-	-	-	(+)	-	-	-	-	+	-	-
N- Acetylglucosamine	++	+	-	+	++	++	++	+	(+)	(+)	++	++	+
Amygdalin	(+)	-	(+)	-	-	-	+	-	-	-	++	(+)	-
Arbutin	(+)	(+)	(+)	-	-	-	-	(+)	-	(+)	++	-	-
Esculin ferric citrate	++	+	(+)	++	(+)	++	++	++	+	++	++	++	+
Salicin	++	-	-	-	-	-	(+)	-	-	-	++	(+)	-
D-cellobiose	++	+	-	++	-	+	++	+	(+)	-	++	(+)	+
D-maltose	(+)	-	-	-	-	-	(+)	-	-	-	(+)	-	-
D-lactose	(+)	-	-	-	-	++	(+)	+	-	-	++	+	-
D-melibiose	-	-	-	-	-	++	(+)	+	-	-	-	-	-
D-saccharose	++	(+)	-	(+)	-	++	++	++	-	-	++	+	-
D-trehalose	++	+	-	-	-	+	++	(+)	-	-	++	-	-

Inulin		-	-	-	+	-	-	-	-	-	-	(+)	-	-
D-melezitose		-	-	-	-	-	(+)	-	-	-	-	++	(+)	-
D-raffinose		(+)	-	-	(+)	-	++	(+)	+	(+)	-	++	(+)	-
Amidon (Starch)		(+)	(+)	-	-	-	-	-	-	-	-	(+)	-	-
Glycogen		++	+	-	(+)	-	+	-	(+)	-	-	++	-	+
Xylitol		-	-	-	-	-	-	-	-	-	-	(+)	-	-
Gentiobiose		-	-	-	-	-	-	-	-	-	-	+	-	-
D-turanose		-	-	-	-	-	(+)	-	-	-	-	++	-	-
D-lyxose		-	-	-	-	-	-	-	-	-	-	(+)	-	-
D-tagatose		-	-	-	-	-	-	-	-	-	-	-	-	-
D-fucose		+	-	-	(+)	-	(+)	(+)	-	(+)	-	++	-	-
L-fucose		-	-	-	-	-	(+)	-	++	-	-	-	(+)	-
D-arabitol		-	-	-	-	-	(+)	(+)	(+)	-	-	++	(+)	+
L-arabitol		-	-	-	-	-	-	-	-	-	-	-	-	-
Potassium gluconate		V	-	(+)	V	-	V	V	V	V	V	V	V	V
Potassium 2-ketogluconate		-	-	-	-	-	-	-	-	-	-	-	-	-
Potassium 5-ketogluconate		-	-	-	V	-	-	V	-	-	V	-	V	-

++ highly positive result; + positive result; (+) weakly positive result; - negative result; V violet result; 1: Strain SW4; 2: *S. leeuwenhoekii* DSM 42122^T; 3: *S. indiaensis* DSM 43803^T; 4: *S. lomondensis* DSM 41428^T; 5: *S. glomeratus* DSM 41457^T; 6: *S. purpurascens* DSM 40310^T; 7: *S. rameus* DSM 41685^T; 8: *S. resistomycificus* DSM 40133^T; 9: *S. iakyrus* DSM 40482^T; 10: *S. spinoverrucosus* DSM 41648^T; 11: *S. niger* DSM 43049^T; 12: *S. muensis* DSM 103493^T; 13: *S. thermodiastaticus* DSM 40573^T.

In summary, genotypic, phenotypic and biochemical analysis supported the reclassification of SW4 as a phylogenetically novel species within the genus *Streptomyces*. The name, *Streptomyces* SW4 sp. nov. with its type strain SW4, has been chosen based on those characteristics.

3.4.1. De novo genome sequencing using Next-generation sequencing (NGS) platforms

In order to support the taxonomic characterization efforts, the genome of SW4 was sequenced by Macrogen (Gasan-dong, Korea) using the PacBio method. Sequencing statistics for PacBio approaches yielded a 169,628 number of reads. The average sequence length was 1,868 kb. 169,628 reads could be merged into 4 contigs, with the largest contig of 5,756,656 bp and the smallest of 249,699 bp. The automated gap filling process resulted in a draft genome size of ~7.4 Mbp with scaffold sequence lengths of 5,756,656 bp, 1,159,340 bp, 309,332 bp and 249,699 bp and a GC content of 73.32%.

Table 21. Whole genome information of *Streptomyces* sp. SW4

Sample Name	SW4
Total Number of Bases	7.475.027
Contigs	4
Total GC%	73.32
Total CDS	9.005
Total tRNA	93
Total rRNA	18
Total Predicted Gene Cluster	17
Well known Gene Cluster (70% similarity)	6
Less known Gene Cluster	10

The GC content in the genome of SW4 (73.32%) is inline with the genus *Streptomyces*. Toribio and co-workers estimated the average GC content of the *Streptomyces* strains was 72 to 73%.[149]

3.4.2. Analysis of the biosynthetic gene clusters

Next, the assembled SW4 genome sequence was analysed in silico for its potential to encode secondary metabolites using the online annotation tool antiSMASH. Apart from identifying secondary metabolite gene clusters, antiSMASH also provides suggestions of homologous gene clusters.

