Investigation of the antibiotic biosynthetic potential and phylogenetic diversity of Indonesian actinomycetes

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Abbreviations and Symbols

Abbreviations

antiSMASH	Antibiotics and secondary metabolite analysis shell
BGC	Biosynthetic gene cluster
dDDH	digital DNA-DNA hybridization
DNA	Deoxyribonucleic acid
ECDC	European Center for Disease Prevention and Control
EMSA	Electromobility shift assay
GGDC	Genome-to-Genome Distance Calculator
GNPS	Global Natural Product Social Molecular Networking
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography coupled to mass spectrometry
HV	Humic acid vitamin
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MS	Mannitol soy flour
NBRC	Biological Resource Center, NITE
NRP	non-ribosomal peptides
OSMAC	One Strain Many Compounds
PCR	Polymerase chain reaction
16S rRNA	16S ribosomal RNA
RT-(q)PCR	(quantitative) reverse transcription PCR
SARP	*Streptomyces* Antibiotic Regulatory Protein
TYGS	Type (Strain) Genome Server

Symbols

^{o}C	Degree in Celsius
µg	Microgram
µl	Microliter
%	Percentage

g	Gram
ml	Milliliter
mg	Milligram
mm	Millimeter
m/z	mass-to-charge ratio

Summary

Actinomycetes are known as a treasure trove for bioactive compounds as they are the origin of about two-thirds of all antibiotics in clinical use. Unique habitats such as marine and unexplored habitats are considered promising sources for sampling new actinomycetes with a high potential to produce new natural products. Indonesia is one of the countries with the most extensive mangrove marine ecosystems and a vast diversity of microbial species. Thus, exploration of Indonesian samples for actinomycetes may lead to the discovery of new antibiotics.

About 422 strains of actinomycetes were isolated from marine sediments of Bali and Lombok Island and soil samples from Enggano Island. The nine most bioactive strains have been prioritized for further drug screening approaches. The nine strains were cultivated on different solid and liquid media using the OSMAC cultivation strategy to assess their biosynthetic capacity to produce natural compounds. A combination of genome mining and mass spectrometry-based (MS) molecular network analysis was applied to identify potential new substances from the nine strains. Several biosynthetic products encoded by the gene clusters of the nine strains were identified, including naphthyridinomycin, amicetin, echinomycin, tirandamycin, antimycin, and desferrioxamine B. Additionally, sixteen putative biosynthesis products and numerous biosynthetic gene clusters were found, which could not be associated with any known compound or biosynthetic gene cluster, respectively, indicating that the nine Indonesian strains have the potential to produce new secondary metabolites.

Furthermore, a regulator-driven strategy was applied to activate (silent) gene clusters in the Indonesian strains. As an example, the SARP-type activator PapR2 was expressed in the Indonesian strain isolate SHP 22-7 to unlock its chemical diversity. Thereby it could be shown that PapR2 expression activates the biosynthesis of the nucleoside antibiotic plicacetin in SHP 22-7. The increased transcription of the plicacetin cluster was detected by RT-PCR analysis and production of the compound was confirmed by HPLC-MS analysis.

Overall, the methodologies described here represent promising strategies for acquiring new natural products from well-known bacterial sources. In addition, the reported unidentified compounds provide a basis for the further characterization and development of these compounds as drug candidates.

Zusammenfassung

Actinomyceten sind bekannt für ihr Potential zur Produktion vieler bioaktive Wirkstoffe und der UUrsprung von etwa zwei Dritteln aller klinisch verwendeten Antibiotika. Einzigartige Lebensräume wie marine und unerforschte Habitate gelten als vielversprechende Quelle für neue Actinomyceten, welche das Potential haben neue Naturstoffe zu produzieren. Indonesien ist eines der Länder mit den ausgedehntesten marinen Mangroven-Ökosystemen und einer großen Vielfalt an mikrobiellen Arten. Die Erkundung von indonesischen Proben nach Actinomyceten könnte also zur Entdeckung neuer Antibiotika führen.

Etwa 422 Actinomyceten-Stämme wurden aus marinen Sedimenten der Inseln Bali und Lombok sowie aus Bodenproben der Insel Enggano isoliert. Die neun bioaktivsten Stämme wurden für weitere Wirkstoff-Screening-Ansätze priorisiert. Die neun Stämme wurden auf verschiedenen festen und flüssigen Medien unter Verwendung der OSMAC-Kultivierungsstrategie kultiviert, um ihr Biosynthesepotential zur Produktion potenziell neuer Naturstoffe zu bewerten. Eine Kombination aus *Genome Mining* und Massenspektrometrie-basierter (MS) molekularer Netzwerkanalyse wurde angewendet, um potenzielle neue Substanzen aus den neun Stämmen zu identifizieren. Es wurden mehrere Biosyntheseprodukte, die von den Genclustern der neun Stämme kodiert werden, gefunden, darunter Naphthyridinomycin, Amicetin, Echinomycin, Tirandamycin, Antimycin und Desferrioxamin B. Zusätzlich wurden sechzehn mutmaßliche Biosyntheseprodukte und zahlreiche Biosynthesegencluster gefunden, die keiner bekannten Verbindung bzw. Gencluster zugeordnet werden konnten, was darauf hindeutet, dass die neun indonesischen Stämme das Potenzial haben, neue Sekundärmetabolite zu produzieren.

Darüber hinaus wurde eine Regulator-basierte Strategie angewandt, um (stille) Gencluster in den indonesischen Stämmen zu aktivieren. Als Beispiel wurde der SARP-Typ Aktivator PapR2 im indonesischen Stammisolat SHP 22-7 exprimiert, um dessen chemische Vielfalt zu erschließen. Dabei konnte gezeigt werden, dass die PapR2-Expression die Biosynthese des Nukleosid-Antibiotikums Plicacetin in SHP 22-7 aktiviert. Die erhöhte Transkription des Plicacetin-Clusters wurde durch RT-PCR-Analyse nachgewiesen und die Produktion der Verbindung durch HPLC-MS-Analyse bestätigt.

Insgesamt stellen die hier beschriebenen Methoden vielversprechende Strategien zur Gewinnung neuer Naturstoffe aus bekannten bakteriellen Quellen dar. Darüber hinaus bieten die berichteten unidentifizierten Verbindungen eine Grundlage für die weitere Charakterisierung und Entwicklung dieser Verbindungen als Arzneimittelkandidaten.

List of publications and personal contributions

Accepted publications and manuscripts

This thesis is based on the following publication and manuscripts

Publication 1:

Ira Handayani, Shanti Ratnakomala, Puspita Lisdiyanti, Fahrurrozi, Mohammad Alanjary, Wolfgang Wohlleben, Yvonne Mast. ***Complete Genome Sequence of Streptomyces sp. Strain BSE7F, a Bali Mangrove Sediment Actinobacterium with Antimicrobial Activities.*** Genome Announcements. Volume 6, Issue 26, e00618-18, 28 June 2018. DOI 10.1128/genomeA.00618-18

Personal contributions:

Experimental design, antimicrobial activity tests, genome sequence submission to NCBI, antiSMASH analysis, writing the manuscript.

Publication 2:

Ira Handayani, Shanti Ratnakomala, Puspita Lisdiyanti, Fahrurrozi, Wien Kusharyoto, Mohammad Alanjary, Regina Ort-Winklbauer, Andreas Kulik, Wolfgang Wohlleben, Yvonne Mast. ***Complete Genome Sequence of Streptomyces sp. Strain SHP 22-7, a New Species Isolated from Mangrove of Enggano Island, Indonesia.*** Microbiology Resource Announcements. Volume 7, Issue 20, e01317-18, 21 November 2018. DOI 10.1128/MRA.01317-18

Personal contributions:

Experimental design, antimicrobial activity tests, genome sequence submission to NCBI, antiSMASH analysis, writing the manuscript.

Publication 3:

Janina Krause, **Ira Handayani**, Kai Blin, Andreas Kulik and Yvonne Mast. ***Disclosing the Potential of the SARP-Type Regulator PapR2 for the Activation of Antibiotic Gene Clusters in Streptomycetes***. Frontiers in Microbiology. Volume 11, Issue 225, 18 February 2020. DOI 10.3389/fmicb.2020.00225.

Personal contributions:

Insertion of the vector pRM4/papR2 in SHP 22-7, bioactivity tests, HPLC analysis, writing a part of results and material and methods of the manuscript.

Publication 4:

Ira Handayani, Hamada Saad, Shanti Ratnakomala, Puspita Lisdiyanti, Wien Kusharyoto, Janina Krause, Wolfgang Wohlleben, Andreas Kulik, Saefuddin Aziz, Harald Gross, Athina Gavriilidou, Nadine Ziemert, Yvonne Mast. ***Mining Indonesian microbial biodiversity for novel natural compounds by a combined genome mining and molecular networking approach.*** Marine Drugs (MDPI). Volume 19, Issue 6, 316, 28 Mei 2020. DOI 10.3390/md19060316.

Personal contributions:

Experimental design, 16S phylogenetic analysis, strain cultivation and extraction of culture broths, antibiotic bioassays, genome sequence submission to NCBI, bioinformatic analysis with antiSMASH and TYGS, writing the manuscript.

1. Introduction

1.1. Drug discovery from microbial natural product producers

Natural resources such as plants, animals, and marine organisms have long been exploited and utilized by humans as sources of medicine for the relief and treatment of various diseases [1,2]. The first record of natural product application in human health date back to ancient Mesopotamia in 2900-2600 BC, which records that around 1000 plants and their derivatives were used as medicines to treat various ailments, from coughs and colds to parasitic infections and inflammation [3]. In the early 1900’s, nearly 80% of all medicines were derived from natural products obtained from plants that were used for health care or to cure diseases such as inflammation, diabetes, malaria, arthritis, cancer, heart and liver disease [4,5]. However, after discovering penicillin from *Penicillium notatum* in 1928 by Alexander Fleming [6], there was a drastic change from plants to microorganisms as a source of natural products. Since then, natural compounds derived from microorganisms have been used in medicine, agriculture, the food industry, and scientific research [7–9], whereby the majority of substances has been applied for antibacterial therapy [10]. Several microbial metabolites also serve as lead compounds in other medical fields, as for example, the fungal metabolite lovastatin, which serves as a lead compound for a series of drugs that lower cholesterol levels. The fungal metabolite cyclosporin is applied to suppress the immune response after transplant surgery, or the macrolide antibiotic rapamycin is used in the treatment of several types of cancer [10].

Microorganisms play an essential role in therapeutic development from natural products and in medical therapy [8]. They have been recognized as a rich source of unique secondary metabolites, valuable for searching drugs and lead compounds [7–9]. Many of the commercially available antibiotics and more than 120 most critical therapeutic agents known to date have been derived from terrestrial microorganisms such as fungi and bacteria [8]. Immunosuppressants such as cyclosporin and mycophenolic acid, antimicrobials such as fusidic acid and griseofulvin, and other new semisynthetic antifungals such as anidulafungin and caspofungin are some examples of commercial therapeutic agents available to date, which are derived from fungi [11]. The antibacterial agent streptomycin, the antifungal metabolite nystatin, and the

anticancer compound doxorubicin are examples of currently available commercial therapeutic agents derived from bacteria [12]. Overall, most of the commercially available therapeutic agents are derived from the bacterial class of actinomycetes. These filamentous bacteria produce more than 10,000 bioactive compounds and about two-thirds of all clinically valuable antibiotics [12,13].

The medicinal potential of actinomycetes was first discovered by Selman Waksman and Albert Schatz in 1943. They found that streptomycin, a natural product from *Streptomyces griseus,* is a potent antibiotic agent to cure tuberculosis, an infectious disease caused by *Mycobacterium tuberculosis* [14]. This finding encourages pharmaceutical companies and researchers to put their large-scale efforts into researching natural microbial products from actinomycetes. The efforts depend mainly on isolating these microorganisms from various environmental samples and screening the desired bioactivity [15]. The approach leads to the golden era of antibiotic discovery during the period from 1940 to 1970. More than 100 new natural substances were discovered during this period and developed into potent antibiotics, including streptomycin, vancomycin, rifamycin, and others [16,17]. However, in recent decades, the number of discoveries, developments, and introductions of new medically relevant drugs to the market has been steadily decreasing. One of the main problems contributing to this decline is the high rediscovery rate of known compounds [18–21]. The loss of interest from the pharmaceutical industry due to large investments in antibiotic development with very high risk, high cost, and small profit potential also contributed to the decline [19,21,22]. Meanwhile, the increasing public health burden caused by antibiotic-resistant pathogenic bacteria and the constant need to find new treatments for non-communicable (chronic) diseases is more significant than ever. These facts indicate that the search for bioactive compounds with novel mechanisms of action or new cellular targets is urgently needed to fill the therapeutic agent pipeline and treat infections with drug-resistant pathogens [23,24].

1.2. Actinomycetes as treasure trove for bioactive secondary metabolites

Actinomycetes are filamentous Gram-positive bacteria with a high guanine and cytosine (GC) content (>70%) in their genomes [25,26]. They live aerobically and are mostly non-pathogenic [27]. Initially, actinomycetes were considered an intermediate form between fungi and bacteria due to their ability to form mycelium and reproduce

by sporulation like fungi [28]. They are mostly mesophilic like other soil bacteria, with optimal growth temperatures ranging between 25°C and 30°C and optimal pH values between 6 and 9 [26]. However, thermophilic actinomycetes can grow at temperatures in the range of 50°C to 60°C [29] and some acidophilic actinomycetes can grow in acidic soils with pH values between 4.5 and 5.5 [30]. Actinomycetes are broadly distributed in terrestrial and aquatic environments, mainly in soil. Generally, they are abundant in cell densities of about 10^6 to 10^9 cells per gram of soil [31], with the largest soil population dominated by the genus *Streptomyces,* which accounts for more than 95% of the actinomycetes strains isolated from soil [32].

The genus *Streptomyces* which belongs to the family *Streptomycetaceae*, is the largest genus of the actinomycetes group with more than 500 species of *Streptomyces* bacteria have been described [33]. One of the most attractive characteristics of *Streptomyces* is its ability to produce a wide variety of secondary metabolites [34]. Another distinctive feature of *Streptomyces* is their complex life cycle, including differentiation into at least three different cell types, closely related to the production of secondary metabolites [35]. The three developmental stages of the *Streptomyces* life cycle consist of (**Figure 1**) [36]: First, the spores germinate to form vegetative hyphae, which grow by elongation of the apical tip, which eventually creates a dense network of vegetative mycelium that scavenges nutrients [36,37]. Second, in response to signals associated with adverse conditions, such as nutrient depletion, the vegetative or substrate mycelium is degraded autolytically by programmed cell death-like (PCD) mechanisms to acquire the building blocks needed to form aerial hyphae [38–40]. The emergence of these aerial hyphae coincides with the initiation of antibiotic production in vegetative cells [36,41] The aerial hyphae formation requires the activity of the *'bld'* gene product, referring to the phenotype of the bald ("hairless") mutant that lacks hairy aerial hyphae [42]. At the final stage of development, aerial hyphae are differentiated into spores through the concurrent events of cell division and cell maturation [36]. The product of the *whi* gene regulates this process. The mutations in this gene result in the mutants failing to form mature pigmented spores [43]. Spores are resistant to a variety of stressful conditions, such as drought and heat. In addition, spores also facilitate the spread of *Streptomyces* to new environments, as all *Streptomyces* cell types are characterized as non-motile [27].

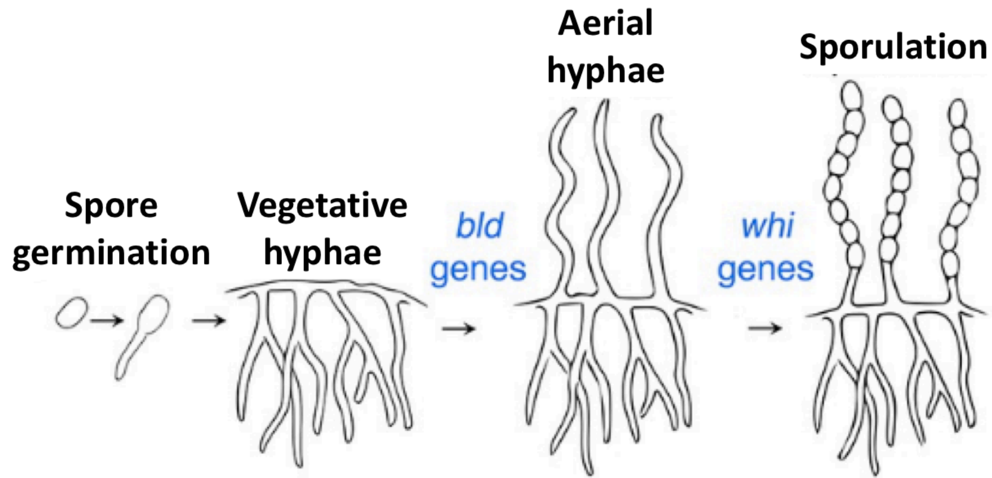


Figure 1. Developmental life cycle of *Streptomyces* [36].

Streptomyces contributes to most bioactive substances used in the pharmaceutical industry. 50% of clinically applied antibiotics [44] and several drugs widely used to combat common diseases, including cancer, are derived from these bacteria [45,46]. Some of the antibacterial agents produced by strains of the genus *Streptomyces*, such as actinomycin D, bleomycin, mitomycin, and plicamycin, are too toxic to be used for the treatment of bacterial infections in humans but, because of their toxicity to cells, have been reinstated as chemotherapeutic drugs [47]. Apart from antibiotics, *Streptomyces* also produce other bioactive compounds, as for example substances with herbicidal activity, such as validamycin A, which is an antifungal agent used to control pathogens in rice and other crops and reduces vegetable seed disease [48], and phosphinothricin tripeptide (PTT), which is a peptide antibiotic with potent herbicide activity derived from *Streptomyces viridochromogenes* [49]. Furthermore, they also produce substances which have e.g. antiparasitic, anti-tumor, immunosuppressant and antimalarial activity such as the antiparasitic ivermectin, the anticancer bleomycin, the immunosuppressant rapamycin, and the antimalarial mollemycin A [9,45,46].

Streptomyces is considered as a treasure trove for many bioactive compounds and secondary metabolites with important clinical applications [34,50]. Secondary metabolite biosynthesis in *Streptomyces* is genetically encoded, whereby the encoding genes usually occur as so-called biosynthetic gene clusters (BGCs), meaning that all genes needed for antibiotic biosynthesis, regulation and self-resistance mechanisms are arranged physically adjacent on the chromosome [51]. With the advances in next-

generation sequencing (NGS) technology, various *in silico* genome mining strategies have been developed and applied to explore the *Streptomyces* genome in terms of biosynthesis capacity [52]. These mining studies have revealed that the *Streptomyces* genome harbors on average 25 with up to 70 BGCs, far more than other actinobacterial genera [52–54]. Only 10 % of BCGs are expressed when the strain is cultured under standard laboratory conditions. These BGCs, are called "silent" or "cryptic" BGCs and have the potential to encode new compounds [55]. Thus, activating these silent gene clusters may unlock the chemical diversity of the actinobacterial strains and increase the discovery of new compounds for medical purposes [56]. In this regards, actinomycetes, especially the genus *Streptomyces*, are still a promising source for discovering new bioactive compounds.