This analysis identified the following 17 biosynthetic gene clusters (Fig. 15). Based on high similarity values (>75%), it could be delineated that the strain is able to produce hopenes, a virginiamycin-like compound, the siderophore desferrioxamine, the pigment melanin and the compatible solute ectoine (BGC is present twice). The remaining BGCs required further analysis. A duplication of the ectoine BGC explains possibly the high salt-tolerance of SW4. This finding is inline with the salinity tolerance experiment, in which SW4 showed to be able to grow at 8% as a maximum concentration of sodium chloride. The contribution of ectoine in response of elevated salitnity is reported not only in plants but also in halophilic bacteria.[155-157]

Overview 1.1 1.2 1.3 1.4 1.5 2.1 2.2 3.1 3.2 4.1 4.2 4.3 4.4 4.5 4.6 4.7 4.8

Identified secondary metabolite regions using strictness 'relaxed'

Region	Type	From	To	Most similar known cluster		Similarity
Region 1.1	terpene	243,190	268,276	hopene	Terpene	76%
Region 1.2	T1PKS	346,322	389,489			
Region 1.3	T1PKS	574,233	616,512	mycotrienin I	NRP + Polyketide	15%
Region 1.4	bacteriocin	729,181	739,396	informatipeptin	RiPP:Lanthipeptide	42%
Region 1.5	terpene	982,707	1,003,662	isorenieratene	Terpene	62%
Region 2.1	NRPS	1	29,314			
Region 2.2	hglE-KS, T2PKS, NRPS, ectoine, PKS-like	33,385	279,463	virginiamycin S1	NRP + Polyketide	83%
Region 3.1	siderophore	79,730	91,258			
Region 3.2	CDPS	182,259	202,947	nogalamycin	Polyketide	30%
Region 4.1	terpene	61,407	83,592	ashimides	NRP	24%
Region 4.2	bacteriocin	88,371	99,692			
Region 4.3	siderophore	461,027	471,717			
Region 4.4	siderophore	3,157,688	3,169,456	desferrioxamin B / desferrioxamine E	Other	83%
Region 4.5	melanin	3,246,841	3,257,350	melanin	Other	80%
Region 4.6	ectoine	4,210,816	4,221,214	ectoine	Other	100%
Region 4.7	T3PKS	4,885,073	4,926,142	flaviolin rhamnoside / 3,3'-diflaviolin / flaviolin	Polyketide:Type III + Saccharide:Oligosaccharide	33%
Region 4.8	indole	5,459,620	5,480,745	5-isoprenylindole-3-carboxylate β-D-glycosyl ester	Other	14%

Figure 16. Biosynthetic gene cluster prediction using AntiSMASH analysis of the whole genome sequence of SW4

The investigation of the pristinamycin gene cluster showed 83% gene homology with the original biosynthetic gene cluster from *Streptomyces pristinaespiralis*. The cluster is also in line with the blueprint corresponding to virginiamycin S biosynthesis. The difference of the two pristinamycin gene clusters lies in the total length of the cluster. *S. pristinaespiralis* consists of around 210 kb, while the corresponding cluster in strain SW4 exhibits only a length of around 153 kb. The presence of an interspace region of around 90 kb in *S. pristinaespiralis* and around 63 kb in SW4, devides the pristinamycin gene clusters differently in a right and left region (Fig. 16).

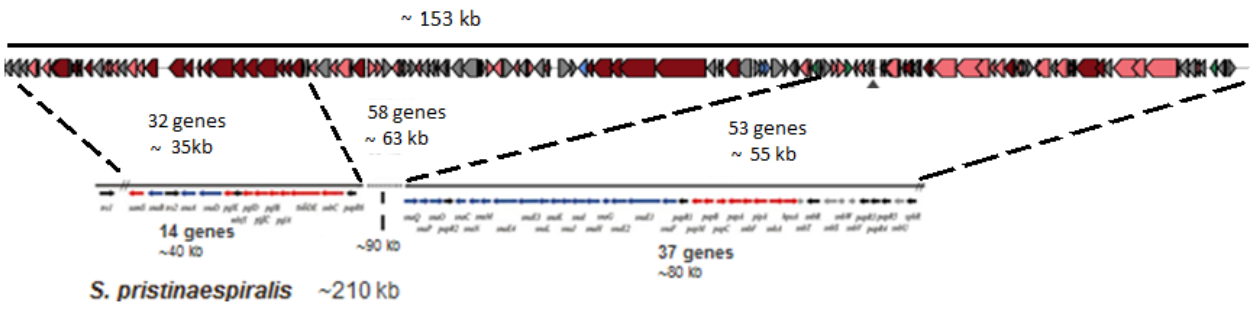


Figure 17. Alignment of the pristinamycin gene clusters of *Streptomyces* sp. SW4 and *S. pristinaespiralis*.

The annotation of each gene in the pristinamycin gene cluster of SW4 revealed a high similarity to the corresponding homologues in *S. pristinaespiralis* and *S. virginae.* Only

two genes in the ORF 06300 and ORF 06305, consisting of 197 and 258 bp respectively did not match with any genes reported from the pristinamycin gene cluster (Table 22).

Table 22. ORFs forming the gene cluster of pristinamycin in SW4

ORF	Size (bp)	AA	ID/SM (%)	Predicted function	Match	Reference
05710	1,210	402	96/98	S-adenosylmethionine synthetase	SamS, *S. pristinaespiralis*	WP_005321878.1
05715	885	294	61/69	PIIA synthetase subunit B	SnaB, *S. pristinaespiralis*	WP_050791658.1
05720	1,278	425	88/92	PIIA synthase subunit A	SnaA, *S. pristinaespiralis*	WP_005321872.1
05725	303	100	85/88	PII peptide synthetase	SnaD, *S. pristinaespiralis*	CBW45640.1
05730	1,086	361	75/84	PII peptide synthetase subunit D	SnaD, *S. pristinaespiralis*	CBW45640.1
05735	897	298	81/88	PII peptide synthetase	SnaD, *S. pristinaespiralis*	CBW45640.1
05740	2,337	778	83/88	PII peptide synthetase	SnaD, *S. pristinaespiralis*	CBW45640.1
05745	300	99	60/60	Hypothetical protein	*S. antimycoticus*	BBJ46506.1
05750	663	221	92/96	PII peptide synthetase	SnaD, *S. pristinaespiralis*	CBW45640.1
05755	180	60	68/74	PII peptide synthetase	SnaD, *S. pristinaespiralis*	CBW45640.1
05760	432	114	75/85	PII peptide synthetase	SnaD, *S. pristinaespiralis*	CBW45640.1
05765	954	318	60/64	PII peptide synthetase	SnaD, *S. pristinaespiralis*	CBW45640.1
05770	213	70	85/92	PLP-dependent aminotransferase	PglE, *S. pristinaespiralis*	WP_106428585.1
05775	483	229	88/94	MbtH family protein	MbtY, *S. pristinaespiralis*	WP_005321855.1
05780	588	195	74/78	Thioesterase type II	PglD *S. pristinaespiralis*	WP_078535553.1
05785	978	137	89/95	Putative pyruvate-dehydrogenase E1 subunit beta	PglC, *S. pristinaespiralis*	ALC18496.1
05790	1,083	360	81/87	Putative pyruvate-dehydrogenase E1 subunit beta	PglC, *S. pristinaespiralis*	ALC18496.1
05795	1350	449	77/86	Enoyl-CoA hydratase/isomerase	PglA, *S. pristinaespiralis*	WP_005321846.1
05800	900	299	81/87	PI synthetase 3 and 4, Dimodular nonribosomal peptide synthase	SnbDE, *S. pristinaespiralis*	CBW45647.1
05805	1,620	540	76/82	PI synthetase 3 and 4	SnbDE, *S. pristinaespiralis*	CBW45647.1
05810	2,073	691	79/85	PI synthetase 3 and 4	SnbDE, *S. pristinaespiralis*	CBW45647.1
05815	1,073	329	85/88	PI synthetase 3 and 4	SnbDE, *S. pristinaespiralis*	CBW45647.1
05820	405	135	75/80	PI synthetase 3 and 4	SnbDE, *S. pristinaespiralis*	CBW45647.1
05825	1,185	395	78/84	PI synthetase 3 and 4	SnbDE, *S. pristinaespiralis*	CBW45647.1
05830	3,087	1,028	79/85	PI synthetase 3 and 4	SnbDE, *S. pristinaespiralis*	CBW45647.1
05835	1,611	536	78/82	PI synthetase 3 and 4	SnbDE, *S. pristinaespiralis*	CBW45647.1
05840	1,743	580	66/72	Pristinamycin I synthase 2	SnbC, *S. pristinaespiralis*	CBW45648.1
05845	2,712	903	83/87	Pristinamycin I synthase 2	SnbC, *S. pristinaespiralis*	CBW45648.1
05850	978	325	79/83	Pristinamycin I synthase 2	SnbC, *S. pristinaespiralis*	CBW45648.1
05855	909	303	81/85	Pristinamycin I synthase 2	SnbC, *S. pristinaespiralis*	CBW45648.1

05860	1,518	505	60/65	Pristinamycin I synthase 2	SnbC, *S. pristinaespiralis*	CBW45648.1
05865	201	66	88/96	Response regulator transcription factor	PapR6, *S. pristinaespiralis*	WP_037777404.1
				21 kb interjaecent region		
06155	1,077	358	85/89	Flavin-dependent oxidoreductase	SnaQ, *S. pristinaespiralis*	WP_005321624.1
06160	756	251	78/84	Thioesterase	SnaP, *S. pristinaespiralis*	WP_037775577.1
06165	1,155	384	81/88	N-methyl-L-tryptophan oxidase	SnaO, *S. pristinaespiralis*	WP_005321620.1
06170	873	290	91/93	SARP-type regulator	PapR2, *S. pristinaespiralis*	WP_005321617.1
06175	476	158	63/77	FMN oxidoreductase	SnaC, *S. pristinaespiralis*	WP_005321616.1
06180	852	283	70/77	4-phosphopantetheinyl transferase	SnaN, *S. pristinaespiralis*	WP_005321615.1
06185	890	124	90/82	Acyltransferase	SnaM, *S. pristinaespiralis*	ALC18597.1
06190	375	154	90/92	Hybrid NRPS/PKS (PKSIV)	SnaE4, *S. pristinaespiralis*	WP_053556633.1
06195	100	33	97/100	Hybrid NRPS/PKS (PKSIV)	SnaE4, *S. pristinaespiralis*	WP_053556633.1
06200	525	174		Hypothetical protein		
06205	918	306	75/80	Hybrid NRPS/PKS (PKSIV)	SnaE4, *S. pristinaespiralis*	WP_053556633.1
06210	1,758	585	61/70	Hybrid NRPS/PKS (PKSIV)	SnaE4, *S. pristinaespiralis*	WP_053556633.1
06215	246	81	80/83	Amino acid adenylation domain	*S. viridodiastaticus*	WP_189912143.1
06220	510	169	60/63	Hybrid NRPS/PKS (PKSIV)	SnaE4, *S. pristinaespiralis*	WP_053556633.1
06225	951	317	86/89	Hybrid NRPS/PKS (PKSIV)	SnaE4, *S. pristinaespiralis*	WP_053556633.1
06230	174	58	65/80	Hybrid NRPS/PKS (PKSIV)	SnaE4, *S. pristinaespiralis*	WP_053556633.1
06235	744	247	84/87	Hybrid NRPS/PKS (PKSIV)	SnaE4, *S. pristinaespiralis*	WP_053556633.1
06240	342	113	77/84	Hybrid NRPS/PKS (PKSIV)	SnaE4, *S. pristinaespiralis*	WP_053556633.1
06245	849	283	72/77	Hybrid NRPS/PKS (PKSIV)	SnaE4, *S. pristinaespiralis*	WP_053556633.1
06250	621	206	69/77	Hybrid NRPS/PKS (PKSIV)	SnaE4, *S. pristinaespiralis*	WP_053556633.1
06255	3,045	1,014	72/77	Hybrid NRPS/PKS (PKSI)	SnaE3, *S. pristinaespiralis*	WP_053556634.1
06260	3,423	1,140	82/85	Hybrid NRPS/PKS (PKSI)	SnaE3, *S. pristinaespiralis*	WP_053556634.1
06265	1,857	618	88/93	Enoyl-CoA hydratase/isomerase	SnaK, *S. pristinaespiralis*	WP_005321605.1
06270	780	259	76/80	Enoyl-CoA hydratase	SnaK, *S. pristinaespiralis*	WP_050791645.1
06275	1,254	417	91/94	Enyol-CoA hydratase	SnaJ, *S. pristinaespiralis*	WP_005321592.1
06280	1,272	423	82/85	Beta-ketoacyl-ACP synthase	SnaH, *S. pristinaespiralis*	ALC18603.1
06285	252	83	83/91	Phosphopantetheine-binding protein	*S. virginiae*	WP_033220286.1
06290	516	171	74/80	Hybrid PKS/NRPS (PKSI)	SnaE2, *S. pristinaespiralis*	WP_053556636.1
06295	564	187	84/91	Hybrid PKS/NRPS (PKSI)	SnaE2, *S. pristinaespiralis*	WP_053556636.1
06300	594	197		Hypothetical protein		
06305	777	258	72/77	Hypothetical protein	*M. tuberculosis*	CNI71997.1