1.3. Isolation of new actinomycetes from regions with high biodiversity

One approach to discover new antibiotics is to search for unknown actinomycetes that may have the potential to produce new natural compounds. Sampling of actinomycetes from conventional environments such as soil often leads to the discovery of already known species that yields known antibiotics [20]. Therefore, drug discovery research efforts by isolating actinomycetes from unique habitats, such as extreme or marine environments is a promising strategy to find new strains as sources for new bioactive compounds [57,58]. The mangrove marine ecosystem is believed to be a rich source of new actinobacterial species that may have the potential to produce new compounds [59–64]. For example, *Saccharomonospora oceani* VJDS-3, isolated from an Indian mangrove ecosystem, is a rare actinobacterial species, which produces the three new bioactive compounds methoxy ethyl cinnamate, 4-hydroxy methyl cinnamate, and 4-methyl benzoic acid [63]. Another example is represented by *Streptomyces sp.* B475, a novel *Streptomyces* species from the Maowei sea mangrove reserve in China that produces several quinoxaline-type antibiotics and their analogues [65]. Furthermore, *Streptomyces qinglanensis* 172205, isolated from a Chinese mangrove soil sample, produced the five new compounds 15R-17,18-dehydroxantholipin, (3E,5E,7E)-3-methyldeca-3,5,7-triene-2,9-dion, and qinlactone A-C, which exhibited strong anti-microbial and antiproliferative bioactivities [66]. In addition to mangrove ecosystems, new actinobacteria are also found in extreme habitats, such as deserts. One example is represented by the strain *Streptomyces asenjonii* KNN 42.F, which was isolated from

a desert soil sample and produced the three new bioactive compounds asenjonamides A-C [67]. The above mentioned examples demonstrate that mangroves and extreme habitat environments can be a promising sources of new natural products.

In this respect Indonesia is an interesting country as it has the largest mangrove forest, with 23% of all mangrove ecosystems worldwide, where around three million hectares of mangrove forests grow along the 95,000 km coastline of Indonesia [68]. Furthermore, Indonesia is one of the most biodiverse countries in the world, which consists of 17,000 islands, with 88,495,000 hectares of tropical forest and 86,700 square kilometers of coral reefs [69,70]. Indonesia is home to many flora and fauna with many species that are endemic and ecologically adapted [71–73]. The diverse habitats of Indonesia, such as acid hot springs, peatland forests, the Thousand Islands reef complex, and Enggano Island, contribute to a wide diversity of microbial species [74–77]. Thus, the unique habitats of Indonesia are promising sources of novel actinomycetal strains, which may produce new bioactive compounds.

1.4. Dereplication strategies using combinatory genome and metabolic mining approaches for drug discovery

An essential part of finding new drug candidates from the vast diversity of forest, soil, or marine biodiversity in current natural product screening programs is the rapid identification of known substances to focus solely on discovering new ones [78]. Dereplication or identification of already known compounds aims to overcome the previously stated challenge of high rediscovery rates prior to the tedious screening, isolation, and purification processes [79–81]. The usefulness of this strategy is to pinpoint known compounds at an early stage of the discovery pathway, thereby reducing the time, effort, and cost involved in drug research [82]. In recent years, integrated genomic and metabolomics mining methods have proven to be an efficient dereplication approach for the identification of new chemical scaffolds [83–86]. Genome mining analyses involve the identification of putative secondary metabolite BGCs by bioinformatic analysis of genome sequences of natural product producers [87,88], e.g. with the help of *in silico* prediction tools, such as antiSMASH [89]. Metabolome mining plays a role in sorting out the chemical substances in extracts of natural product producers through their mass fragmentation patterns. It is known that metabolites with similar chemical structures tend to produce similar mass fragmentation patterns in mass spectrometric (MS) analysis [90]. The Global Natural

Product Social (GNPS) computing platform applies this knowledge to group structurally related entities, which often derive from a similar biosynthetic origin, as a united set of molecular family clusters [91]. The GNPS platform has proven its effectiveness in managing large numbers of samples allowing dereplication and identification and/or tentative structural classification [90]. The combinatory use of two genomic and metabolomics computational tools strengthens the rapid identification of novel chemical compounds. The finding of the antibacterial agent thiomarinol from *Pseudoalteromonas luteoviolacea* [83] is one example of the successful use of this method [86]. Another example is the discovery of microviridin 1777, a chymotrypsin inhibitor derived from *Microcystis aeruginosa* EAWAG 127a [92].

1.5. Activation of silent gene clusters in actinomycetes

The latest bioinformatic analysis of genome sequences from actinomycetes revealed a striking difference between the genetic potential to produce secondary metabolites, reflected by the occurrence of numerous biosynthetic gene clusters (BGCs), and the true natural compound production capacity of the strains when grown under standard laboratory conditions [55]. This fact is attributed to the presence of cryptic or silent BCGs that are not expressed when cultured under standard laboratory conditions [55]. It is estimated that only 10% of the clusters are expressed; thus, these bacteria still hold the genetic potential to produce new bioactive substances [93]. The activation of silent clusters opens up the possibility to exploit chemical diversity in the tested organisms, thus allowing for the discovery of new molecules for medical uses [56].

Several efforts to activate silent cluster expression have been reported e.g., involving genetic- and cultivation-driven approaches [94]. The “One Strain Many Compound (OSMAC)” concept is a culture-based approach and is a well-established strategy, which was introduced nearly two decades ago [95]. This concept still leads to the discovery new compounds, as exemplified by the identification of the new aromatic polyketide lugdunomycin from *Streptomyces* sp. QL37 [96] or the eudesmane sesquiterpenoid and a new homolog of virginiae butanolides (VB-E) from the strain *Lentzea violacea* AS 08 [97]. This strategy involves the variation of media compositions as the basis to test for different natural compound production profiles since it is known that actinomycetes produce different compounds under different cultivation conditions [95]. In line with the OSMAC concept, elicitor screening approaches have recently

been proposed [98]. This concept intends to mimic natural trigger molecules, which induce the expression of biosynthesis pathways. In this study, the combination of high throughput elicitor filtering (HiTES) and a matrix laser desorption/ionization mass spectrometry (MALDI-MS) was carried out for rapid identification of cryptic peptides natural product. This study led to the discovery of a cryptic depsipeptide antibiotic cinnapeptide from *S. ghanaensis* [98]. The co-cultivation approaches represent another culture-based activation strategy, which attempts to induce the expression of the seemingly silent gene clusters by growing two or more microorganisms in one cultivation chamber [99]. The presence of predators and antagonists is supposed to activate a defense mechanism in the antibiotic producer strain resulting in antibiotic secretion [99]. This approach led to the isolation of a novel indole alkaloid metabolite from co-cultures of *Streptomyces* sp. MA37 and *Pseudomonas* sp., which were not previously observed in monocultures of the individual strain [100].

Besides the above-mentioned culture-based activation efforts, a genetic-based approach by heterologous expression of target BGCs has been also developed. The heterologous expression strategy is used to activate target BGCs, which are not expressed in the natural hosts by cloning the target BGCs to heterologous hosts [101]. This method allows optimization of product yields, functional elucidation of silent gene clusters, and production of novel derivatives [102]. One example of a successful BGC activation by heterologous expression strategy is a new isomer of verticilactam production in *Streptomyces avermitilis* strain SUKA17 [103]. The use of heterologous transcriptional regulators is another promising strategy to activate secondary metabolites biosynthetic pathways. Recently, Martinez-Burgo et al. (2019) demonstrated that conserved transcriptional activators might be used to induce BGC expression in foreign *Streptomyces* strains. In the study, the expression of the non-native transcriptional activator PimM, a PAS-LuxR type regulator from *Streptomyces natalensis* in *Streptomyces clavuligerus* led to the induction of the expression of the biosynthetic pathways for clavulanic acid, cefamycin C, and tunicamycin synthesis [104]. In this respect, *Streptomyces* Antibiotic Regulatory Protein (SARP)-type regulators can also be used to activate BGC expression in foreign *Streptomyces* strains since they are well-known activators of antibiotic biosynthesis in actinomycetes. In addition, SARP regulators have only been found in actinomycetes, especially in the genus *Streptomyces*, where the encoding genes occur in an abundance of 98% [105].

SARP-genes are present in BGCs encoding for different types of secondary metabolites, such as type I- [106–108] and type II-PKS polyketides cluster [108–111], ribosomal [112,113] and non-ribosomal peptides (NRP) cluster [114], hybrid polyketides-peptide cluster [115–120], β-azachinone [121–123] and azoxy cluster [124]. The known SARP regulators show a comparable protein architecture and bind to similar recognition sequences at the DNA. Their regulatory function can be replaced by other SARP regulators, as exemplified by Garg and Parry (2010), who showed that overexpression of the SARP gene *vlmI* from the valanimycin producer *Streptomyces viridifaciens* in the *redD* mutant of *S. coelicolor* M512 restores undecylprodigiosin (RED) production, suggesting that *vlmI* can complement the *redD* mutation [125]. Another typical SARP-type regulator is PapR2 from the pristinamycin producer *Streptomyces pristinaespiralis*. Mast et al. (2015) showed that PapR2 is an essential activator for pristinamycin biosynthesis since a *S. pristinaespiralis papR2* deletion mutant does not produce pristinamycin anymore. In contrast, overexpression of *papR2* in *S. pristinaespiralis* led to an increased pristinamycin production [119]. The PapR2 target genes have been determined in the pristinamycin producer, and a conserved PapR2 binding site was proposed with the aid of electromobility shift assay (EMSA) and reverse transcription PCR (RT-PCR) analysis [119]. Thus, overexpression of the SARP gene *papR2* from *S. pristinaespiralis* in foreign *Streptomyces* strains may lead to the induction of silent gene clusters in the non-native PapR2 producer.

2. Aim of the work

Actinomycetes isolated from marine habitats and unexplored habitats have been reported as promising sources for structurally unique and diverse novel natural substances. Indonesia is a country with incredible biodiversity and unique ecosystems. Thus, samples from biodiverse habitats of Indonesia are expected to be a promising source for unknown actinomycetal strains, which may have the potential to produce novel bioactive compounds. This study aims to explore the biosynthetic capacity of selected Indonesian actinomycetes isolated from three different locations in Indonesia. For this purpose, 422 Indonesian actinomycetes strain isolates, which have previously been obtained from Lombok, Bali, and Enggano Islands and analyzed for antibacterial activity served as a basis. Based on bioactivity-guided studies the most bioactive strain isolates shall be prioritized for a combinatory genome mining and molecular network approach to assess the biosynthetic capacity of the strains to produce new natural compounds. Furthermore, the feasibility of a regulator-driven approach to activate antibiotic gene clusters in the Indonesian strains shall be tested. For this purpose, the SARP-type regulator PapR2 from *S. pristinaespiralis* will be heterologously expressed in the Indonesian strain SHP 22-7 as test organism with the aim to induce secondary metabolite biosynthetic pathways in this strain.

Results and discussion

Results of the following publication and manuscripts are summarized and discussed in the "Result and Discussion" section:

Ira Handayani, Shanti Ratnakomala, Puspita Lisdiyanti, Fahrurrozi, Mohammad Alanjary, Wolfgang Wohlleben, Yvonne Mast. ***Complete Genome Sequence of Streptomyces sp. Strain BSE7F, a Bali Mangrove Sediment Actinobacterium with Antimicrobial Activities.*** Genome Announcements. Volume 6, Issue 26, e00618-18, 28 June 2018. DOI 10.1128/genomeA.00618-18.

Ira Handayani, Shanti Ratnakomala, Puspita Lisdiyanti, Fahrurrozi, Wien Kusharyoto, Mohammad Alanjary, Regina Ort-Winklbauer, Andreas Kulik, Wolfgang Wohlleben, Yvonne Mast. ***Complete Genome Sequence of Streptomyces sp. Strain SHP 22-7, a New Species Isolated from Mangrove of Enggano Island, Indonesia.*** Microbiology Resource Announcements. Volume 7, Issue 20, e01317-18, 21 November 2018. DOI 10.1128/MRA.01317-18.

Janina Krause, **Ira Handayani**, Kai Blin, Andreas Kulik and Yvonne Mast. ***Disclosing the Potential of the SARP-Type Regulator PapR2 for the Activation of Antibiotic Gene Clusters in Streptomycetes***. Frontiers in Microbiology. Volume 11, Issue 225, 18 February 2020. DOI 10.3389/fmicb.2020.00225.

Ira Handayani, Hamada Saad, Shanti Ratnakomala, Puspita Lisdiyanti, Wien kusharyoto, Janina Krause, Wolfgang Wohlleben, Andreas Kulik, Saefuddin Aziz, Harald Gross, Athina Gavriilidou, Nadine Ziemert, Yvonne Mast. **Mining Indonesian microbial biodiversity for novel natural compounds by a combined genome mining and molecular networking approach.** Marine Drugs (MDPI). Volume 19, Issue 6, 316, 28 Mei 2020. DOI 10.3390/md19060316.

3. Results and Discussion

3.1. Isolation and characterization of Indonesian actinomycetes

3.1.1. Isolation of actinomycetes from terrestrial and marine habitats of Indonesia

Bioprospecting from unique habitats has proven efficient for obtaining novel bioactive compound producers [57,58]. In this regard, Indonesia, which is rich in biodiverse habitats, is expected to be a source for finding potential untapped actinomycetal strains that can produce new bioactive secondary metabolites. In the frame of cooperative work with partners from the Indonesia Institute for Sciences (LIPI), soil samples from terrestrial and marine habitats from three different geographic locations in Indonesia have been taken to isolate actinomycetes. Soil samples from terrestrial habitats were collected from the island of Enggano (5° 22' 57.0792" S, 102° 13' 28.2792" E), Indonesia. This island was chosen as a sampling site for terrestrial environmental samples, as it is a remote island with rich biodiversity and many endemic species. Marine samples were collected from marine sediments from Bali Island (8 ° 43 '5.5 "South Latitude, 115 ° 10' 7.8" East Longitude) and Lombok Island, West Nusa Tenggara (8 ° 24'17.133 '' South Latitude, 116 ° 15 '57.228 ' 'E), Indonesia. Isolation of actinomycetes from the Indonesian samples was performed by the Indonesian cooperation partners and performed using standard isolation protocols as described previously [126–130]. From these isolation efforts, 422 strains from three different areas of Indonesia have been collected. Samples from Enggano Island accounted for the highest number of isolates of actinomycetes strains (56.2%) from all sampling locations, followed by sediment samples from Lombok Island (37.2%) and the island of Bali (6.6%). From these results, it can be concluded that the untapped pristine island of Enggano is more promising for sampling actinomycetes.

3.1.2. Preliminary antibiotic screening based on bioactivity

A preliminary screening has been carried out by the Indonesian partners with the 422 actinomycetes isolates by antibacterial bioassays using an agar plug diffusion bioassay. From this screening, twenty strain isolates that showed the largest diameter of the inhibition zone in the agar plug bioassay were prioritized for further research. A

list of the twenty priority strains, their isolation sources, and isolation methods are shown in Table 1.

Table 1. Indonesian strain isolate, isolation method, source of isolation, and most closely related species (%) based on 16S rRNA gene sequence phylogenetic analysis with EzTaxon (modified from Handayani et al., 2021).

No.	Strain (*Streptomyces* sp.)	Isolation method	Source of isolation	Most closely related species based on 16S analysis
1	SHP 22-7	phenol	Soil under a Ketapang tree (*Terminalia catappa*) from Desa Meok, Enggano Island	*Streptomyces rochei* NBRC 12908^T (99.59%)
2	SHP 20-4	phenol	Soil under a Kina tree (*Cinchona* sp), Desa Banjarsari, Enggano Island	*Streptomyces hydrogenans* NBRC 12908^T (99.68%)
3	SHP 2-1	phenol	Soil under a Hiyeb tree (*Artocarpus elastica*) near Bak Blau water spring, Desa Meok, Enggano Island	*Streptomyces griseoluteus* NBRC 13375^T (98.96%)
4	DHE 17-7	dry heat	Soil under a Ficus tree (*Ficus* sp), Desa Boboyo, Enggano Island	*Streptomyces lannensis* TA4-8^T (99.78%)
5	DHE 12-3	dry heat	Soil under a Cempedak tree (*Artocarpus integer*), Desa Boboyo, Enggano Island	*Streptomyces coerulescens* ISP 51446^T (98.87%)
6	DHE 7-1	dry heat	Soil under a Terok tree (*Artocarpus elastica*), desa Boboyo, Enggano Island	*Streptomyces adustus* WH-9^T (99.59%)
7	DHE 6-7	dry heat	Soil under forest Snake fruit tree (*Salacca* sp.), Desa Malakoni, Enggano Island	*Streptomyces parvulus* NBRC 13193^T (98.55%)
8	DHE 5-1	dry heat	Soil under a Banana tree (*Musa* sp.), Desa Banjar sari, Enggano Island	*Streptomyces parvulus* NBRC 13193^T (99.79%)

9	BSE 7-9	NBRC medium 802	Mangrove sediment near plant rhizosphere, Kuta, Bali Island	*Streptomyces bellus* ISP 5185 ^{T} (99.06%)
10	BSE 7F	NBRC medium 802	Mangrove sediment near plant rhizosphere, Kuta, Bali Island	*Streptomyces matensis* NBRC 12889^{T} (99.72%)
11	I1	HV	Sea sand from Cemara Beach, West Lombok	*Isoptericola hypogeus* HKI 0342 (T) (76.14%)
12	I2	HV	Sea sand from Kuta Beach, South Lombok	-
13	I3	HV + chlorine 1%	Mangrove sediment from Pantai Tanjung Kelor, Sekotong, West Lombok Island	*Streptomyces longispororuber* NBRC 13488^{T} (99.23%)
14	I4	HV + chlorine 1%	Mangrove sediment from Pantai Tanjung Kelor, Sekotong, West Lombok Island	*Streptomyces griseoincarnatus* LMG 19316^{T} (99.89%)
15	I5	HV + chlorine 1%	Mangrove sediment from Pantai Tanjung Kelor, Sekotong, West Lombok Island	*Streptomyces viridodiasticus* NBRC13106^{T} (99.31 %)
16	I6	HV + chlorine 1%	Mangrove sediment from Pantai Tanjung Kelor, Sekotong, West Lombok Island	*Streptomyces spongiicola* HNM0071^{T} (99.78 %)
17	I7	HV + chlorine 1%	Mangrove sediment from Pantai Tanjung Kelor, Sekotong, West Lombok Island	-
18	I8	HV	Sea sands from Pantai Koeta, Lombok Island	*Streptomyces smyrnaeus* SM3501^{T} (98.44 %)
19	I9	HV	Sea sands from Pantai Koeta, Lombok Island	*Streptomyces gancidicus* NBRC 15412^{T} (98.82 %)

20	I10	dry heat	Leaf litter under a cocoa tree Desa Genggelang, North Lombok	*Streptomyces antibioticus* NBRC 12838 (T) (100 %)

3.1.3. Taxonomic classification of twenty Indonesian actinomycetes based on 16S rRNA gene sequence phylogeny

The twenty prioritized isolates were investigated regarding their phylogenetic relationships by comparative 16S rRNA gene sequence analysis. For this purpose, the 20 strains were grown individually for two days in 50 ml of R5 medium at 28°C [13]. The genomic DNA was isolated using the Nucleospin® Tissue kit from Macherey-Nagel (catalog number 740952.50) following the standard protocol from the manufacturer. The genomic DNA was applied as a PCR template to amplify the 16S rRNA gene using polymerase chain reaction (PCR). PCR was performed with the 16S rRNA gene-specific primers 27Fbac (5'-AGAGTTTGATCMTGGCTCAG-3 ') and 1492Runi (5'-TACGGTTACCTTACGACTT-3'). The individual PCR amplification from the 20 strains was sub-cloned into the cloning vector pDrive (Qiagen) using the basic DNA manipulation procedure as described previously by Sambrook et al. [131]. The resulting pDrive constructs with the 16S gene fragments were sequenced at MWG Eurofins (Ebersberg, Germany) with the 27Fbac primer. The generated 16S sequences, which ranged between 547 to 997 bp of the expected full-length 16S rRNA sequence, were analyzed using the EzTaxon database (https://www.ezbiocloud.net) [132] to determine the strains phylotype.