06310	702	234	90/93	Hybrid PKS/NRPS (PKSI)	SnaE2, *S. pristinaespiralis*	WP_053556636.1
06315	1,887	628	76/82	Hybrid PKS/NRPS	*S. virginiae*	BAF50727.1
06320	720	239	53/59	Hybrid PKS/NRPS (PKSI)	SnaE2, *S. pristinaespiralis*	WP_053556636.1
06325	1,599	532	66/71	Hybrid PKS/NRPS (PKSI)	SnaE2, *S. pristinaespiralis*	WP_053556636.1
06330	330	110	86/92	Hybrid PKS/NRPS (PKSI)	SnaE2, *S. pristinaespiralis*	WP_053556636.1
06335	438	145	54/59	Hybrid PKS/NRPS (PKSI)	SnaE2, *S. pristinaespiralis*	WP_053556636.1
06340	183	61	80/91	Hybrid PKS/NRPS (PKSI)	SnaE1, *S. pristinaespiralis*	WP_053556637.1
06345	2,967	988	71/77	Hybrid PKS/NRPS (PKSI)	SnaE1, *S. pristinaespiralis*	WP_053556637.1
06350	1,230	409	48/56	Hybrid PKS/NRPS (PKSI)	SnaE1, *S. pristinaespiralis*	WP_053556637.1
06355	1,146	381	54/59	Hybrid PKS/NRPS (PKSI)	SnaE1, *S. pristinaespiralis*	WP_053556637.1
06360	3,150	1,049	73/78	Hybrid PKS/NRPS (PKSI)	SnaE1, *S. pristinaespiralis*	WP_053556637.1
06365	1,800	599	66/73	Hybrid PKS/NRPS (PKSI)	SnaE1, *S. pristinaespiralis*	WP_053556637.1
06370	4,314	1,437	67/73	Hybrid PKS/NRPS (PKSI)	SnaE1, *S. pristinaespiralis*	WP_053556637.1
06375	110	38	84/91	Hybrid PKS/NRPS (PKSI)	SnaE1, *S. pristinaespiralis*	WP_053556637.1
06380	1,206	401	78/87	Hybrid PKS/NRPS (PKSI)	SnaE1, *S. pristinaespiralis*	WP_053556637.1
06385	1,176	391	76/86	Branched chain alpha-ketoacyl- dehydrogenase	SnaF, *S. pristinaespiralis*	WP_005321577.1
06390	516	171	94/96	Branched chain alpha-ketoacyl- dehydrogenase	SnaF, *S. pristinaespiralis*	WP_005321577.1
06395	882	293	73/84	Branched chain alpha-ketoacyl- dehydrogenase	SnaF, *S. pristinaespiralis*	WP_005321577.1
06400	762	254	78/82	AfsR/SARP type regulator	PapR, *S. pristinaespiralis*	WP_005321575.1
06405	957	318	75/80	N(5)-glutamine methyltransferase	PapM, *S. pristinaespiralis*	AAC44869.1
06410	315	104	75/80	Chorismate mutase	PapB, *S. pristinaespiralis*	AAC44864.1
06415	864	287	69/76	Prephenate dehydrogenase	PapC, *S. pristinaespiralis*	WP_053556639.1

ID/SM, % identity/similarity of amino acid sequences; ORF, open reading frame; AA, amino acids; red, gene for PI biosynthesis; blue, genes for PII biosynthesis; *S. pristinaespiralis, Streptomyces pristinaespiralis* HCCB 10218; *S. viridodiastaticus, Streptomyces viridodiastaticus* ATCC 25518; *S. antimycoticus, Streptomyces antimycoticus* NBRC 100767; *M. tuberculosis, Mycobacterium tuberculosis* 401416; *S. virginiae, Streptomyces virginiae* ATCC 19817.

Regarding the high similarity of the composition and the arrangement of pristinamycin gene cluster fom SW4 and *S. pristinaespiralis,* the biosynthetic pathway of pristinamycin I and II probably similar.