EzTaxon analysis disclosed that all 17 strain isolates, namely *Streptomyces* sp. SHP 22-7, SHP 20-4, SHP 2-1, DHE 17-7, DHE 12-3, DHE 7-1, DHE 6-7, DHE 5-1, BSE 7-9, BSE 7F, I3, I4, I5, I6, I8, I9, and I10 belonged to the genus *Streptomyces* with 16S gene sequence similarities, which ranged between 98.44% - 100% of the nearest predicted neighbors (**Table 1**). This taxonomic classification allows further prioritization. The 16S gene sequence of strain *Streptomyces* sp. I10 showed 100% similarity to *Streptomyces antibioticus,* a known producer of the peptide antibiotic actinomycin and thus most likely represents the same species; due to the high similarity valued to a producer strain of a known substance, this strain was not followed up in further experiments. Within the EzTaxon analysis, only strain I1 was assigned to

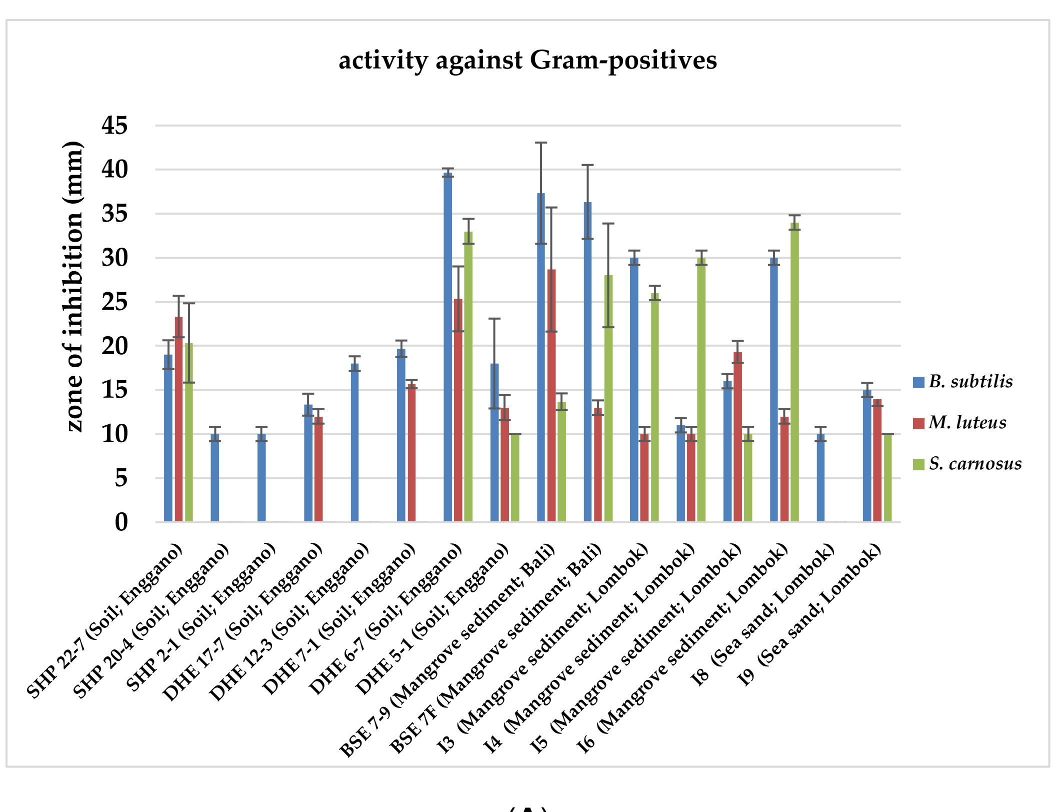
the genus *Isoptericola*, which is a rare actinomycete. However, the similarity of strain I1 to the *Isoptericola* genus was only 76.14%, thus making its classification questionable. In addition, the glycerin stock for I2 was found to be contaminated. Furthermore, the top-hit taxon for strain I7 could not be identified, and this is presumably due to the type of strain is not present in EzBioCloud. Therefore, *Streptomyces* sp. I1, I2, and I7 were also excluded in the subsequent studies because they could not be clearly assigned to actinomycetes. From this taxonomic study, 16 strains were selected for further investigation. The sixteen strains were *Streptomyces* sp. SHP 22-7, SHP 20-4, SHP 2-1, DHE 17-7, DHE 12-3, DHE 7-1, DHE 6-7, DHE 5-1, BSE 7-9, BSE 7F, I3, I4, I5, I6, I8, and I9.

3.1.4. Prioritization of producer strains based on bioactivity data

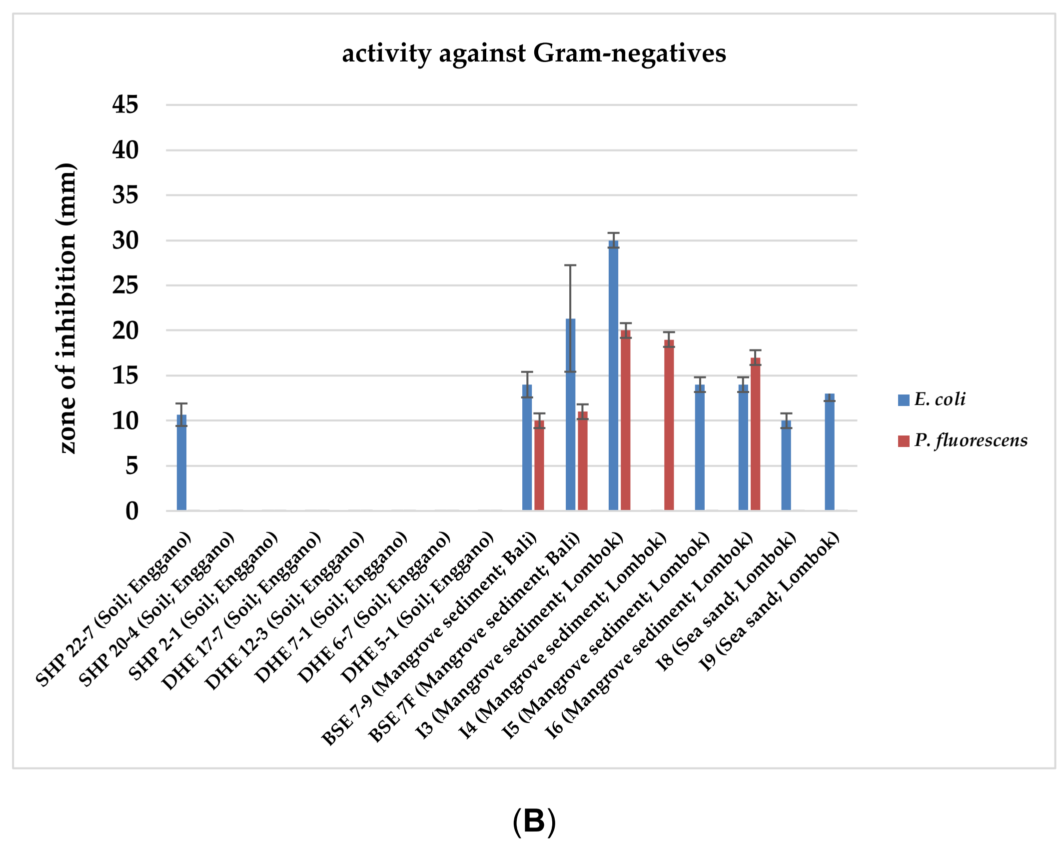
For further study, sixteen representatives from the taxonomic analysis were investigated for their microbial activity using an agar plug diffusion bioassay. Gram-positive bacteria (*Bacillus subtilis*, *Micrococcus luteus*, and *Staphylococcus carnosus*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas fluorescens*) were used as test organisms. Within the framework of further activity screenings, the sixteen isolates were evenly distributed on the surface of a mannitol soy flour (MS) agar plate (20 g mannitol, 20 g soy flour (full fat), 16 g agar in 1 liter of distilled water) [80]. The agar plates were incubated for ten days at 28°C. The ten-day inoculum was cut aseptically with a 9 mm diameter cork borer and placed on the bioassay test plate. The antimicrobial activity emanating from the strain isolate was determined by measuring the inhibition zone diameter (mm) around the agar plug. All of the bioassays were carried out as three independent biological replicates. This analysis found that all tested isolates showed bioactivity against *B. subtilis* (**Figure 2A**), and nine strains exhibited additional activity against Gram-negative test strains (**Figure 2B**). In addition, four isolates, namely BSE 7-9, BSE 7F, I3, and I6, showed activity against both, Gram-positive and negative test bacteria (**Figure 2**).

Nine strains were prioritized based on the bioactivity profile for further investigation. Strains SHP 22-7, BSE 7-9, BSE 7F, I3, I4, I5, and I6, were selected since they displayed antimicrobial activity against Gram-positive and Gram-negative organisms (**Figure 2A, B**). Whereas DHE 17-7 and DHE 7-1 were chosen because they showed bioactivity against at least two different Gram-positive tests strains. However, DHE 6-

7 and DHE 5-1 were not selected for further analysis, although they showed activity against all Gram-positive test strains since both organisms displayed a close phylogenetic relationship similarity of 98.55% for DHE 6-7 and 99.79% for DHE 5-1 with *Streptomyces parvulus* (**Table 1**). *Streptomyces parvulus* is a known producer of the peptide antibiotic actinomycin D [133]. Actinomycin D as *m/z* 1255.8 $[M+H]^+$ and *m/z* 1253.7 $[M+H]^-$ (**Figure 3C**) with a retention time (RT) of 12.0 min (**Figure 3A, B**) was detected as a product in an initial effort by HPLC-MS analysis of the methanol extract of culture samples from DHE 6-7 and DHE 5-1. Thus, both strains were not further analyzed.

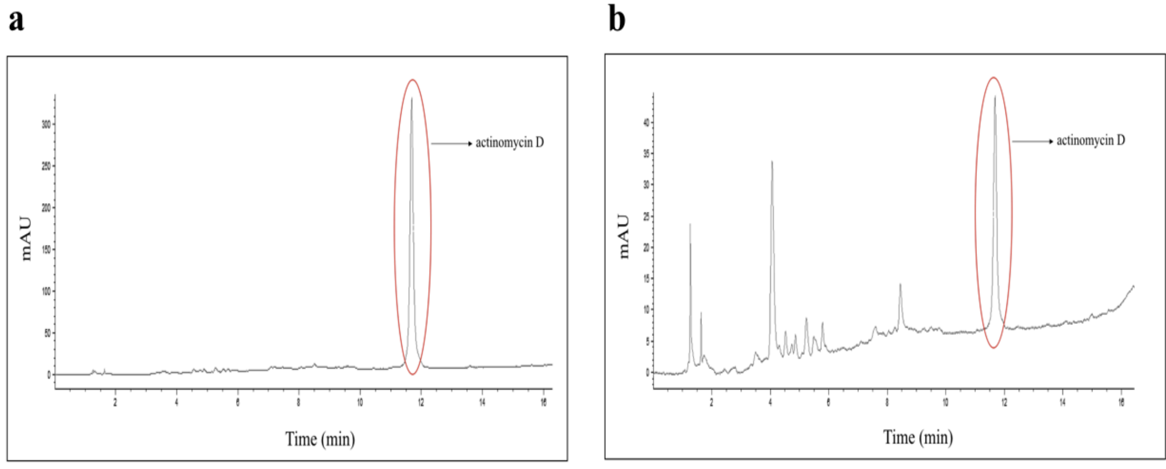


(**A**)



(**B**)

Figure 2. Graphical presentation of results from antimicrobial bioassays with 16 Indonesian actinomycetes strain samples against Gram-positive (A) and Gram-negative test strains (B). Inhibition zone diameters of agar plug test assays are given in mm. Agar plugs were used after ten days of growth of the respective actinomycetes strains. Data shown are the result of three independent biological replicates. [134]



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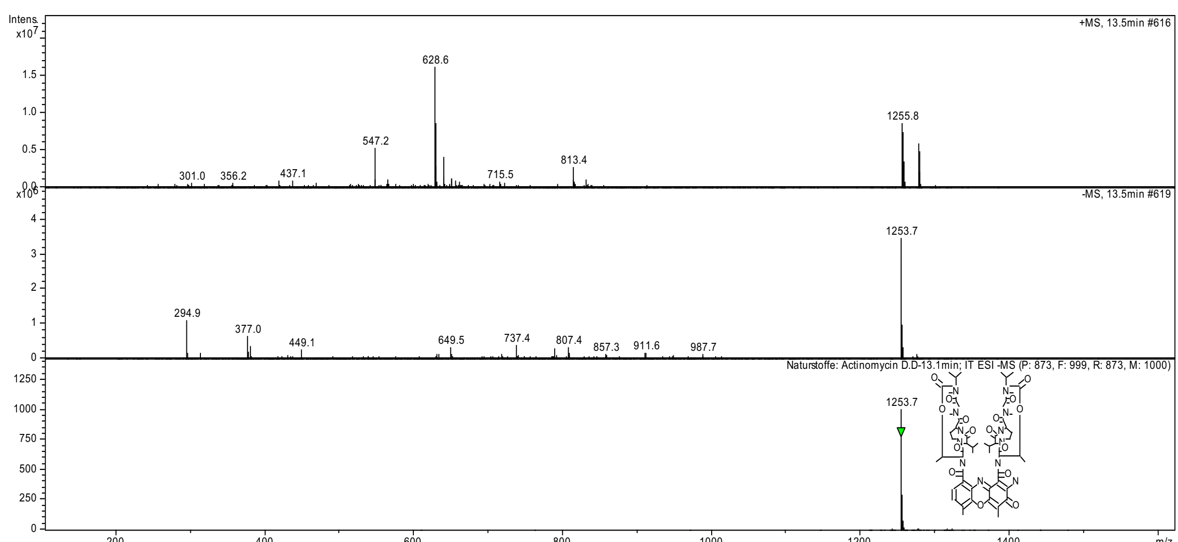


Figure 3. Actinomycin D production in DHE 6-7 (a) and DHE 5-1 (b) at a retention time of 12 min. Peaks in HPLC spectrum representing actinomycin are marked with red color. Mass spectra of actinomycin D as *m/z* 1255.8 $[M+H]^+$ and *m/z* 1253.7 $[M+H]^-$ detected in DHE 6-7 (c) [134].

3.1.5. Taxonomic classification of nine prioritized Indonesian actinomycetes strains based on phylogenomic analysis

The nine prioritized strains, namely SHP 22-7, BSE 7-9, BSE 7F DHE 17-7, DHE 7-1, I3, I4, I5, and I6, were investigated for their taxonomic classification by phylogenomic analysis based on their complete genome sequence to understand their phylogenetic relationships better. For this purpose, the genomic DNA from each of the nine strains was isolated using the genomic tip 100/G kit from Qiagen (catalog number 10243) following the standard protocol provided by the manufacturer. Then, a 10-20 kb paired-end library was constructed for genome sequencing. The genome sequencing was performed with the Pacific Biosciences RS II (PacBioRSII) technology (Macrogen, South Korea) [135–137].

The genome sequences were analyzed with the Type (Strain) Genome Server (TYGS) (https://tygs.dsmz.de) to investigate the phylogenetic relationship of the strains [138]. The TYGS tool allows phylogenomic analysis based on the genome sequences of strains to identify the closest related strains based on digital DNA-DNA hybridization (dDDH). The dDDH values are calculated by the Genome-to-Genome Distance Calculator (GGDC) 2.1 (http://ggdc.dsmz.de), which is implemented in the TYGS tool

[139]. TYGS phylogenomics analysis revealed that all nine isolates belonged to the genus *Streptomyces*. In addition, the TYGS data showed that the dDDH values between nine Indonesian strains and their closest relatives ranging from 31.4% (*Streptomyces* sp. I4) and 51.5% (*Streptomyces* sp. I6) (using the GGDC distance formula d_4) (**Table 2**), which is below the threshold value of 70% used for species delineation [140,141]. Thus, it proposed that the nine isolates were a new collection of *Streptomyces* species.

A phylogenomic tree of the nine Indonesian strains and their closest related strains was generated. The phylogenomic tree shows that the strain SHP 22-7 and DHE 17-7, which were both isolated from soil samples obtained from different locations of Enggano Island, belong to the same clade (clade A) (**Figure 4**) and most likely represent similar species with a dDDH value of 86.7% (**Table 2**). Both strains are closely related to *Streptomyces luteus* TRM 45540, a strain which has been isolated from a soil samples from China [142]. All strains isolated from mangroves, either from Lombok Island sediments (*Streptomyces* sp. I3, I4, and I5) or Bali Island sediments (BSE 7F, and BSE 7-9), belonged to clade B, indicating a close relationship between the five isolates (**Figure 4**). In addition, dDDH analysis showed that BSE 7F is closely related to BSE 7-9 with a value of 95.7% and thus most likely represents the same subspecies (**Table 2**), while *Streptomyces* sp. I3 and I4 probably represent the same species having a dDDH score of nearly 100% (Table 2). The five mangrove strains were closely related to the type strain *S. capillispiralis* DSM 41695 isolated from a Swedish soil sample [143].

The phylogenomic analysis also revealed that two isolates, namely DHE 7-1 isolated from a soil sample from Enggano Island and *Streptomyces* sp. I6, isolated from a mangrove sample from Lombok Island, belonged to a different clade (clade C and D, respectively) (**Figure 4**). The closest related type strain of DHE 7-1, with a dDDH value of 32.3% was *Streptomyces bungoensis* DSM 41781, a strain which was isolated from a soil sample from Japan [144], whereas the closest neighbor of *Streptomyces* sp. I6 with a dDDH value of 51.5% (**Table 2**) is *Streptomyces spongiicola* HNM0071, which was isolated from a marine sponge sample collected from China [145]. In summary, both 16S rRNA gene-based phylogenetic and phylogenomic studies revealed that the nine priority isolates belonged to the genus *Streptomyces* and, based on dDDH analysis, were identified as new species.

Table 2. Data from pairwise comparisons between genome sequences from the nine Indonesian strains and their closest related strains based on dDDH analysis [134]. “Query strain” refers to analyzed strain, and “subject strain” refers to most closely related Indonesian strain sample. Degree of relatedness is given as dDDH distance formula d4 as previously described by Meyer-Kolthoff et al. [66].