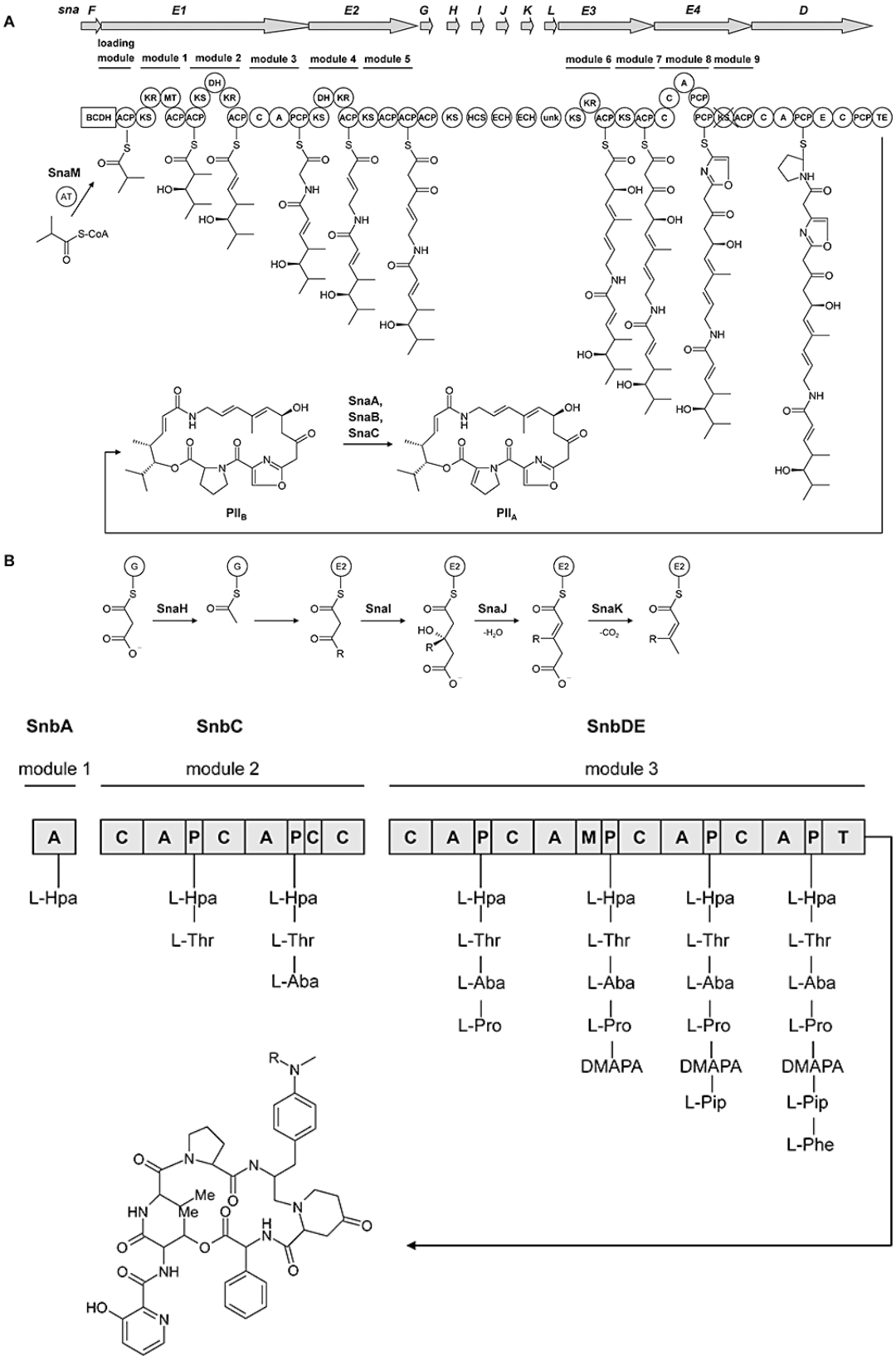


Figure 18. Biosynthetic pathway of pristinamycin I and II from *S. pristinaespiralis.* Adopted from Mast *et al.*[158]

3.4.3. Chemical investigation of the strain *Streptomyces* sp. SW4

To verify pristinamycin production by strain SW4, bacteria had to be cultivated on small scale. As a positive control, *Streptomyces pristinaespiralis* wildtype and Synercid, a commercial drug containing a 30:70 combination of quinupristin (pristinamycin I A, a group B streptogramin) and dalfopristin (pristinamycin II A, a group A streptogramin) were included. The cultivation and isolation procedure of pristinamycin from *Streptomyces pristinaespiralis* was adapted, as a similar product was expected. For the isolation of the putative pristinamycin-like compounds, SW4 was cultured as described. Pre-cultures were prepared in 100 ml HT7T medium; main cultures comprised 100 ml HT7T broth per flask and were inoculated with 2 ml pre-culture. Cultures were incubated at 30°C for 7 days. To enhance the production of pristinamycin, gamma butyrolactone and resin were added after 24h incubation. Whole broth of cultures were extracted twice with ethyl acetate.The dry crude extract was redisolved and analyzed using standard LC-MS conditions and bioassays.

The presence of gamma butyrolactone and resin XAD-16 in the culture medium after 24 h incubation, induced an elevated secondary metabolism, which can be delineated by the presence of additional peaks in the HPLC profile in both strains SW4 and *S. pristinaspiralis* (Fig. 18). A major peak in the crude extract of SW4 at t_R = 30 min, shows double intensity after addition of inducer and resin to the culture medium. The addition of inducer to *S. pristinaspiralis* also leads to an enhanced peak at t_R = 30 min in HPLC profile. Since the HPLC profile refer to the total metabolite produced by bacteria during cultivation, the presence of gamma butyrolacton probably is a responsible of activating several biosynthetic gene clusters in *Streptomyces*. Since the function of resins is to absorb several substances inhibitory to bacterial growth, the presence of XAD-16 in the culture medium probably leads to the disruption of a negative feedback loop and therefore to an increase of pristinamycin production.

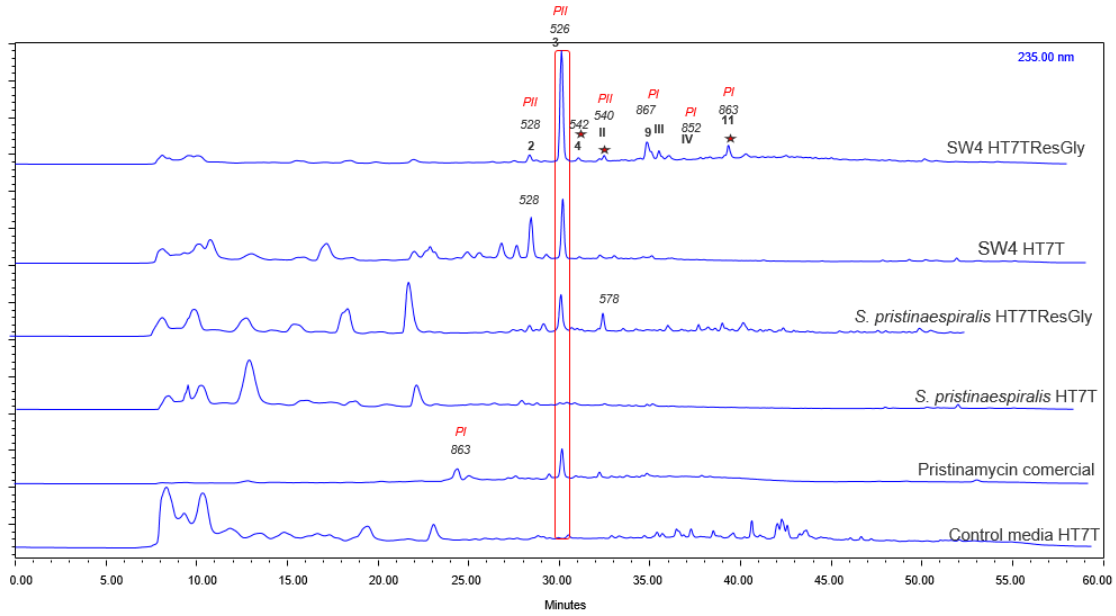


Figure 19. Comparison HPLC profiles of *Streptomyces* sp. SW4 and *Streptomyces pristinaespiralis*. Commercial pristinamycin use as a positive control. The effect of additional gamma butyrolactone and resin in the culture also analyzed. The abbreviatons are explained in Tabel 23.

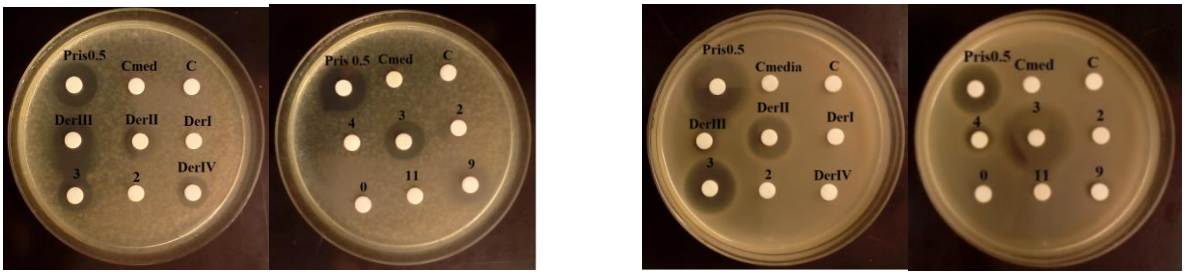
The LC/MS run revealed that strain SW4 is able to produce pristinamycin II with the mass of 526 Da as the major compound. Beside this compound, several derivates thereof were observed pristinamycin IA with the mass of 867 Da, pristinamycin IC with the mass of 852 Da and osteogrysin G with the mass of 528 Da. Furthermore, three unknown derivates were also found, which showed the masses 542 Da, 540 Da, and 863 Da, respectively (Table 23).

Table 23. Chemical compounds detected from SW4 cultivation

Code	t_R (min)	Calculated [*m/z*] M+H	Possible formula	Name
2	29	528	$C_{28}H_{37}N_3O_7$	Ostreogrycin G
3	30	526	$C_{28}H_{35}N_3O_7$	Pristinamycin IIA
4	31	542	unknown	Unknown derivative
DerII	32	540	unknown	Unknown derivative
9	34.5	867	$C_{45}H_{54}N_8O_{10}$	Pristinamycin IA
DerIII	35.5	867	$C_{45}H_{54}N_8O_{10}$	Pristinamycin IA

DerIV	39	852	$C_{44}H_{52}N_8O_{10}$	Pristinamycin IC
11	40	863	unknown	Unknown derivative

The respective compounds were collected and evaluated in an antimicrobial bioassay. Each compound was collected with HPLC and tested the activity against *E.coli* and *B. subtilis*. Each compound showed a different bioactivity profile (Fig. 19).



A B

Figure 20. Bioassay of HPLC fraction of crude extract of *Streptomyces* sp. SW4 against *B. subtilis* (A) and *E. coli* (B). Compound no. 3 (526 Da) and Der II (540 Da) exhibited similar inhibition zones comparable with the Synercid standard concerning the growth of *B. subtilis* and *E. coli*. Compound no. 4 (542 Da) inhibited only *E. coli* slightly. Compound no. 11 (863 Da) showed no inhibition zone with both bacteria.

The production of pristinamycin is usually a mixture of two different chemical classes of components, pristinamycin I (~867 Da) and pristinamycin II (~ 526 Da) in a ratio of 30:70.[158] Each compoud alone exhibits a moderate bacteriostatic activity. The mixture of pristinamycin componds represent a synergistic combination of bactericidal activity against a wide rage of Gram-positive bacteria and Gram-negative bacteria.[159]

Up-scaling of the production of pristinamycin from SW4 were performed using a similar protocol as employed before for the main cultures. A batch of main culture consisted of 30 Erlenmeyer flasks containing 100 ml medium HT7T broth. Cultures were incubated at 30°C for 7 days and twice extracted with ethyl acetate followed by VLC separation into 7 fractions. The purification workflow is shown in Figure 20.