Query Strain	Subject Strain	dDDH (d_4, in %)
I3	I4	99.6
BSE 7-9	BSE 7F	95.7
DHE 17-7	SHP 22-7	86.7
I4	I5	82.6
I3	I5	82.5
BSE 7F	I5	78.4
BSE 7-9	I5	78.4
BSE 7-9	I4	77.2
BSE 7F	I4	77.2
BSE 7F	I3	77
BSE 7-9	I3	77
I6	*Streptomyces spongiicola* HNM0071	51.5
SHP 22-7	*Streptomyces luteus* TRM 45540	43.6
DHE 17-7	*Streptomyces luteus* TRM 45540	40.3
DHE 7-1	*Streptomyces bungoensis* DSM 41781	32.3
I3	*Streptomyces capillispiralis* DSM 41695	31.5
BSE 7-9	*Streptomyces capillispiralis* DSM 41695	31.5
I5	*Streptomyces capillispiralis* DSM 41695	31.5
I4	*Streptomyces capillispiralis* DSM 41695	31.4
BSE 7F	*Streptomyces capillispiralis* DSM 41695	31.4

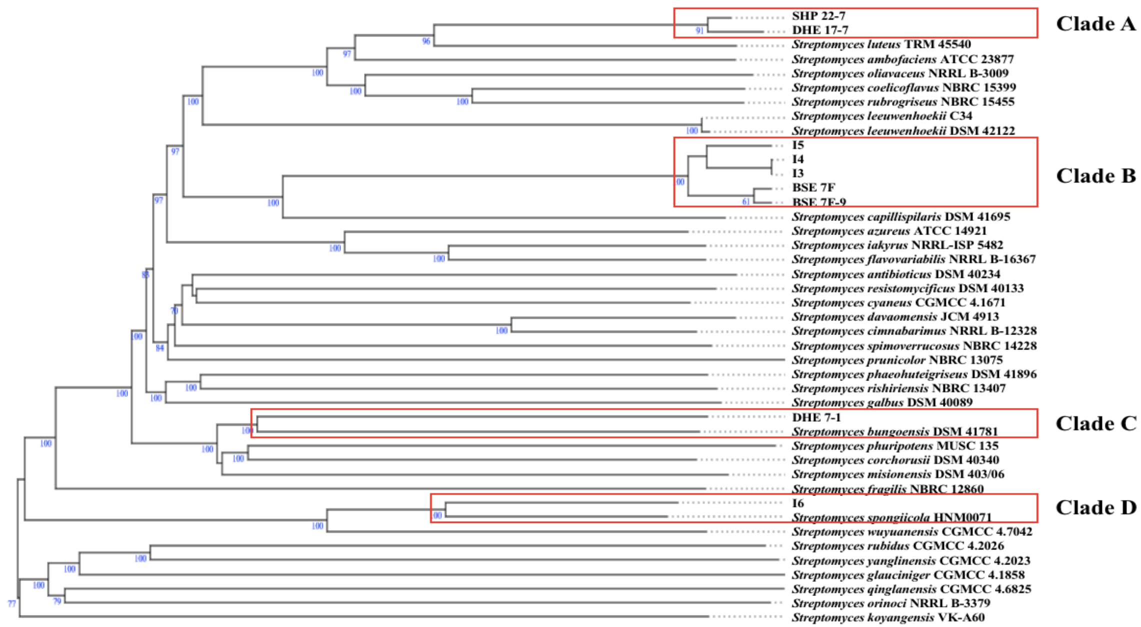


Figure 4. Whole-genome sequence-based phylogenetic tree generated with the TYGS web server for nine Indonesian *Streptomyces* isolates (highlighted by red boxes) and closely related type strains. Tree inferred with FastME from GBDP distances was determined from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d5. The numbers above branches indicate GBDP pseudo-bootstrap support values >60% from 100 replications, with an average branch support of 84.4%. The tree was rooted at the mid-point.

3.1.6. Genetic potential of nine Indonesian actinomycetes for secondary metabolite biosynthesis

The genome sequences of the nine Indonesian actinomycetes were analyzed using the antiSMASH version 5.0 platform (https://antismash. secondarymetabolites.org) to determine the genetic potential of these strains to produce secondary metabolites. The antiSMASH results showed that several BGCs identified from nine Indonesian isolates showed high similarity (>60%) with known BGCs (**Table 3**). For example, all strains contained BGCs encoding compounds commonly produced by streptomycetes, such as desferrioxamine, hopene, and spore pigments. These results align with previous observations that reported that these BGCs were present in most of the analyzed *Streptomyces* genomes [53]. Ectoine and geosmin gene clusters were uncovered in all Indonesian isolates except for *Streptomyces* sp. I6 (**Table 3**). An albaflavenone BGC was found in all nine Indonesia actinomycetes strains, excluding *Streptomyces* sp. I6 and DHE 7-1. Interestingly, all strains isolated from mangroves, namely BSE 7F, BSE 7-9, I3, I4, and I5, had two ectoine BGC. The additional ectoine BGC probably contributes to this organism in its adaptation to high salinity environments in mangrove ecosystems to prevent osmotic stress.

Aborycin and alkylresorcinol BGCs were exclusively found in the five *Streptomyces* mangrove strains. Meanwhile, amicetin, candicidin, coelichelin, fluostatin M-Q BGCs were only observed in the SHP 22-7 and DHE 17-7, which have been isolated from soil. Candicidin, as an example of a fungicide [146], is most likely produced by terrestrial streptomycetes as a defense mechanism of these bacteria against local fungal competitors. Coelichelin is a peptide siderophore that may be required for the soil-living streptomycetes to absorb poorly soluble environmental Fe^{3+} [147], which is rare and highly contested by other microorganisms in the soil. The finding of the same BGC composition in strains originating from the same habitat, such as soil or mangrove forests, probably due to the fact that each biosynthetic product has a specific biochemical relationship in its respective environment. *Streptomyces* sp. I6 was the only strain from nine examined strains contained BGCs encoding for staurosporine, scabichelin, echinomycin, flaviolin, and tirandamycin. Likewise, I6 and DHE 7-1 were the only strains containing isorenieratene BGC among the nine strains (**Table 3**). The two strains, I6 and DHE 7-1, were observed phylogenetically apart from the other strains (**Figure 4**). From the phylogenomic and gene cluster analysis, it can be

concluded that phylogenetically related isolates tend to have similar biosynthetic units known as BGCs formed by environmental conditions. In addition, phylogenetically unique organisms harbor more unique clusters and thus have the potential to encode for novel substances. This finding is in line with the observation from Meij et al., who described that environmental conditions have an essential role in managing the formation of secondary metabolites in actinomycetes [148].

The genome sequences of the nine Indonesian strains were additionally analyzed using the BiG-SCAPE software (https://bigscape-corason.secondarymetabolites.org/) to obtain a more detailed picture of the distribution of BGC among the strains. BiG-SCAPE enables rapid computation and visual exploration of BGC similarities by grouping BGCs into gene cluster families (GCFs) based on their sequence and Pfam protein family similarity [149]. This analysis revealed eight unique GCFs that cannot be associated with any known BGCs and may potentially encode new substances. One of the eight unique BGCs is the ectoine-butyrolactone-NRPS-T1PKS GCF with less than 60% similarity to known BGCs in the antiSMASH database. The list of predicted BGCs of nine Indonesian strains derived from the antiSMASH analysis is available at the supplementary materials section of Handayani et al [134]. The ectoine-butyrolactone-NRPS-T1PKS BGC, which showed some similarity to the polyoxypeptin (48%) and aurantimycin A (51%) BGCs, was distributed among the strains I3, I4, I5, and BSE 7F isolated from mangroves (**Figure 5**). In addition, the polyketide type III (T3PKS) GCF, which showed low BGC similarity (7–8%) to the herboxidiene BGC (**Figure 5**), was distributed among the strains DHE 17-7, SHP 22-7, and DHE 7-1, isolated from soil. These data indicate that phylogenetically related strains originating from the same environmental habitat tend to have a similar BGC composition profile. Furthermore, it confirms that phylogenetically unique organisms have more unique BGCs that could potentially code for new substances. From these observations, it can be concluded that sampling from unique environments is a necessary undertaking, as this can lead to the discovery of phylogenetically unique species, which have a higher potential to generate new natural compounds, as previously stated by Hug et al. [20].

Table 3. Presence (grey color) and absence (white color) of BGCs in nine Indonesian strains as predicted by antiSMASH analysis with similarity above 60%.

Clade	Isolates	desferrioxamin B	spore pigment	hopene	ectoine	geosmin	albaflavenone	amicetin	candicidin	coelichelin	fluostatins M-Q	sapB	streptothricin	levorin	aborycin	alkylresorcinol	naphthyridinomycin	venezuelin	resistomycin	rhizomide	sstaurosporine	scabichelin	echinomycin	flaviolin	isorenieratene	tirandamycin
A	SHP22-7	grey	grey	grey	grey	grey	grey	grey	grey	grey	grey	white	white	white	white	white	white	white	white	white	white	white	white	white	white	white
	DHE17-7	grey	grey	grey	grey	grey	grey	grey	grey	grey	grey	grey	grey	grey	white	white	white	white	white	white	white	white	white	white	white	white
B	BSE7F	grey	grey	grey	grey	grey	grey	white	white	white	white	white	white	white	grey	grey	grey	grey	grey	white	white	white	white	white	white	white
	BSE7-9	grey	grey	grey	grey	grey	grey	white	white	white	white	white	white	white	grey	grey	grey	grey	grey	white	white	white	white	white	white	white
	I5	grey	grey	grey	grey	grey	grey	white	white	white	white	white	white	white	grey	grey	white	grey	grey	white	white	white	white	white	white	white
	I4	grey	grey	grey	grey	grey	grey	white	white	white	white	white	white	white	grey	grey	grey	grey	white	white	white	white	white	white	white	white
	I3	grey	grey	grey	grey	grey	grey	white	white	white	white	white	white	white	grey	grey	grey	white	white	grey	white	white	white	white	white	white
C	DHE7-1	grey	grey	grey	grey	grey	white	white	white	white	white	white	white	white	white	white	white	white	white	white	white	white	white	white	grey	white
D	I6	grey	grey	grey	white	white	white	white	white	white	white	grey	white	white	white	white	white	white	white	white	grey	grey	grey	grey	grey	grey

(Column group header: known compound predicted by antiSMASH)

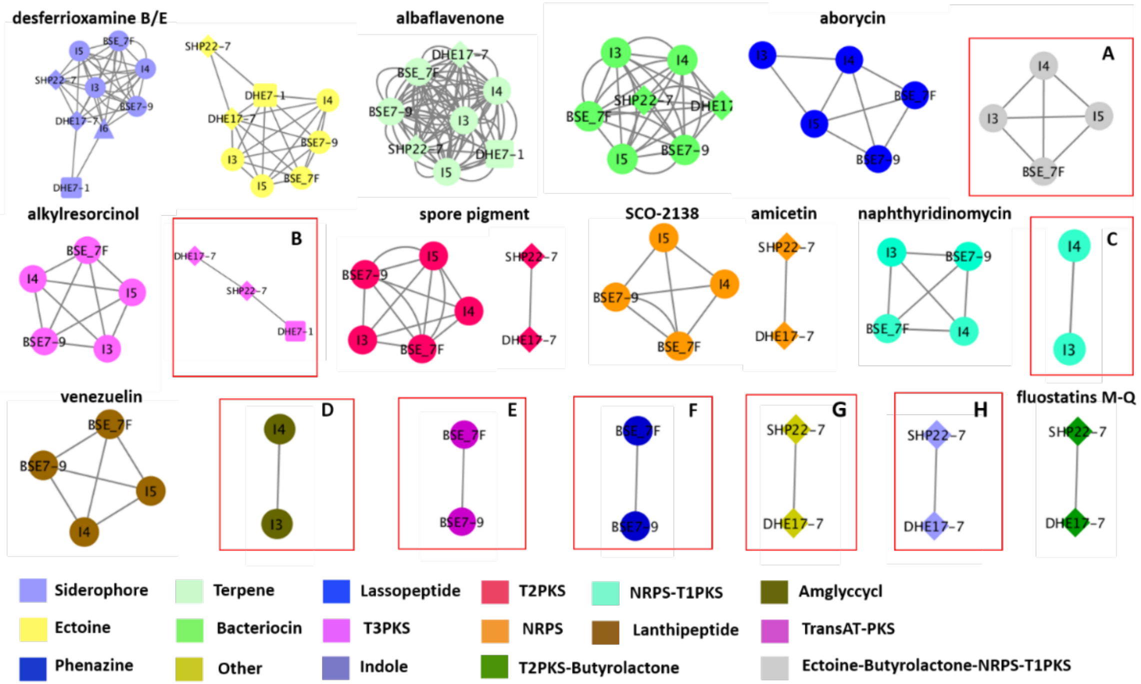


Figure 5. Similarity network of the predicted biosynthetic gene clusters (BGCs) of the nine Indonesian *Streptomyces* strains. Shared similar BGCs are indicated by a connected lines. Each node represents a specific BGC type (labeled with different colors). The shape node represents the same species, i.e., clade A (SHP 22-7 and DHE 17-7) indicated with diamond, clade B (I4, I5, BSE 7F, and BSE 7-9) shown with ellipse, clade C (DHE 7-1) with a cube, and clade D (I6) indicated with a triangle. Eight unique GCFs with similarities less than 60% are highlighted by red boxes: (A) Ectoine-butyrolactone-NRPS-T1PKS; (B) T3PKS; (C) Otherks-T1PKS, (D) Amglyccyc; (E) TransAT-PKS; (F) Phenazine; (G) Other; and (H) Indole [134].

3.2. Exploration of the metabolic capacity of nine Indonesian actinomycetes

3.2.1. Exploitation of the biosynthetic potential by applying the OSMAC strategy

In order to draw conclusions regarding the biosynthetic capacity of the nine isolates prioritized in the context of bioactivity, various growth media following the OSMAC strategy were screened to determine optimal production conditions. The determination of optimal production conditions was carried out by cultivating each of the nine strains, namely SHP 22-7, DHE 17-7, DHE 7-1, BSE 7-9, BSE 7F, I3, I4, I5, and I6 in twelve different liquid cultivation media (SGG, YM, OM, R5, MS, TSG, NL19, NL300, NL330, NL500, NL550, and NL800). The media compositions can be found in the Table S2 of Handayani et al. [134]. Cell culture samples were harvested at time points of 48, 72, 96, and 168 h. The cell cultures were each extracted with ethyl acetate, concentrated in a vacuum, and then redissolved in methanol. The methanolic extracts were tested in bioassays against a selected panel of test strains *B. subtilis*, *S. carnosus*, *M. luteus*, *E. coli*, and *P. fluorescens*. Samples, which yielded the largest inhibition zone in the bioactivity test were designated as the samples cultivated under optimal growth conditions. (**Table 4**).

In addition, it is hypothesized that actinomycetes as soil organisms grow and develop better on solid nutrient substrates and that healthy, well-grown cultures produce more diverse compounds [150]. Thus, to fully explore the biosynthetic potential of the actinomycetes of the nine strains for the production of novel secondary metabolites, we also applied the extraction of antibiotics from solid media. For this purpose, the nine isolates were spread individually on agar plates containing the respective growth media as mentioned above. The agar plates were then incubated for 7-10 days at 28°C until spores formed. The agar with the grown cultures was squeezed and concentrated. The aqueous phase from the solid medium extract was utilized for bioactivity tests and further chemical analysis.

In this exploratory study of biosynthetic potential, we observed that *Streptomyces* sp. I3 and I4 have the same optimal cultivation parameters. Both strains showed promising potential when grown in liquid NL550 medium for up to 72 hours and mannitol soya (MS) agar medium cultivated for 168 hours. This similar production behavior is

explained by the observation that *Streptomyces* sp. I3 and I4 most likely represent the same species as suggested above. Furthermore, MS medium was observed as the optimal production medium for most of the nine Indonesian strain isolates (*Streptomyces* sp. I3, I4, I5, and I6) (**Table 4**). MS is known as the most suitable medium for streptomycetes in terms of spore isolation [13]. Thus, it is supports the hypothesis that strains can produce better when they show healthy growth and development.

Table 4. List of optimal culture conditions (media, time point) and bioactivity profile of nine Indonesian strain isolates. The plus symbol indicates the tested strain has antimicrobial activity against Gram-positive bacteria. While the minus character suggests, the tested strain has antimicrobial activity against Gram-negative bacteria [134].

Strain	Optimal production in liquid medium	Optimal production in solid medium	Optimal incubation time (h) in liquid	Antimicrobial activity against Gram (+)/(-) test bacteria
I6	R5	MS	48	(+), (-)
DHE 7-1	OM	OM	48	(+)
DHE 17-7	SGG	SGG	48	(+)
I3	NL550	MS	72	(+), (-)
I4	NL550	MS	72	(+), (-)
I5	MS	MS	96	(+), (-)
SHP 22-7	NL300	NL300	96	(+), (-)
BSE 7-9	NL19	NL19	96	(+), (-)
BSE 7F	NL500	NL500	168	(+), (-)

3.2.2. Identification of natural compounds and their coding gene clusters in nine Indonesian *Streptomyces* strains

With the help of Harald Groß´s group, our collaboration partner in the Department of Pharmaceutical Biology (University of Tuebingen), a combination of genomic and metabolic mining approaches was applied as a dereplication strategy. This strategy was conducted to identify the bioactive substances of the nine Indonesian isolates, which were grown under the various conditions mentioned earlier, and to associate the

biosynthetic pathways to the compounds. While genome mining performed to identify the BGCs responsible for compound production, metabolomic mining was accomplished to determine the bioactive compounds generated by the nine isolates under various conditions.

This strategy led to the detection of several known compounds and their encoding BGCs, such as desferrioxamine B with m/z 656.2830 $[M - 2H + Fe]^+$ in *Streptomyces* sp. I3, I4, I6, and SHP 22-7. Naphthyridinomycin with *m/z* 418.1980 $[M+H]^+$ was disclosed in *Streptomyces* sp. I3, I4, I5, BSE 7F, and BSE 7-9 isolated from mangroves. Next, amicetin with *m/z* 619.3100 $[M+H]^+$ was found in SHP 22-7 and DHE 17-7 derived from Enggano soil. In addition, antimycin with *m/z* 535.2659 $[M+H]^+$ was discovered in BSE 7F. Furthermore, echinoserine with *m/z* 1137.4504 $[M+H]^+$, echinomycin with *m/z* 1101.4279 $[M+H]^+$, tirandamycin with *m/z* 337.1650 $[M+H]^+$ and staurosporine with *m/z* 467.2070 $[M+H]^+$ were discovered in *Streptomyces* sp. I6 (**Table 5**).

We observed that naphthyridinomycin compounds were only detected in extract samples from strains originating from mangroves, such as BSE 7F, BSE 7-9, I3, I4, and I5 (**Table 5**). Meanwhile, amicetin was only found as a biosynthetic product from isolates SHP 22-7 and DHE 17-7 taken from soil samples of Enggano Island (**Table 5**). Furthermore, we also found that *Streptomyces* sp. I6 produces echinomycin (**Table 5**), a substance also reported as a biosynthetic product of the closely related strain *Streptomyces spongiicola* HNM0071, which was originally isolated from a marine sponge [145]. The observation that naphthyridinomycin, amicetin, and echinomycin, are biosynthetic products of closely related strains obtained from similar habitats underlines our assumption that phylogenetically related strains are likely to produce similar compounds as a result of the action of these strains on changing environmental conditions of their natural habitats. In particular, isolates of *Streptomyces* sp. I3 and I4 originating from the same habitat most likely represent the same species, as indicated by the nearly 100% dDDH value, the high similarity of BGC composition, and similar chemical compounds production profiles of the two strains (see above). In this context, it is worth to mention that current drug research often addresses the dereplication of known compounds during drug screening strategies [82,151,152]. However, what is also noteworthy is the fact that the problem of dereplication of producer strains is also an important issue, as shown in the current study. Therefore, it is necessary to make

efforts in making phylogenetic profiles at the beginning of the strategy to sort out already known producer strains.