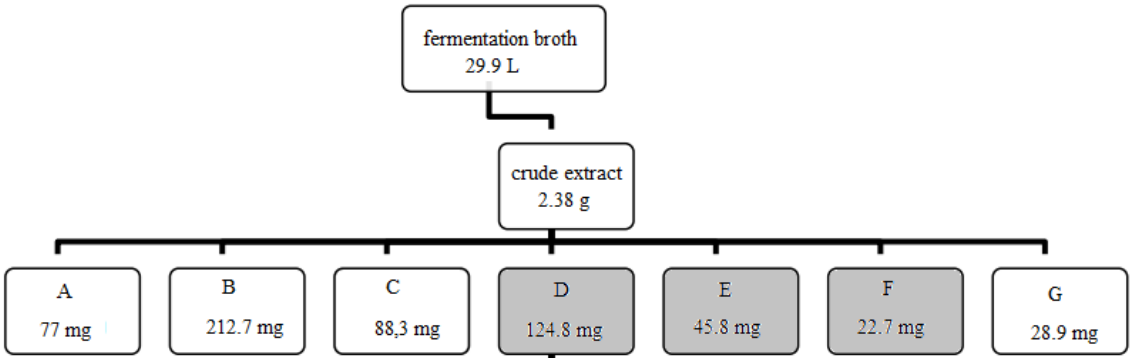


Figure 21. Fractionation scheme of SW4
Crude extract was obtained from 9 L fermentation broth, VLC separation generated 7 fraction (A-G). Fractions containing pristinamycin are highlighted in grey (supported by LC-MS and bioassay analysis). Yields are given in grams (g) or milligrams (mg).

All fractions were subjected to LC-MS for mass analysis and were additionally tested against *B. subtilis* and *E.coli* for antimicrobial activity. Mass analysis was then compared to the bioassay results. The mass of interest, i.e. that of pristinamycin IIA (m/z ~526 Da $[M+H]^+$) and pristinamycin IA (m/z ~866 Da $[M+H]^+$) were extracted from the total ion count (TIC). Three fractions showed a clear peak at ~526 and ~866 Da, which was strongly concentrated in fractions D – F (70-90% methanol). The intensity of peak ~526 Da is four times higher than the intensity of peak possing a mass of ~866 Da. In fraction G, the concentration of pristinamycin visibly decreased.

Based on bioassay data and LC-MS investigations (i.e. peak intensity), fraction E was selected for further purification using HPLC. Fraction E was separated into 8 subfractions. A closer look at the masses corresponding to the peaks at these retention times, revealed that the peak around 18 min represents pristinamycin derivatives A1 (m/z 508.3 $[M+H]^+$) and A2 (m/z 540.3 $[M+H]^+$), while the peak at t_R = 20 min represents pristinamycin derivatives A3 (m/z 526.3 $[M+H]^+$) and A4 (m/z 510.8 $[M+H]^+$), respectively.

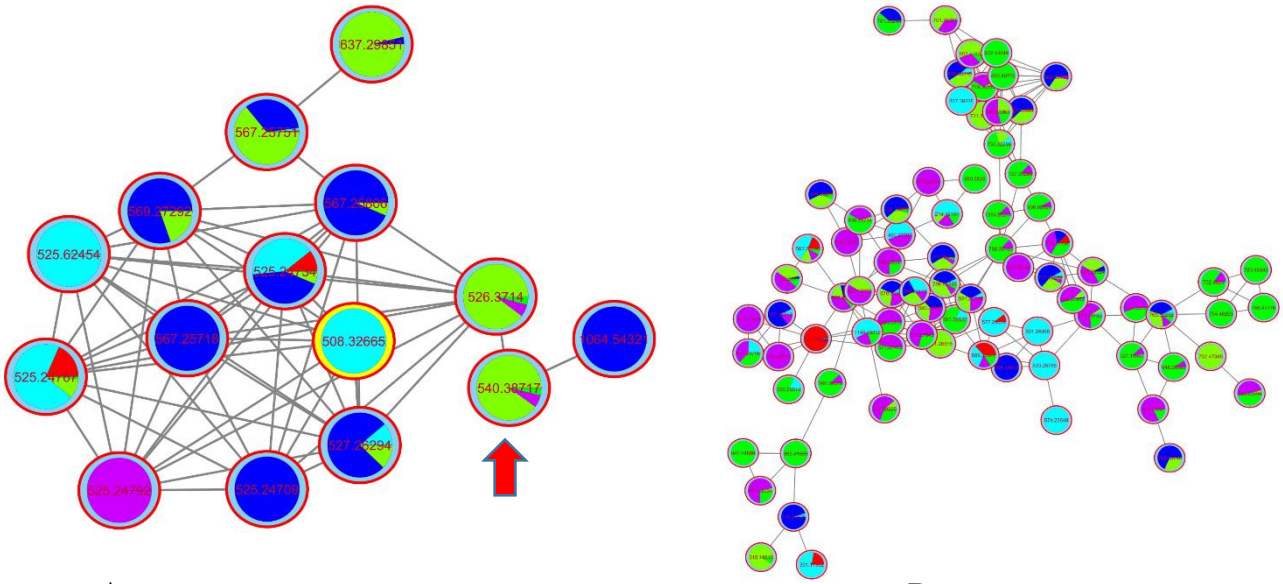


Figure 22. Molecular network of compounds related to pristinamycin from SW4. A: pristinamycin II; B: pristinamycin I. Red: 70% A methanol fraction; light blue: 70% B methanol fraction; dark blue: 80% A methanol fraction; green: 80% B methanol fraction; purple: 90% methanol fraction. Red arrow indicates one of the new derivatives.

Considering the GNPS output of the pristinamycin molecular family, it is evident that SW4 can deliver a suite of related entities (Fig. 21). One of these congeners with molecular $[M+H]^+$ 540 Da was speculated to be a derivative of the known pristinamycin IIA with an extra 14 Da in the form of an additional CH_2 taking into account the molecular formula prediction.

As a result of the hybride nature of pristinamycin scaffold as NRPS/PKS product, it was challenging to envision where such as sctructural modification can occur due to the crowdedness of their comparative MS/MS spectra.

3.5. Discussion and Outlook

SW4 was shown to be a bacterium to produce potent antibacterial agents. The strain SW4 was identified to be a member of the genus *Streptomyces* mainly by the strong agreement concerning the GC content, morphological and physiological characteristics. Phylogenetic analyses (16S rRNA) confirmed that SW4 should be grouped together with *S. leeuwenhoekii* DSM 42122^T, *S. indiaensis* DSM 43803^T and *S. lomondensis* DSM 41428^T. However, some phenotypic characteristics such as substrat mycelium color and

aerial mycelium color after growth in different media, differ. Growing on ISP2 agar, SW4 looks a purplish pink concerning the substrat mycelium and white in aerial mycelium, while substrat mycelium color of *S. leeuwenhoekii* DSM 42122^T, *S. indiaensis* DSM 43803^T, and *S. lomondensis* DSM 41428^T are grey, white and yellow green , respectively.

SW4 shows a different color in aerial mycelium, substrat mycelium and soluble pigments when grown in different media. Variation of color indicates a response of a bacterium to the changing environmental conditions by the production of suitable secondary metabolites. As common for *Streptomyces* spp., the genome of SW4 contains several BGCs encoding secondary metabolites. However, many secondary metabolites are produced only under specific culture conditons such as, special growth time, temperature, pH, aeration rate, specific media contents, or the presence of inducers.[160]

Due to its cellular fatty acid and cell wall quinones profile, SW4 shows similarities to the twelve reference type strains in genus *Streptomyces*, but differ more in their value ranges. Regarding the cellular fatty acid profile of SW4, anteiso-$C_{15:0}$, iso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$ are consist as a major cellular fatty acid. This composition is similar with *S. leeuwenhoekii* DSM 42122^T, *S. indiaensis* DSM 43803^T, and *S. lomondensis* DSM 41428^T but differ in their value ranges.

When comparing the GC contents, it appeared that all related type strains of this genus show ~70-73 mol% GC (determined by tygs data base). As to the genome sequences of the SW4 strain and the determined GC content, SW4 actually exhibits 73.32 mol% GC. However, digital DNA-DNA sequence similarity of all related type strains to SW4 are below 70%.

With this information, it is very likely that SW4 forms a completely new species or type strain. Nevertheless, before jumping to conclusions and proposing an incorrect classification, additional and alternative tests should be considered.

Furthermore, chemotaxonomic considerations (similar compounds, different strains) could be included in the polyphasic taxonomy approach. Some genome sequences of *Streptomyces* type strains are available and were reviewed also with respect to their secondary metabolite profiles. As SW4 encodes 17 biosynthetic gene clusters, it was postulated, that related strains also produce similar secondary metabolites.

The question as to whether strain SW4 produce new metabolites could be answered in the affirmative. In the genome of SW4 two hybrid NRPS-PKS and one NRPS gene clusters were annotated. One cluster showed elements potentially encoding a virginiamycin or pristinamycin-like compound. Comparison of pristinamycin gene clusters amongst relative strains could therefore reveal the potential of this isolate to produce a new derivative of pristinamycin.

Pristinamycin is an antibiotic and was firstly reported to be produced from *Streptomyces pristinaespiralis*. Like other members of the streptogramin- type family, such as virginiamycin, mikamycin, vernamycin and others, pristinamycin is a mixture of two different chemical classes of components, pristinamycin I and pristinamycin II in a specific ratio. Pristinamycin I belongs to the B group of streptogramins and is structurally a branched cyclic hexadepsipeptide, while pristinamycin II belongs to the A group of streptogramins and has the overall structure of a polyunsaturated macrolactones but represents hybrid between polyketides and polypeptides.[158]

The presence of inducer and resin in the cultivation medium leads to an increase of the production of pristinamycin in strain SW4. This finding is in line with the report that gamma butyrolactone is a hormone-like signal molecule and the majority *Streptomyces* species produce this molecule. The presence of gamma butyrolactone regulatory systems are important concerning the regulation of secondary metabolites in *Streptomyces*.[161] The production of pristinamycin also increase with the additional adsorbent resin in the batch fermentation of SW4. This finding correlates with the report that the presence of resin can eliminate the growth inhibition and feedback regulation of pristinamycin to the mycelial growth of *Streptomyces pristinaespiralis*.[162]

Many gene clusters in SW4 besides pristinamycin are still left but not expressed. The use of quorum sensing substances apparently only increased the production of pristinamycin, especially pristinamycin IIA or virginiamycin S. Other derivates of pristinamycin were still produced only in low concentrations. To enhance the production of pristinamycin derivates, genetic engineering such as heterologous expression and regulator manipulation is needed.

Screening and isolation novel bacteria from hotspot areas such as Indonesia is still very important to enable the discovery of novel bioactive compounds and taxonomically

novel strains. The application of an antibiotic screening step is still beneficial to keep focus on highly potential isolates. Meanwhile, using bioassay guiding method to select potential bacteria for new compounds could be end up with known compounds. However, a combination of molecular taxonomy using 16S rRNA and metabolomic profiling by using LC and HR/MS in conjuction with metabolomics network software in the primary dereplication step would have been more promising to get novel compounds.

In this work, we were not able to isolate new derivates due to their low production rate. However, we able to find a new species from Nusakambangan island.

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Publications and conference participations

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Sept. 27th-28th 2017, in Tübingen, Germany:
Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM) – poster presentation: **Aziz, S.**, Mast, Y., Wohlleben, W., & Gross, H. "Exploration of novel antibiotics from Indonesian soil bacteria-in their biosynthetic and genomic context"

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