Furthermore, we found that DHE 17-7 produces ECO-0501, a type I PKS product, which so far has so only been reported as a product of *Amycolatopsis orientalis* ATCC 43491 (**Table 5**) [153]. However, we were unable to identify the BGC encoding the production of ECO-0501 in the genome of strain DHE 17-7 by antiSMASH analysis. By searching manually, we found a potential candidate gene cluster (cluster region 24), which might code for this substance. Cluster region 24 codes for a predictive type I PKS BGC showing some similarities (<55%) with BGCs encoding structurally related macrolactam compounds, such as vicenistatin, sceliphrolactam, and streptovaricin (**Figure 6**). Besides, we also observed that the compound MY336-a was detected as product from BSE 7-9, but the BGC encoding the biosynthesis of this compound could not be clearly identified. MY336-a was previously identified as a product from *Streptomyces gabonae*. The BGC encoding the biosynthesis of MY336-a is not described [154]. Since this compound is relatively small with m/z 253.13182 $[M + H]^+$, and the origin of its biosynthesis is unclear, it is rather unreliable to assign specific gene sequences as a potential coding region for this substance.

Interestingly, we disclosed that the ferrioxamine molecular family was only detectable for the strains grown on solid media (**Tables 5** and **6**). In addition to the identified known compounds described above, samples grown on solid media uniquely generated novel molecular family, such as a group of putative new peptides with m/z 598.2834 $[M + 2H]^{2+}$, 662.8048 $[M + 2H]^{2+}$, and 727.3259 $[M + 2H]^{2+}$, which cannot be attributed to any known compounds. The compounds were detected in strains I3, I5, and BSE 7F (**Figure 7**, **Table 6**), indicating that the cultivation conditions had a substantial effect on the chemical profile. The potential BGC associated with the biosynthesis of this novel peptide is likely a bacteriocin gene cluster showing 42-57% similarity to the BGC informatipeptin BGC (**Tables S6**, **S8**, and **S9** of Handayani et al [134]), which could be identified in the genomes of the three strains. Alternatively, the three strains also shared the NRPS/ectoine/butyrolactone/other/T1PKS gene cluster (**Tables S6**, **S8**, and **S9** of Handayani et al [134]), which is also plausible if a peptide group might be encoded from this region. A similar cluster of NRPS/ectoine/butyrolactone/other/T1PKS was also observed in regions 21 and 22 for the phylogenetically related strains of BSE 7-9, but no respective compound was

identified (**Table S10** of Handayani et al [134]). A further example of the impact of cultivation methods on chemical profiles is represented by a group of additional unknown features of isolate SHP 22-7, designated as a putative new compound group I, which were exclusively produced by strains grown on solid media. The compound of group I consists of four putative novel compounds with *m/z* 821.3349 $[M + H]^+$, *m/z* 734.3031 $[M + H]^+$, *m/z* 679.2430 $[M + H]^+$, and *m/z* 647.2710 $[M + H]^+$ (**Table 6**, **Figure 8**). The genome of the SHP 22-7 strain consists of 13 BGCs (cluster region 3, 4, 5, 7, 8, 11, 12, 14, 15, 20, 21, 22, 23, and 25) with less than 50% similarity to already known BGCs. The potential BGCs linked to the biosynthesis of group I compounds probably derive from one of the thirteen unique BGCs of SHP 22-7 mentioned above.

In the same context, the DHE 17-7 strain also provided several putative new compounds (group II compounds) with masses ranging from 435–474 Da, which were only detected when the strains were grown in liquid media (**Table 6**, **Figure 9**). Five BGCs (region 10, 16, 17, 22, and 28) are present in the genome of strain DHE 17-7, which showed no similarity to any known BGCs in the antiSMASH database and nine BGCs (regions 3, 4, 6, 11, 13, 19, 24, 25, and 26) have less than 50% similarity. Thus, the unknown metabolites are most likely encoded by any of the unique BGCs of DHE 17-7 mentioned above (**Table S4** of Handayani et al [134]).

Next, we investigated the relationship between growth conditions and compound production. Chiani et al. showed that complex nitrogen sources such as soybean meal and corn steep liquor could promote ferrioxamine production in streptomycetes [155,156]. Interestingly, in this study, we found that ferrioxamine B/D and its analogs were detected mainly for strains grown on non-liquid media (**Table 5** and **Table 6**), such as soy flour-containing MS agar, which was observed for *Streptomyces* sp. I3, I4, and I6. In addition, ferrioxamine B/D was also discovered in SHP 22-7 grown on NL300 solid agar containing cottonseed powder. Furthermore, ferrioxamine analog was identified in DHE 17-7 cultivated on SGG consisting of corn steep powder (**Table S2** of Handayani et al. [134]). The appearance of ferrioxamines only in samples grown on solid media is possibly related to the fact that iron (Fe^{3+}) is more evenly distributed in liquid media than solid media. Thus, cells grown on solid media may be exposed to localized iron depletion conditions, leading to ferrioxamine biosynthesis [147].

In addition to ferrioxamine and its analogs, further known but also unknown compounds were only detected in samples of strains cultivated on solid media, namely, the three already known substances echinomycin, staurosporine, and tirandamycin for *Streptomyces* sp. I6; three putative new peptidic compounds for *Streptomyces* sp. I3, I5, and BSE 7F, and a putative new compound I for *Streptomyces* sp. SHP 22-7 (**Tables 5** and **6**). In addition, we also found several unknown and known compounds in samples of strains grown exclusively in liquid media, such as amicetin (SHP 22-7 and DHE 17-7), antimycin and its analogs (BSE 7F), ECO-0501 (DHE 17 -7), and a putative novel compound group II for *Streptomyces* sp. DHE 17-7 (**Tables 5** and **6**). These results show that the cultivation conditions significantly affect the formation of substances. Therefore, both liquid and solid cultivation approaches should be employed to fully explore the biosynthetic potential of actinomycetes and thereby increase the opportunities of discovering new compounds.

In conclusion, the integration of GNPS and gene cluster network strategies revealed several potential new compounds from *Streptomyces* sp. I3, I4, I5, I6, BSE 7F, BSE 7-9, and DHE 17-7. Furthermore, some of these compounds can be associated with possible coding BGCs, and some of them are predicted to be encoded by unique BGCs. Therefore, new Indonesian isolates are a valuable source for further drug research and development approaches. In addition, we conclude that the combination of phylogenomic, GNPS, and gene cluster networking analysis is an efficient strategy for prioritizing phylogenetically unique producer strains, which have the biosynthetic capacity to produce novel compounds.

Table 5. Correlation between known compounds and BGC distribution in the nine Indonesian strain isolates. A checkmark (√) indicates identified BGC in the strain studied, a question mark (?) indicates that BGC is not identified in the strain studied, and a minus sign (-) indicates the compound is not present in the medium [134].

Ion cluster name (Ion Formula)	*m/z* measured	Adduct	Main producer and media type		BGC identified
			Solid	Liquid	
Ferrioxamine D1 ($C_{27}H_{48}N_6O_9$)	656.2830	[M − 2H + Fe]$^+$	SHP 22-7; I3; I4; I6	-	√
Naphthyridinomycin A ($C_{21}H_{28}N_3O_6$)	418.1980	[M + H]$^+$	I3; I4; I5	BSE 7F; BSE 7-9; I5	√
Amicetin ($C_{29}H_{43}N_6O_9$)	619.3100	[M + H]$^+$	-	SHP 22-7; DHE 17-7	√
Antimycin A2 ($C_{27}H_{39}N_2O_9$)	535.2659	[M + H]$^+$	-	BSE 7F	√
Echinoserine ($C_{51}H_{69}N_{12}O_{14}S_2$)	1137.4504	[M + H]$^+$	I6	-	√
Echinomycin	1101.4279	[M + H]$^+$	I6	-	√

($C_{51}H_{65}N_{12}O_{12}S_2$)					
Tirandamycin A ($C_{18}H_{25}O_6$)	337.1650	$[M + H]^+$	I6	-	√
Staurosporine ($C_{28}H_{27}N_4O_3$)	467.2070	$[M + H]^+$	I6		√
ECO-0501 ($C_{46}H_{69}N_4O_{10}$)	837.5022	$[M + H]^+$	-	DHE 17-7	?
MY336-a ($C_{13}H_{19}NO_4$)	253.13182	$[M+H]^+$	-	BSE 7-9	?

Table 6. Overview of analogs and putative new compounds identified for the nine Indonesian *Streptomyces* strains. A minus sign (-) indicates that the compound is not present in the medium [134].

Ion clusterdescription	*m/z* measured	Adduct	Main producer and media type	
			Solid	Liquid
Ferrioxamine analogs	627.3303	$[M - 2H + Fe]^+$	I3; I4; I6; DHE 17-7	-
	788.3753	$[M - 2H + Fe]^+$	BSE 7-9	-
	840.4060	$[M - 2H + Fe]^+$	-	BSE 7-9
	640.2520	$[M - 2H + Fe]^+$	-	DHE 17-7
	654.2685	$[M - 2H + Fe]^+$	DHE 17-7	-
Putative new peptides	598.2834	$[M + 2H]^{2+}$	I3, I5, BSE 7F	-
	662.8048	$[M + 2H]^{2+}$	I3, I5, BSE 7F	-
	727.3259	$[M + 2H]^{2+}$	I3, I5, BSE 7F	-
Putative new compound group I	821.3349	$[M + H]^+$	SHP 22-7	-
	734.3031	$[M + H]^+$	SHP 22-7	-
	679.2430	$[M + H]^+$	SHP 22-7	-
	647.2710	$[M + H]^+$	SHP 22-7	-
Putative new compound group II	435.2774	$[M + 2H]^{2+}$	-	DHE 17-7
	442.2857	$[M + 2H]^{2+}$	-	DHE 17-7
	449.2934	$[M + 2H]^{2+}$	-	DHE 17-7
	474.2833	$[M + 2H]^{2+}$	-	DHE 17-7

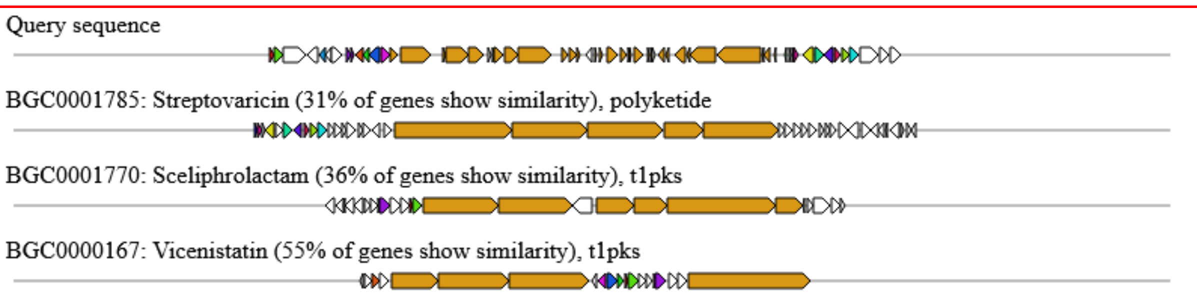


Figure 6. Cluster similarity between the DHE 17-7 gene region 24 (query sequence) and the streptovaricin, sceliphrolactam and vicenistatin cluster [134].

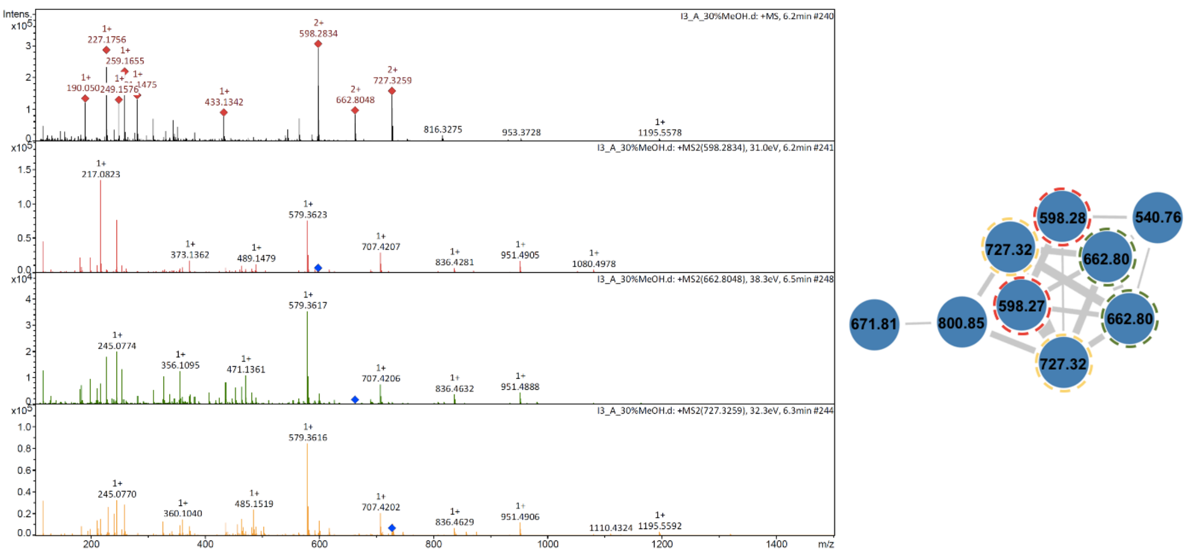


Figure 7. Comparative positive MS^2 of some unknown compounds of likely novel peptides with m/z 598.2834 $[M + 2H]^{2+}$, 662.8048 $[M + 2H]^{2+}$, and 727.3259 $[M + 2H]^{2+}$ identified by Hamada Saad from pharmacy group [134].

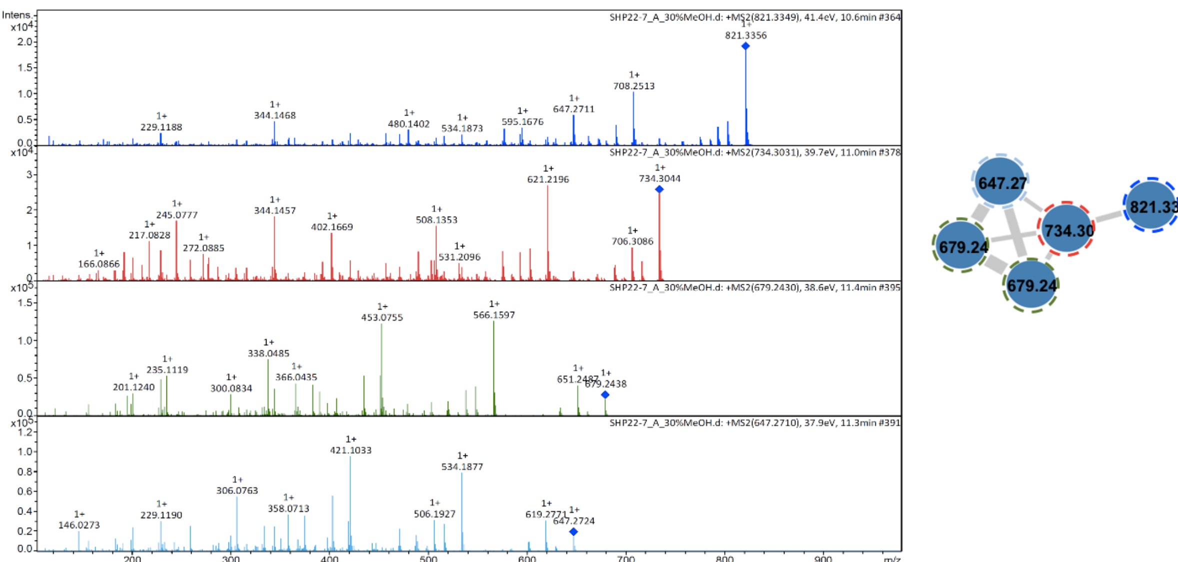
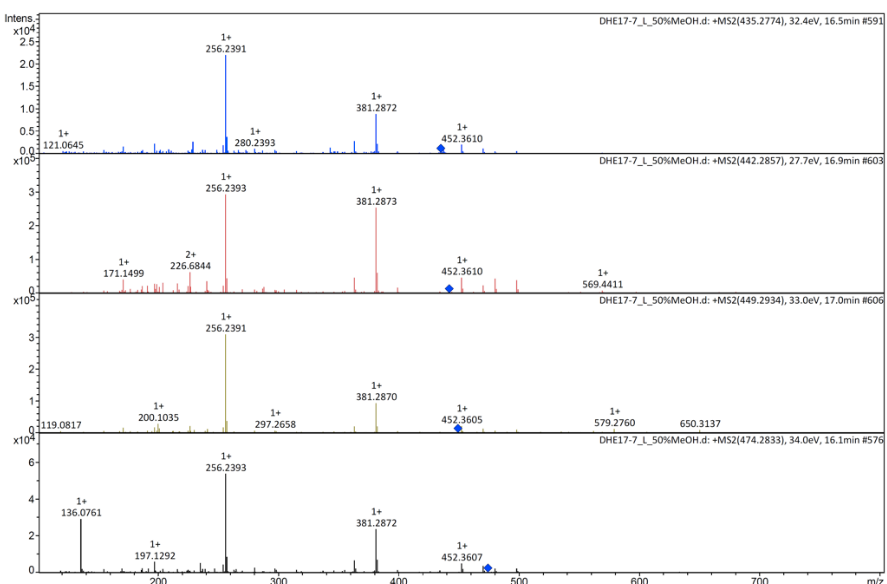


Figure 8. Comparative positive MS^2 of some unknown compounds with m/z 821.3349 $[M + H]^+$, m/z 734.3031 $[M + H]^+$, m/z 679.2430 $[M + H]^+$, and m/z 647.2710 $[M + H]^+$ from isolate SHP 22-7 observed by Hamada Saad of group pharmacy [134].



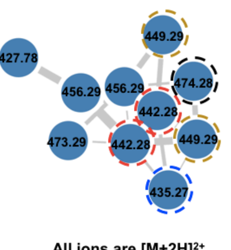


Figure 9. Comparative positive MS^2 of some unknown compounds with masses ranging from 435–474 Da from isolate DHE 17-7 detected by Hamada Saad of group pharmacy [134].

3.2.3. Activation of silent gene clusters through overexpression of the SARP-type regulator PapR2

Activating silent gene cluster expression to find new antibiotics following the OSMAC approach is a thoroughly successful but entirely non-specific activation effort. This accounts for the weakness of the OSMAC approach, whereby significant analytical effort is required to identify products from silent gene clusters. Thus, it would be highly advantageous to have a more general activation strategy that targets a defined set of BGCs, which will address this issue. In this regard, we applied the SARP-guided method as a proof of concept that SARPs can be used as an activator of specific antibiotic gene clusters in actinomycetes since they are exclusively found in actinomycetes, especially in streptomycetes and known to act as activators of particular pathways of secondary metabolite biosynthesis.

In order to demonstrate that the (SARP)-type regulator can be used as an activator, the SARP-type regulator PapR2 from *S. pristinaespiralis* were heterologous expressed in the novel Indonesian strain of *Streptomyces* sp. SHP 22-7 to study the effect of *papR2* gene expression on transcriptional activation of biosynthetic pathways and production of secondary metabolites in SHP 22-7. Using the bioinformatics tool PatScan, Janina Krause found multiple *papR2*-like consensus binding sequences in five of the 25 BGC predictions from SHP 22-7, i.e., clusters 3, 5, 9, 10, and 15 (Table

1 from Krause et al. [94]). Therefore, SHP 22-7 was selected as a candidate strain for the SARP-type regulator PapR2 activation.

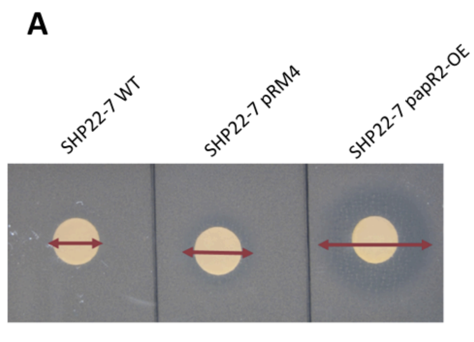
For *papR2* expression studies in SHP 22-7, the blank vector pRM4 and the *papR2* overexpression construct pRM4/papR2, which harbors the *papR2* gene under the control of the constitutive *ermE** promoter, were each transferred into SHP 22-7, yielding the strains SHP 22-7/pRM4 and SHP 22-7/papR2-OE, respectively. Both strains, as well as the wild-type SHP 22-7 (WT) were used in antibacterial bioassays to determine the effect of *papR2* overexpression on antibiotic production. For this purpose, SHP 22-7/papR2-OE, SHP 22-7/pRM4, and the SHP 22-7 WT were grown in NL19 medium. The cell culture samples were taken at 168 hours and extracted with ethyl acetate. Then, the ethyl acetate extracts were concentrated in a vacuum and then redissolved in methanol. The methanolic extracts were applied for antibacterial bioassays using *B. subtilis* as test organism. Filter discs with 5 µl of kanamycin (50 µg/ml) and methanol were used as positive and negative controls, respectively. All bioassays were performed as ten independent biological replicates. In this study, we observed that the inhibition zone of the SHP 22-7/papR2-OE extract samples were significantly larger on average against *B. subtilis* than extracts from SHP 22-7/pRM4 or SHP 22-7/WT (**Figure 10A**). The extracts of SHP 22-7/papR2-OE showed an inhibition zone. 11.7 ± 2.1 mm, whereas extracts of SHP 22-7/pRM4 and SHP 22-7/WT displayed a smaller inhibition zone of 8.3 ± 2.6 and 7.3 ± 2, respectively (**Figure 10B**). These data suggest that *papR2* expression in SHP 22-7 leads to significantly increased antibiotic activity.

In order to find out what substance is responsible for the increase in antibiotic activity in the presence of *papR2* overexpression in SHP 22-7, the methanolic extracts from cell culture samples SHP 22-7/papR2-OE, SHP 22-7/pRM4 , and SHP 22-7/WT were analyzed by HPLC-MS. In the HPLC analysis, amicetin [retention time (RT) 4.0 min], plicacetin (RT 4.8 min), and plicacetin isomers (RT 5.6 min) were found in all three samples (**Figure 11A**). The identity of the compounds was verified by MS/MS analysis with amicetin at m/z 617.1 $[M-H]^+$, plicacetin at m/z 516.1 $[M-H]^+$, plicacetin isomers at m/z 516.1 $[M-H]^+$) (**Figure 11B**). From the comparison between the HPLC spectra of samples SHP 22-7/papR2-OE, SHP 22-7/pRM4, and SHP 22-7/WT, it was found that there was an increase in peak intensity, especially for plicacetin (mAU 605) and plicacetin isomer (mAU 259) in sample SHP 22-7/papR2-OE compared to samples

SHP 22-7/pRM4 [plicacetin (mAU 120), plicacetin isomer (mAU 97)] and SHP 22-7/WT [plicacetin (mAU 66), plicacetin isomer (mAU 57)] (**Figure 11C**). We conclude from the above results that the increased bioactivity of the SHP 22-7/papR2-OE samples against *B. subtilis* arose from the increased production of the nucleoside antibiotic plicacetin, which was the result from the overexpression of *papR2*. The increased production of plicacetin at the SHP 22-7/papR2-OE suggests that the expression of the SARP activator PapR2 can induce or increase antibiotic production in foreign streptomycetes.

To prove that the antibiotic amicetin(-like) BGC was transcribed in the presence of *papR2* gene expression, Janina Krause performed RT-PCR using the RNA isolated from the SHP 22-7/papR2-OE, SHP 22-7/pRM4, and SHP 22-7/WT cell pellets were used as a template in the RT-PCR experiments and served as a negative control (Figure 5A of Krause et.al [94]). cDNA was used in conjunction with the 16S primer as a positive control (Figure 5B of Krause et.al [94]). For cluster-specific transcriptional analysis, cDNA was generated with the primer pairs cl3fw/rv, cl6fw/rv, cl9fw/rv, cl10fw/rv, and cl15fw/rv (Supplementary Table S1 of Krause et.al [94]), paralleling the predicted biosynthetic genes from cluster 3, 6 , 9, 10, and 15 respectively. The transcriptional analysis revealed the presence of a stronger signal for cluster 9 amplification in the SHP 22-7/papR2 -OE sample than the SHP 22-7/pRM4 and SHP 22-7/WT samples, in which there was almost no signal at all (Figure 5C of Krause et.al [94]). Meanwhile, for clusters 3, 6, 10, and 15, there was no difference in signal intensity (Figure 5C of Krause et.al [94]). Thus, these data confirm that PapR2 activates the transcription of the amicetin gene cluster of SHP 22-7 located in region 9.

In summary, the SARP-guided method offers the possibility to selectively activate antibiotic gene clusters with the help of precise knowledge of regulatory mechanisms in actinomycetes. Thus, the activation of BGCs by SARP-type regulators represents a promising strategy to be applied to exploit the biosynthetic potential of actinomycetes.



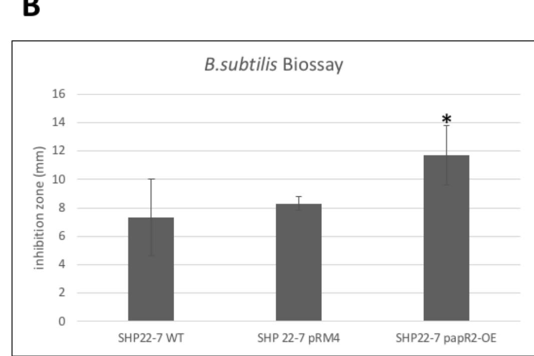


Figure 10. Filter disk diffusion assay with one representative example of culture extracts from SHP 22-7 WT, SHP 22-7/pRM4, and SHP 22-7/papR2-OE, respectively, against *B. subtilis*.; red arrow indicates inhibition zone (**A**). Graphical representation of the inhibition zone diameters (mm) from 10 independent biological replicates (n = 10), ∗ indicates significance (**B**) [94].

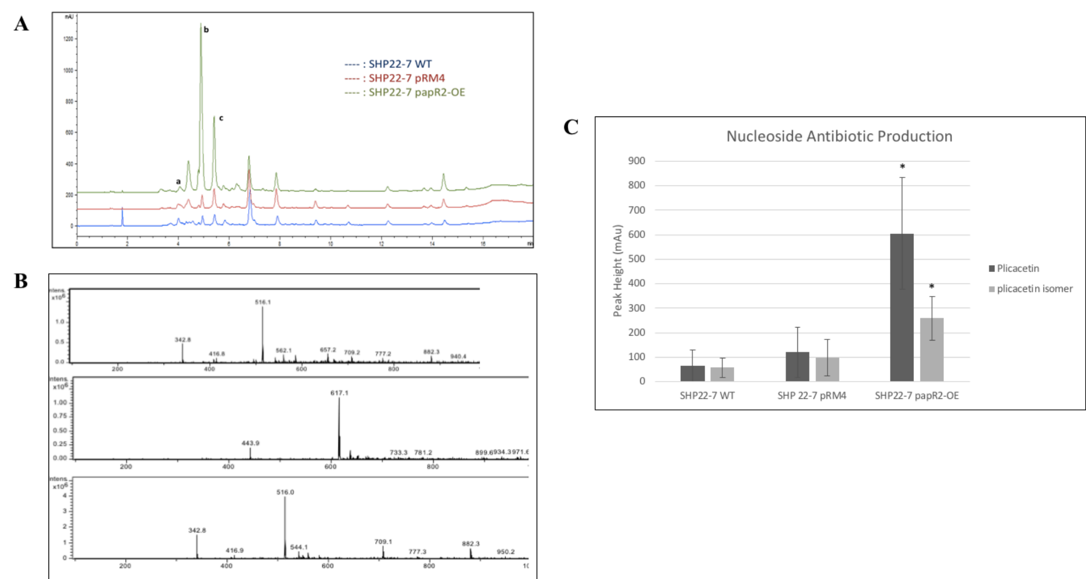


Figure 11. Plicacetin and plicacetin isomer production in the strains SHP 22-7 WT, SHP 22-7/pRM4, and SHP 22-7/papR2-OE, respectively. HPLC profile of all three strains. Peaks in the HPLC spectrum representing amicetin are marked with a, plicacetin marked with b, and plicacetin isomer marked with c (**A**). Mass spectra of plicacetin (upper), amicetin (middle), and a plicacetin isomer (lower) (**B**) isolated from SHP 22-7 WT. Graphical presentation of production levels of plicacetin and plicacetin isomer in each strain. n = 10; ∗ means significance (**C**) [94].

Concluding Remarks

Isolation of novel actinomycetal strains from marine and unexplored habitats has been reported as an effective strategy for acquiring new natural products as applicable drug candidates for combating drug-resistant pathogens [57,58]. Indonesia is one of the most biodiverse countries with the most extensive mangrove marine ecosystem and a wide diversity of microbial species. Indonesian soil samples are likely to be a promising source for new actinomycetal strains. As a proof of concept, soil samples from the Bali and Lombok marine ecosystems and the Enggano habitats in Indonesia were collected and pre-screened with several test organisms to isolate new actinomycetes. About 422 strains of actinomycetes have been isolated from these three unique Indonesia regions and screened for their antimicrobial activity. With the help of the integrated genomics and metabolomics strategy, sixteen compounds that are not associated with any known compounds were identified in in the nine most potent Indonesian antibiotic-producing strains. The phylogeny-related genome mining studies found that the nine strains were proposed as a new collection of *Streptomyces* species. The study also found that phylogenetically related species tend to have similar BGC compositions, and phylogenetically unique species have more unique BGCs that could potentially code for novel substances. These data show that the Indonesian habitat can indeed be considered as a promising source for acquiring novel actinomycetal strains that have a high potential to produce new bioactive natural products.

When cultivating the nine strains in various liquid and solid media following the OSMAC method, it was found that the culture conditions significantly affected the ability of these strains to produce certain compounds. Thus, the combination of the two cultivation methods, solid and liquid cultivation, is a suitable method to optimize the full biosynthetic potential of actinomycetes in terms of producing new compounds. In addition, metabolomic data indicate that the ability of a strain to produce certain compounds can be affected by environmental conditions in which the producing strain originates.

Furthermore, in the study of the SARP-guided method on the activation of silent gene clusters in Indonesian strains, it was found that the expression of the SARP PapR2 activator could increase the production of plicacetin in the SHP 22-7 strain.

This result suggests that SARP-guided BGC activation is a promising strategy for selectively activating the antibiotic (silent) gene clusters of actinomycetes, thus unlocking their chemical diversity.

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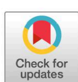
Appendix

Accepted publication 1

Ira Handayani, Shanti Ratnakomala, Puspita Lisdiyanti, Fahrurrozi, Mohammad Alanjary, Wolfgang Wohlleben, Yvonne Mast. ***Complete Genome Sequence of Streptomyces sp. Strain BSE7F, a Bali Mangrove Sediment Actinobacterium with Antimicrobial Activities.*** Genome Announcements. Volume 6, Issue 26, e00618-18, 28 June 2018. DOI 10.1128/genomeA.00618-18

PROKARYOTES



Complete Genome Sequence of *Streptomyces* sp. Strain BSE7F, a Bali Mangrove Sediment Actinobacterium with Antimicrobial Activities

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ABSTRACT The strain *Streptomyces* sp. BSE7F, a novel *Streptomyces* strain isolated from Indonesian mangrove sediment, displays antimicrobial activities against Gram-positive bacteria, Gram-negative bacteria, and yeast. Bioinformatic analysis of the genome sequence revealed the occurrence of 22 biosynthetic gene clusters disclosing the secondary metabolite capacity of strain BSE7F.

Actinomycetes have proven to be an excellent source of antibiotics (1, 2). In recent years, bioprospecting of rare habitats has turned out to be an efficient way to discover novel antibiotic producers (3). Indonesia is one of the most species-rich countries in the world (4). This biodiversity may also be reflected by microbial species diversity. Thus, Indonesian soils should serve as an excellent source of novel antibiotic producers. However, due to the overexploitation of terrestrial actinomycetes, the search for new antibiotics is more promising in unique environments. One such habitat is represented by the mangrove ecosystem (5). Many rare and novel actinobacterial species have been isolated from mangrove samples and have been shown to be potent producers of new bioactive secondary metabolites (6–10).

In this study, a novel *Streptomyces* strain, designated BSE7F, was isolated from a mangrove sediment sample from Bali, Indonesia. BSE7F exhibits antimicrobial activity against selected Gram-positive bacteria (*Bacillus subtilis*, *Micrococcus luteus*, and *Staphylococcus carnosus*), Gram-negative bacteria (*Escherichia coli*), and yeast (*Saccharomyces cerevisiae*). Here, we present the annotated genome sequence of strain BSE7F and report on its biosynthetic potential for antibiotic production.

The BSE7F genome was sequenced using PacBio RS II technology. The total genome size of BSE7F is 7,510,161 bp with five contigs and a G+C content of 72.3%. Using CheckM marker gene analysis (11), genome completeness was determined to be 98.3% with 0.4% contamination. Genome annotation with Prokka version 1.12b (12) identified 6,880 coding sequences (CDSs), 79 tRNAs, and 21 rRNAs on the BSE7F genome. Phylogenetic analysis of the whole-genome sequence using RAxML Web servers (13) revealed that BSE7F is closely related to *Streptomyces* sp. HNS054 (14). Average nucleotide identity (ANI) searches were performed using MASH (15) against RefSeq (16) genomes, which resulted in one match with an ANI over 95%. This was confirmed via JSpeciesWS (17), which showed a 97.1% ANI to *Streptomyces* sp. HNS054. An ANI higher than 95% indicates that BSE7F belongs to the same species as *Streptomyces* sp. HNS054, a strain originally isolated from a marine sponge (14).

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In order to identify the antibiotic gene clusters (BGCs) that are responsible for the bioactivity of BSE7F, the genome was analyzed using antiSMASH version 4.0 (18), which predicted 22 BGCs. Seven of these matched known clusters for desferrioxamine B (19), venezuelin (20), albaflavenone (21), alkylresorcinol (22), naphthyridinomycin (23), and ectoine (twice) (24) with 100% similarity and three clusters encoding resistomycin (25), hopene (26), and spore pigment biosynthesis with >80% similarity. The remaining clusters were predicted to encode 3 polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) hybrids, 2 terpenes, 2 bacteriocins, 1 NRPS, 1 phenazine, 1 lassopeptide, 1 siderophore, and 1 aminoglycoside/aminocyclitol-terpene hybrid. Overall, the discovery of several unknown putative BGCs in BSE7F is providing a strong basis for further experimental studies on the antibiotic production capacity of BSE7F, which may lead to the discovery of novel natural compounds. These preliminary studies suggest that Indonesia's mangrove soil is a promising source of novel bioactive compound producers.

Accession number(s). This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number QEQV00000000. The version described in this paper is the first version, QEQV01000000.

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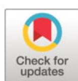
GENOME SEQUENCES



Complete Genome Sequence of *Streptomyces* sp. Strain SHP22-7, a New Species Isolated from Mangrove of Enggano Island, Indonesia

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ABSTRACT *Streptomyces* sp. SHP22-7 is a novel strain isolated from a mangrove sample on Enggano Island, Indonesia. Here, we present the 7.9-Mbp genome sequence of SHP22-7, which will provide insight into its natural compound biosynthetic potential.

Enggano Island is one of the outlying islands of Indonesia, located in the southwest of Bengkulu City with the coordinates 5°31′13″ latitude and 102°16′0″E longitude. It is a desert island 100 km from Sumatra Island and unique in terms of its high number of endemic species and richness in biodiversity (1, 2). An expedition to Enggano Island in 2015, coordinated by the Indonesian Institute of Sciences (LIPI), disclosed many new species of plants (3), animals (4), and microorganisms (5, 6). One of the microbial species that was isolated from a mangrove soil sample obtained from Enggano Island during that expedition is *Streptomyces* sp. SHP22-7, a potential antibiotic producer. As a general basis for further studies on the antibiotic production capacity of SHP22-7, we present here its complete genome sequence and bioinformatic analysis results.

For genome isolation, SHP22-7 was cultivated for 2 days in 50 ml of R5 medium (7) at 30°C. Genomic DNA was extracted and purified using the Genomic-tip 100/G kit from Qiagen (catalog number 10243). The genomic DNA isolation procedure was carried out following the standard protocol provided by the manufacturer. For proper cell lysis, achromopeptidase (5 mg/ml; Sigma) was added to the cells. For genome sequencing, a 10-kb paired-end library was constructed, and sequencing was performed with the Pacific Biosciences RS II platform. The genome was assembled using Hierarchical Genome Assembly (HGAP) V.3.0 (8). Altogether, 201,312 filtered reads (N_{50}, 8,282 bp) were assembled to a nucleotide draft sequence of 7,899,734 bp with a 6-fold coverage. The total genome consists of 146 contigs with an average G+C content of 72.20%. Genome annotation was performed with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (9). We observed 4,602 coding sequences (CDSs), 63 tRNAs, and 18 rRNA genes on the SHP22-7 genome. Using RaxML Web servers (10), we found that SHP22-7 is closely related to *Streptomyces* sp. CC71 (11). A MASH (12) analysis against all RefSeq (13) genomes yielded average nucleotide identity (ANI) estimates, which indicate that SHP22-7 is 95% similar to *Streptomyces* sp. CC71. This was confirmed with JSpeciesWS tree (14) with an ANI of 97.91%. The ANI higher than 95% indicates that SHP22-7 belongs to the same species as *Streptomyces* sp. CC71, a strain isolated from

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a sediment sample of the Cuatro Cienegas Basin, which is an oasis in the Chihuahuan desert in the north of Mexico (11).

The genome sequence of SHP22-7 was analyzed for secondary metabolite-specific biosynthesis gene clusters (BGCs) using AntiSMASH 4.0 (15), which predicted 25 BGCs. Three of the BGCs matched known clusters for albaflavenone (16), desferrioxamine B (17), and ectoine (18) with 100% similarity. Another three BGCs showed >80% similarity to clusters encoding amicetin (19), hopene (20), and candicidin (21) biosynthesis. The remaining clusters putatively encode 3 polyketides, 3 nonribosomal peptides, 2 nonribosomal peptide-polyketide hybrids, 2 terpenes, 2 bacteriocins, 2 lanthipeptides, 1 siderophore, 1 melanin, and 1 indole. Overall, the genome sequence of SHP22-7 provides useful information to further explore uncharacterized secondary metabolites from that strain.

Data availability. This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number QXMM00000000. The version described in this paper is version QXMM01000000. Raw sequencing data are available under SRA accession number PRJNA489221. The genes used for phylogenomic analysis are available at https://figshare.com/articles/concatMLST_fasta/7228880. Alignments were done using a concatenated supermatrix of all genes accessible at https://figshare.com/articles/mlst_aligned_zip/7229099. For all software, default settings were used.

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Appendix

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Disclosing the Potential of the SARP-Type Regulator PapR2 for the Activation of Antibiotic Gene Clusters in Streptomycetes

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Streptomyces antibiotic regulatory protein (SARP) family regulators are well-known activators of antibiotic biosynthesis in streptomycetes. The respective genes occur in various types of antibiotic gene clusters encoding, e.g., for polyketides, ribosomally and non-ribosomally synthesized peptides, or β-lactam antibiotics. We found that overexpression of the SARP-type regulator gene *papR2* from *Streptomyces pristinaespiralis* in *Streptomyces lividans* leads to the activation of the silent undecylprodigiosin (Red) gene cluster. The activation happens upon the inducing function of PapR2, which takes over the regulatory role of RedD, the latter of which is the intrinsic SARP regulator of Red biosynthesis in *S. lividans*. Due to the broad abundance of SARP genes in different antibiotic gene clusters of various actinomycetes and the uniform activating principle of the encoded regulators, we suggest that this type of regulator is especially well suited to be used as an initiator of antibiotic biosynthesis in actinomycetes. Here, we report on a SARP-guided strategy to activate antibiotic gene clusters. As a proof of principle, we present the PapR2-driven activation of the amicetin/plicacetin gene cluster in the novel Indonesian strain isolate *Streptomyces* sp. SHP22-7.

Keywords: actinomycetes, *Streptomyces*, antibiotic, regulator, SARP, silent gene cluster

INTRODUCTION

In 2018, the WHO warned that the dramatic increase of antibiotic resistances coupled with the scarcity of new antibiotics will lead to a global health crisis in the 21st century (World Health Organization, 2015). Even nowadays, infections that are caused by drug-resistant pathogens are suggested to account for 700,000 deaths worldwide annually (United Nations Foundation and the Wellcome Charitable Trust, 2016). According to the World Bank Group "by 2050, drug-resistant infections could cause global economic damage on par with the 2008 financial crisis" (Worldbank, 2016). Thus, there is a substantial need for new antibiotics in order to combat drug-resistances. Bacteria have long been recognized as a prolific source for antibiotics

(Newman and Cragg, 2016). Especially actinomycetes are potent producers of bioactive molecules, as they provide up to 70% of all medically important antibiotic agents (Tanaka and Omura, 1990). The capability to produce these natural compounds is genetically encoded in the actinomycetes genome, whereby the respective genes usually are organized as biosynthetic gene clusters (BGCs). In recent years, genomic analyses of actinomycetes have revealed the presence of numerous "silent" or "cryptic" BGCs, meaning that these clusters remain silent or are only weakly expressed under standard lab conditions. Indeed it is estimated that actinomycetes encode ∼10-times the number of secondary metabolites than anticipated from prior fermentation studies (Baltz, 2017). Thus, these microorganisms still hold the genetic potential to produce new bioactive compounds. Consequently, there are several attempts to activate silent gene cluster expression in order to find new antibiotics. However, most of these activation efforts are either (a) completely unspecific in terms of the BGC(s) to be activated [e.g., by adding general elicitors to the cell culture, co-cultivation approaches, or strain-cultivations following the "one strain-many compounds" (OSMAC) strategy] or (b) they are absolutely specific for the BGC of interest (e.g., heterologous expression of the BGC, introduction of an artificial promoter in front of the BGC, or manipulation of a cluster-situated regulator) as reviewed in Ochi and Hosaka (2013) and Zhu et al. (2014). Both approaches have their drawbacks, as there are either major analytical efforts to identify the product from the silent gene cluster [in terms of (a)] or tedious genetic engineering efforts to manipulate the producer strain [in terms of (b)]. Thus, it would be highly beneficial to have a more general activation strategy that targets a defined set of BGCs, which would tackle both issues. In a recent study from Martínez-Burgo et al. (2019) it has been shown that conserved pathway-specific activators can be used to activate BGC expression in a foreign *Streptomyces* strain. In this study the heterologous expression of the PAS-LuxR type regulator gene *pimM* from *Streptomyces natalensis* in *Streptomyces clavuligerus* led to the activation of clavulanic acid, cephamycin C, and tunicamycin production.

Here we demonstrate that *Streptomyces* Antibiotic Regulatory Protein (SARP)-type regulators can be used as activators of certain antibiotic gene clusters in actinomycetes and describe a genome-based approach to screen for SARP-activated gene clusters. SARPs have exclusively been found in actinomycetes, especially in streptomycetes, where they act as pathway-specific activators of secondary metabolite biosynthesis (Bibb, 2005). They are known to be associated with various antibiotic gene clusters, encoding type I- (Bate et al., 2002; Takano et al., 2005; Novakova et al., 2011) and type II-PKS derived polyketides (Lombó et al., 1999; Sheldon et al., 2002; Aigle et al., 2005; Novakova et al., 2011), ribosomally (Widdick et al., 2003; Wu et al., 2018) and non-ribosomally synthesized peptides (Ryding et al., 2002), hybrid polyketide-peptide compounds (Pulsawat et al., 2009; Suzuki et al., 2010; Xie et al., 2012; Salehi-Najafabadi et al., 2014; Mast et al., 2015; Ye et al., 2018), β-azachinones (Santamarta et al., 2002; Rodríguez et al., 2008; Kurniawan et al., 2014), and azoxy compounds (Garg et al., 2002). SARP genes usually are located within the BGC they are regulating. The encoded SARP gene products are characterized by a winged helix-turn-helix (HTH) DNA-binding motif at the N-terminus that binds to a conserved recognition sequence within the major groove of the target DNA (Wietzorrek and Bibb, 1997; Liu et al., 2013). The DNA recognition sequence constitutes direct heptameric repeat sequences followed by 4 bp spacers, which are often localized between the −10 and the −35 promoter element of the respective target DNA. Such a localization has already been described for the SARP type regulator AfsR from *Streptomyces coelicolor*, which binds to a recognition sequence 8 bp upstream of the −10 element (Tanaka et al., 2007). Also the SARP regulators Aur1PR4 from *Streptomyces aureofaciens* and FdmR1 from *Streptomyces griseus* bind to heptameric repeat sequences, which are located 8 bp upstream of the −10 region (Chen et al., 2008; Rehakova et al., 2013). ActII-ORF4 from *S. coelicolor* interacts with the −35 element for transcriptional activation (Arias et al., 1999). DnrI from *Streptomyces peucetius* and SanG from *Streptomyces ansochromogenes* bind to interaction sites that occur within the −35 element (Sheldon et al., 2002; He et al., 2010). It is suggested that in general the SARP binding site overlaps with the −35 region of the target promoter, which is a binding region of the majority of repressors but not activators. Thus, SARP-driven transcriptional activation has been proposed to occur via a novel mechanism (Tanaka et al., 2007). The C-terminal bacterial activation domain (BTAD) of the SARP protein activates the transcription of the target genes by recruiting the RNA polymerase (RNAP) to the respective promoter, where a ternary DNA–SARP–RNAP complex is formed allowing for transcriptional initiation (Tanaka et al., 2007). "Small" SARP-type activators only contain the HTH DNA binding and BTAD domain, whereas "large" SARPS carry additional domains at the C-terminal side of the protein. These domains include a domain of unknown function belonging to the P-loop NTPase family, and one or more copies of a tetratricopeptide repeat (TPR) motif (Liu et al., 2013). A typical "small" SARP-type activator is represented by PapR2, which has been identified as the major activator of pristinamycin biosynthesis in *Streptomyces pristinaespiralis* (Mast et al., 2015). A *papR2* deletion mutant is unable to produce any pristinamycin, depicting that PapR2 is essential for pristinamycin biosynthesis (Mast et al., 2015). In contrast, overexpression of *papR2* in *S. pristinaespiralis* leads to an increased pristinamycin production, which shows that PapR2 has an activating function (Mast et al., 2015). With the help of electromobility shift assays (EMSAs) and (quantitative) reverse transcription PCR [RT-(q)PCR] analysis the PapR2 target genes have been identified in the pristinamycin producer and a conserved PapR2 binding site was proposed (Mast et al., 2015).

In this study, we report on the potential of SARP-type regulators as genetic engineering devices for the activation of (silent) BGCs in actinomycetes. SARP-type regulators are present predominantly in actinomycetes with an abundance of 98% in the genus *Streptomyces.* We demonstrate that the SARP-type regulator PapR2 activates the silent undecylprodigiosin (Red) gene cluster in *Streptomyces lividans.* Additionally, we provide evidence for a PapR2-guided activation of a BGC in the poorly studied Indonesian strain isolate *Streptomyces* sp.

SHP22-7 (SHP22-7), which yielded an increased production of the nucleoside antibiotic plicacetin.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Cultivation Conditions

The bacterial strains and plasmids used in this study are listed in **Supplementary Table S1**. For routine cloning strategies *Escherichia coli* Novablue (Novagen) was used. *S. lividans* T7 (Fischer, 1996) and SHP22-7 (Handayani et al., 2018) were applied for antibiotic production analysis, generation of overexpression strains, and transcriptional analysis. Cloning procedures and strain cultivation were carried out as described before (Mast et al., 2015). For cultivation and isolation of RNA, *Streptomyces* strains were grown in 100 ml of R5 medium in 500-ml Erlenmeyer flasks (with steel springs) on an orbital shaker (180 rpm) at 28°C (Kieser et al., 2000). For isolation of genomic DNA and protoplast transformation experiments, strains were grown in 100 ml of S-medium (Kieser et al., 2000). Kanamycin (50 μg/ml), apramycin (50 μg/ml), or thiostrepton (20 μg/ml) were used for selection when appropriate. For antibiotic production experiments with *S. lividans*, strains were grown in YEME medium as reported before (Mast et al., 2015). For antibiotic production experiments with SHP22-7, strains (SHP22-7*papR2-OE*; references: SHP22-7*pRM4* and SHP22-7 WT) were grown in 50 ml NL410 medium consisting of glucose (10 g l^{-1}), glycerol (10 g l^{-1}), oat meal (5 g l^{-1}), soy flour (10 g l^{-1}), yeast extract (5 g l^{-1}), Bacto casamino acids (5 g l^{-1}), $CaCO_3$ (1 g l^{-1}), and distilled water (pH was adjusted to 7.0 with NaOH) as a preculture. After 3 days, 10 ml of preculture was transferred to 100 ml of sterile main culture medium NL19, consisting of mannitol (20 g l^{-1}), soy flour (20 g l^{-1}), and distilled water (pH adjusted to pH 7.5 with NaOH). Cells were grown for 168 h at 28°C.

Molecular Cloning

Basic procedures for DNA manipulation were performed as described previously (Sambrook et al., 1989; Kieser et al., 2000). Primers used for PCR were obtained from MWG Biotech AG (MWG, Ebersberg, Germany) and are listed in **Supplementary Table S1**.

Construction of the papR2 Overexpression Strain SHP22-7papR2-OE

For *papR2* overexpression experiments with SHP22-7, the *papR2* gene was isolated as a *Nde*I/*Hin*dIII-fragment from plasmid pGM190/papR2 (Mast et al., 2015) and was cloned into the *Nde*I/*Hin*dIII restriction site of the integrative expression vector pRM4. In the resulting overexpression construct pRM4/papR2, the *papR2* gene is under control of the constitutive promoter of the erythromycin resistance gene *ermEp**. pRM4/papR2 was transferred to SHP22-7 by protoplast transformation. Transformants were selected with apramycin (50 μg/ml), which resulted in the overexpression strain SHP22-7*papR2-OE*. Strain SHP22-7*pRM4*, harboring the empty pRM4 vector, was generated by protoplast transformation accordingly and served as a reference.

PapR2 Protein Expression in *S. lividans*

For *papR2* overexpression experiments *SLpapR2-OE* precultures were grown in 100 ml of YEME liquid medium for 2 days at 28°C. Five milliliters of preculture was used as inoculum for 100 ml YEME liquid medium as main culture with thiostrepton (12.5 μg/ml) as inductor for gene expression (Mast et al., 2015). The main culture was cultivated for 3 days at 28°C. PapR2 protein purification was carried out as reported before (Mast et al., 2015).

Spectrophotometrical Analysis for Red Detection

For Red detection, culture supernatant from *SLpapR2-OE* and *SLpGM190* (reference) was treated as reported in Onaka et al. (2011) and absorption was measured with a Hitachi U-2000 spectrophotometer.

Sample Treatment for SHP22-7 Bioassays and Compound Detection

For SHP22-7 compound detection and bioassay tests, 5 ml culture samples of SHP22-7*papR2-OE* (references: SHP22-7*pRM4* and SHP22-7 WT) was extracted with 5 ml ethyl acetate for 30 min at RT. Ethyl acetate samples were concentrated *in vacuo* completely and then redissolved in 0.75 ml of methanol. Methanolic extracts were used for bioassays and high-performance liquid chromatography/mass spectrometry (HPLC–MS) analysis.

Bioassays

Antibiotic activity was analyzed in disc diffusion assays using *Bacillus subtilis* ATCC6051 as test organism. Thirty microliters of methanolic extract from three independent biological samples of SHP22-7 WT, SHP22-7*pRM4*, and SHP22-7*papR2-OE*, respectively, was pipetted on a filter disc, which was placed on a *B. subtilis* test plate. Five microliters of kanamycin (50 μg/ml) was applied as a positive control and 30 μl of methanol as negative control to test the functionality of the *B. subtilis* bioassay plates. The plates were incubated overnight at 37°C. Antibiotic activity was quantified by measuring the diameter of the inhibition zone around the filter discs. The bioassay was carried out as 10 independent biological replicates.

HPLC and HPLC–MS Analysis for Amicetin/Plicacetin Detection

High-performance liquid chromatography analyses were performed with a HP1090M system with ChemStation 3D software rev. A.08.03 (Agilent Technologies, Waldbronn, Germany) on a Nucleosil C18 column (5 μm, 125 mm × 3 mm) fitted with a precolumn (20 × 3 mm) and with a flow rate of 850 μl min^{-1}. Chromatography was done by linear gradient elution from 95.5 solvent A (water with 0.1% phosphoric acid) to 100% solvent B [acetonitrile (ACN)] over 15 min. The injection volume was 5 μl. Multiple wavelength monitoring was

performed at 210, 230, 260, 280, 310, 435, and 500 nm. UV-Vis spectra were measured from 200 to 600 nm. The evaluation of the chromatograms (210 nm only) was done by means of an in-house HPLC–UV–Vis database.

High-performance liquid chromatography–mass spectrometry analysis of amicetin/plicacetin was performed with an Agilent 1200 series chromatography system (binary pump, high performance autosampler, DAD-detector) coupled with an LC/MSD Ultra Trap System XCT 6330 (Agilent Technologies, Waldbronn, Germany). The sample (5 μl) was injected on a Nucleosil 100 C18 column (3 μm, 100 × 2 mm) fitted with a precolumn (3 μm, 10 × 2 mm) at a flow rate of 400 μl/min and a linear gradient from 100% solvent A (0.1% HCOOH in water) to 100% solvent B (0.06% HCOOH in ACN) over 15 min at 40°C. UV–Vis-detection was done at 220, 260, 280, 360, and 435 nm. Electrospray ionization was performed in positive and negative ultra-scan mode (alternating) with a capillary voltage of 3.5 kV and a drying gas temperature of 350°C. Detection of *m/z* values was conducted with Agilent DataAnalysis for 6300 Series IonTrap LC/MS Version 3.4 (Bruker Daltonik). Upon HPLC–MS analyses amicetin, plicacetin, and plicacetin isomer were identified by comparisons of their UV/visible spectra, retention times, and molecular masses with authentic standards, as *m/z* 617.1 $[M\text{-}H]^-$ and *m/z* 516.1 $[M\text{-}H]^-$, respectively.

AntiSMASH Analysis

With the webtool antiSMASH whole genomes can be scanned for the occurrence of BGCs. Gene cluster similarity is given in % and indicates the number of similar genes to a known cluster. Genes are similar if a BLAST-alignment results in an *e*-value $<10^{-5}$ and the sequence identity is >30%. Additionally, the shortest alignment must enclose >25% of the sequence. If all genes of a known cluster can be found in the query cluster, the similarity of the sequences is 100%. The similarity lowers if less genes of the known cluster can be found in the query cluster (Medema et al., 2011).

PatScan Analysis

PatScan analysis (Blin et al., 2018) was performed with the *S. lividans* T7 genome (GenBank Accession Number ACEY00000000) and the PapR2 consensus sequence 5′-GTCAGSS-3′ using the software at https://patscan.secondarymetabolites.org/.

PapR2 Electromobility Shift Assays

For EMSAs with Red-specific promoter regions, 182 bp DNA fragments of the upstream regions of *redP* and *redQ* were amplified by PCR from genomic DNA of *S. lividans* T7 with primer pairs PredPfw/rv and PredQfw/rv, respectively (**Supplementary Table S1**). For EMSAs with the *pliA* promoter region, a 230 bp DNA fragment of the upstream region of *pliA* was amplified by PCR from genomic DNA of strain SHP22-7 as template and primer pair PpliAfw/rv (**Supplementary Table S1**). Promoter DNA amplificates included a 16 bp Cy5 adapter sequence, each at the 3′- and 5′-end, which was added via the respective primer sequences. The generated amplificates were used as templates in a second PCR approach together with a Cy5 primer (**Supplementary Table S1**) in order to conduct Cy5 labeling of the promoter regions. Promoter labeling and PapR2 EMSAs were carried out with variable concentrations of PapR2 protein sample as reported before (Mast et al., 2015). To verify the specificity of the PapR2-DNA binding, an excess of unlabeled, specific, and non-specific DNA, respectively, was added to the EMSA mixture as described previously (Mast et al., 2015). DNA bands were visualized by fluorescence imaging using a Typhoon Trio™ Variable Mode Imager (GE Healthcare).

Transcriptional Analysis by Reverse Transcription Analysis (RT-PCR)

SLpGM190 and *SLpapR2-OE*, as well as SHP22-7, SHP22-7*pRM4*, and SHP22-7*papR2-OE* were each grown under *papR2*-overexpression conditions as described above. Thirty milliliters of each cell culture was harvested after 48 h. Cell disruption was carried out with glass beads (150–212 μm; Sigma) at 6,500 rpm, 1 × 20-30 s, using a Precellys Homogenizer (Peqlab). Total RNA was isolated as described previously (Sambrook et al., 1989) and served as the basis for RT-PCR experiments. DNA was removed by digestion with DNase (Thermo Fisher Scientific) and absence of DNA was verified via PCR analysis. RNA concentrations and quality were checked using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). cDNA from 3 μg RNA was generated with random primers, reverse transcriptase, and cofactors (Fermentas). For RT-PCRs, primers were used that amplify cDNA of 200–250 bp from internal gene sequences. PCR conditions were 98°C for 5 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and a final cycling step at 72°C for 5 min. As a positive control, cDNA was amplified from the 16S rRNA transcript, which is transcribed constitutively. To exclude DNA contamination, negative controls were carried out by using total RNA as a template for each RT-PCR reaction. At least three independent biological replicates have been tested.

Transcriptional Analysis by Real-Time qPCR

Real-time qPCR analysis was applied for quantitative cDNA determination. PCR reactions were run with SYBR® Green Supermix (BioRad) on an iQ5 Multicolor Real-Time-PCR Detection System (BioRad). The SYBR® Green Dye shows increased fluorescence when bound to double-stranded DNA. The fluorescence is measured at 494 and 521 nm and gives the proportional amount of generated dsDNA. cDNA was generated from cultures of *SLpGM190* and *SLpapR2-OE* as described above. The primer pairs redPintfw/rv and redQintfw/rv, respectively (**Supplementary Table S1**), which amplify fragments of about 180 bp from internal gene sequences, were used together with cDNA as template in qPCR reactions. *hrdB* was used as housekeeping gene in each experiment in order to standardize the results by eliminating variation in RNA and cDNA quantity and quality. Each reaction mixture of 10 μL volume contained 5 μL SYBR® Green Supermix (BioRad), 3.85 μL nuclease-free water, 0.2 μL of each primer, and 0.75 μL template. PCR conditions were 98°C for 5 min, followed by 35 cycles of

95°C for 20 s, and 57°C for 30 s. To determine amplification specificity, melting curve analyses were performed after the last cycle, showing in all cases one single peak. Results were analyzed using the $\Delta\Delta$CT-method (Livak and Schmittgen, 2001). Changes in gene expression are represented in relation to the data from samples of *SLpGM190*. Data are presented as the results from six independent biological replicates.

Database Analysis

FASTA sequences of SARP proteins were extracted from the antiSMASH database version 2 (Blin et al., 2019) by querying for all genes that hit the antiSMASH smCoG (secondary metabolite clusters of orthologous groups) profile SMCOG1041 (transcriptional regulator, SARP family). These hits were checked for the presence of at least one out of four SARP-related profiles from the PFAM database: PF00486.27 (Trans_reg_C, the HTH-style DNA binding domain), PF93704.16 (BTAD, the transcriptional activator), PF00931.22 (NB-ARC, a domain of unknown function found, e.g., in *Saccharopolyspora erythraea* SARPs), and PF13424.6 (TPR_12, a TPR found in many larger SARPs). Sequences that did not hit at least one of these domains were discarded. Sequences were then annotated by which of the four profiles were hit and grouped by taxonomic order and BCG type.

RESULTS

PapR2 Induces Expression of the Silent Red Gene Cluster in *S. lividans*

Streptomyces lividans is a widely used heterologous host strain, which under specific laboratory conditions does not exhibit production of the two pigmented secondary metabolites actinorhodin and undecylprodigiosin (Hu et al., 2002; Martinez et al., 2005; Rodríguez et al., 2013). In frame of analyzing the regulatory role of PapR2 in *S. pristinaespiralis*, the *papR2* gene was heterologously expressed in *S. lividans* T7 for protein purification purposes (Mast et al., 2015). For overexpression experiments the *S. lividans* strain *SLpapR2-OE* was used, which harbors the *papR2* gene under control of the thiostrepton-inducible promoter P_{tipA} on the replicative medium-copy plasmid pGM190 (Mast et al., 2015). *S. lividans* strain *SLpGM190* was used as a reference, containing the pGM190 empty vector. After 2–3 days of growth in YEME liquid medium with thiostrepton as inductor for gene expression, the whole *SLpapR2-OE* culture, as well as the culture supernatant showed an intensive red pigment formation, which was not observed for samples of *SLpGM190* (**Figure 1A**). This phenomenon was also observed on R5 agar with thiostrepton, where *SLpapR2-OE* mycelium was intensively red colored after 3–4 days of cultivation, whereas *SLpGM190* mycelium was not (**Supplementary Figure S1**). *S. lividans* is known to harbor a Red BCG, which remains silent under normal growth conditions (Horinouchi et al., 1986). Red biosynthesis has mainly been studied in *S. coelicolor*; however, since *S. coelicolor* and *S. lividans* are very closely related species, knowledge on Red biosynthesis and regulation can be transferred to *S. lividans* (van Wezel et al., 2000; Lewis et al., 2010).To investigate, whether the red color of the *SLpapR2-OE* cultures originates from the formation of the Red metabolite, we performed spectrophotometrical analysis. A pH shift was carried out with the culture supernatant of *SLpapR2-OE* and the absorption maxima of the sample was determined by using a Hitachi U-2000 spectrophotometer. Spectrophotometrical analysis led to the detection of the Red-specific spectral absorption maxima (Onaka et al., 2002) at 533 and 468 nm under acidic and basic conditions, respectively (**Figure 1B**), which proved that the *SLpapR2-OE* samples contained Red (**Figure 1C**). These data suggested that the overexpression of *papR2* in *S. lividans* induced Red biosynthesis.

PapR2 Mimics the Function of the *S. lividans* SARP-Type Regulator RedD

In *S. lividans* Red biosynthesis is under control of the SARP-type regulator RedD, which directly activates the Red biosynthetic genes (Takano et al., 1992; White and Bibb, 1997). An amino acid sequence comparison using BLASTP revealed that PapR2 and RedD are highly similar to each other (44% identity, 55% similarity) (Mast et al., 2015). This amino acid sequence similarity was even higher for the HTH motif of the protein (66% identity, 75% similarity). Thus, we suspected that PapR2 may substitute for the function of RedD and activates Red biosynthesis in *S. lividans*. In order to identify potential SARP-type binding motifs within the *S. lividans* Red BGC, the genome was analyzed with the bioinformatic tool PatScan, which allows for the identification of specific sequence patterns in a given genome sequence (Blin et al., 2018). PatScan analysis was performed with the previously described PapR2 consensus motif (5'-GTCAGSS-3') (Mast et al., 2015) as sequence pattern on the *S. lividans* genome sequence. Thereby, two highly conserved PapR2-like motifs were identified within the intergenic region of the Red-specific biosynthetic genes *redP* (SCO5888) and *redQ* (SCO5887) with each 100 and 96.5% identity, respectively (**Figure 2A**). *redP* encodes a 3-ketoacyl-acyl carrier protein synthase, whereas *redQ* codes for an acyl carrier protein, both of which have been shown to be involved in Red biosynthesis (Mo et al., 2008). To analyze the functionality of the identified SARP motifs, EMSAs were performed with the PapR2 protein and the upstream regions of *redP* (P*redP*, 182 bp) and *redQ* (P*redQ*, 182 bp), respectively, harboring the PapR2 consensus sequence. EMSAs showed that PapR2 specifically binds to the P*redP* and P*redQ* fragment, respectively (**Figure 2B**), revealing the functionality of the identified SARP-type motifs.

To confirm the regulatory effect of PapR2 on the transcription of the Red BGC, we performed RT-PCR and quantitative qPCR experiments. For these studies *SLpapR2-OE*, as well as the reference strain *SLpGM190* were grown in R5 medium. After 72 h of cultivation samples were harvested for RNA isolation. For each strain six biological replicates were performed. Isolated RNA was used as a template in RT-PCR experiments as a negative control (**Figure 3A**), whereas cDNA was used together with 16S primers as a positive control (**Figure 3B**). For *red* gene-specific transcriptional analysis, isolated RNA was used as template for RT-PCR experiments with the primer pairs redPintfw/rv

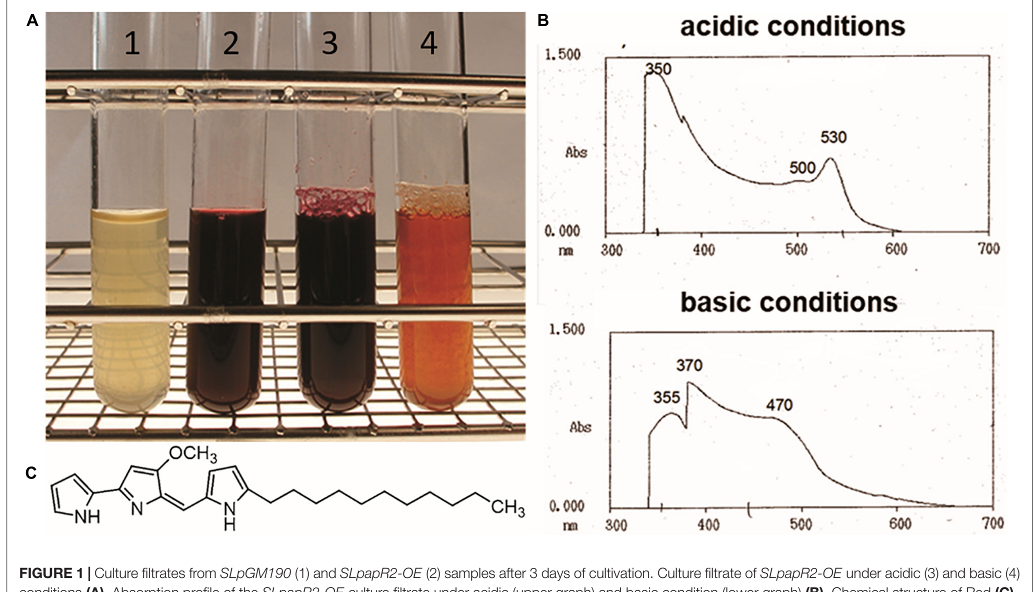

FIGURE 1 | Culture filtrates from *SLpGM190* (1) and *SLpapR2-OE* (2) samples after 3 days of cultivation. Culture filtrate of *SLpapR2-OE* under acidic (3) and basic (4) conditions **(A)**. Absorption profile of the *SLpapR2-OE* culture filtrate under acidic (upper graph) and basic condition (lower graph) **(B)**. Chemical structure of Red **(C)**.

and redQintfw/rv, which annealed to internal parts of *redP* and *redQ*, respectively. The transcriptional analysis revealed that there are stronger signals for the *redP* and *redQ* cDNA amplificates in *SLpapR2-OE* samples compared to *SLpGM190* samples (**Figures 3C,D**, respectively). In order to quantify the amount of *redP* and *redQ* RT-qPCR was performed. Based on the threshold cycle it was calculated that *redP* and *redQ* transcripts were increased to 57- and 492-fold, respectively, in samples of *SLpapR2-OE* compared to samples of *SLpGM190* (**Supplementary Figure S2**). Thus, transcriptional analyses demonstrated that PapR2 activates the transcription of the Red biosynthetic genes *redP* and *redQ*. Due to the intensive Red production of strain *SLpapR2-OE* together with the data obtained from PapR2 EMSA studies and transcriptional analysis, we propose that overexpressed PapR2 in *S. lividans* takes over the regulatory function of RedD and activates the transcription of the Red biosynthetic genes, which leads to the formation of the red colored secondary metabolite Red.

PapR2 Expression Improves Antibiotic Activity of SHP22-7

In order to study the general application of SARP-type regulators for activation of antibiotic biosynthesis, we exemplary tested strain SHP22-7 as a host for *papR2* expression. SHP22-7 is a novel strain isolate from a soil sample of the unique desert island Enggano, Indonesia (Handayani et al., 2018). SHP22-7 shows broad-spectrum antibacterial activity against Gram-positive and Gram-negative bacteria, including *E. coli*, *Staphylococcus carnosus*, *Micrococcus luteus*, and *B. subtilis*. The SHP22-7 genome has been sequenced recently and antiSMASH analysis led to the identification of 25 potential secondary metabolite gene clusters (Handayani et al., 2018; **Table 1**). Four of the gene clusters (clusters 3, 6, 10, and 15) contain SARP genes, which makes SHP22-7 a good candidate strain for a SARP-guided activation. For *papR2* expression studies in SHP22-7, the *papR2* gene was cloned into the integrative plasmid pRM4 under control of the constitutive *ermE** promoter, resulting in construct pRM4/papR2. The pRM4 vector was used as expression plasmid in these analyses to avoid addition of thiostrepton as inductor (see above), which would influence subsequent antibacterial bioassays. The plasmid was transferred to SHP22-7 by protoplast transformation. The resulting expression strain SHP22-7*papR2-OE*, as well as the two reference strains SHP22-7*pRM4*, which harbors the empty pRM4 vector, and the SHP22-7 wild-type (WT) were used for antibacterial bioassay studies. SHP22-7*papR2-OE*, SHP22-7pRM4, and SHP22-7 WT were each cultivated in NL19 medium and samples were taken at 168 h. Methanolic culture extracts were applied for antibacterial bioassays using *B. subtilis* as test organism. Filter discs with kanamycin and methanol served as positive and negative control, respectively. Bioassays were carried out as 10 independent biological replicates. Thereby, significantly larger inhibition zones against *B. subtilis* were observed on average with extracts from SHP22-7*papR2-OE* compared to extracts from SHP22-7*pRM4* or SHP22-7 WT (**Figure 4A**). The extract of SHP22-7*papR2-OE* caused a zone of inhibition of 11.7 ± 2.1 mm, whereas the extracts of SHP22-7*pRM4* and SHP22-7 WT yielded smaller

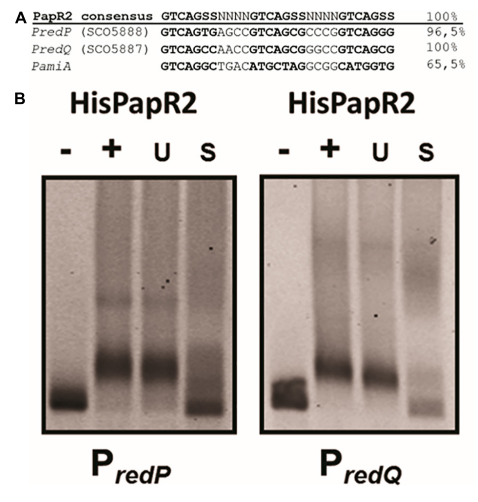

FIGURE 2 | SARP binding sequences within the upstream region of the RED-biosynthetic genes *redP* (SCO5888), *redQ* (SCO5887), and *pliA* **(A)**. EMSAs with undefined HisPapR2 concentrations and Cy5-labeled promoter regions of *redP* and *redQ*. – indicates negative control without protein, + indicates addition of HisPapR2 protein. The specificity of the reaction was checked by the addition of 500-fold specific (S) and unspecific (U) unlabeled DNA **(B)**.

inhibitions zones of 8.3 ± 2.6 and 7.3 ± 2.7 mm, respectively (**Figure 4B**). These data showed that *papR2* expression in SHP22-7 leads to a significantly improved antibiotic activity.

PapR2 Activates Transcription of Cluster 9 in SHP22-7

To identify the substance that is responsible for the antibiotic activity of SHP22-7 but avoid elaborative chemical analytics in the first place, we screened the SHP22-7 genome sequence for the occurrence of PapR2-like consensus sequences by using the PatScan tool. Here, we only considered motifs that were located within intergenic regions of genes from suggested SHP22-7 BGCs. The motif search led to the identification of PapR2-like consensus sequences within 5 of the 25 predicted BGCs from SHP22-7. These included clusters 6 and 10 (NRPS-like gene cluster), cluster 15 (type 2 PKS-butyrolactone-like gene cluster), as well as clusters 3 and 9 ("other" type of gene cluster) (**Table 1**). In order to find out which of the five gene clusters is activated by PapR2, comparative RT-PCR analysis was carried out with SHP22-7*papR2-OE*, SHP22-7pRM4, and SHP22-7 WT in four independent biological replicates. Cells were grown under antibiotic production conditions and samples were harvested at 168 h of cultivation. Two samples were collected for each strain and growth time point, whereby one sample was used for RT-PCR analysis and the second one for HPLC–MS analysis (see the section "SARP-Type Regulatory Genes Are Widespread in BGCs From Diverse Actinobacteria"). RT-PCR was carried out with RNA isolated from cell pellets of SHP22-7*papR2-OE*, SHP22-7*pRM4*, and SHP22-7 WT, respectively. Isolated RNA was used as a template in RT-PCR experiments as a negative control (**Figure 5A**), whereas cDNA was used together with *16S* primers as a positive control (**Figure 5B**). For cluster specific transcriptional analysis, cDNA was generated with primer pairs cl3fw/rv, cl6fw/rv, cl9fw/rv, cl10fw/rv, and cl15fw/rv (**Supplementary Table S1**), each aligning to a predicted biosynthesis gene of clusters 3, 6, 9, 10, and 15, respectively. The transcriptional analysis revealed that there is a stronger signal for the cluster 9 amplificate in the SHP22-7*papR2-OE* samples compared to the samples of SHP22-7*pRM4* and SHP22-7 WT, where there is nearly no signal at all (**Figure 5C**). This difference in signal intensity could not be observed for clusters 3, 6, 10, and 15 (**Figure 5C**). Thus, these data suggested that PapR2 activates the transcription of cluster 9 in SHP22-7*papR2-OE.*

Cluster 9 Resembles an Amicetin Gene Cluster

In order to deduce for what type of metabolite cluster 9 encodes for, we analyzed the cluster sequence region in detail. The antiSMASH output predicted a 75% similarity of cluster 9 to the plicacetin/amicetin gene cluster from *Streptomyces vinaceusdrappus* NRRL 2363 (**Table 1**). The cluster similarity describes the number of genes with a similarity above the ClusterBlast threshold of 30% sequence similarity at over 25% coverage. Manually cluster analysis by sequence alignments yielded a 100% sequence similarity since the sequence region from *amiA–amiD* was not recognized by antiSMASH to be part of the amicetin BGC (**Figures 6A** vs. **B**). Plicacetin (**Figure 6C**) and amicetin (**Figure 6D**) are disaccharide pyrimidine nucleoside antibiotics with a broad-spectrum antibacterial (especially against *Mycobacterium tuberculosis*) and antiviral activity. They act as peptidyl transferase inhibitors and thus inhibit protein synthesis. Amicetin consists of the two deoxysugar moieties, D-amosamine and D-amicetose, as well as cytosine, *p*-aminobenzoic acid (PABA), and a terminal methylserine moiety, whereby the latter moiety is missing in plicacetin (Zhang G.G. et al., 2012; Korzybski et al., 2013). The gene cluster analysis of SHP22-7 revealed that the PapR2-like motif is located directly in front of the *orf pliA* (**Figure 6B**) and shows a rather weak sequence identity of 65.5% to the PapR2 consensus motif (**Figure 2A**). The same motif is present upstream of *amiA* in *S. vinaceusdrappus.* The *pliA* gene presents 99.75% gene nucleotide sequence identity to *amiA* of *S. vinaceusdrappus*, which translates to a 100% amino acid sequence identity among the predicted gene products. In *S. vinaceusdrappus amiA* is the first gene of the amicetin BCG and encodes a putative 4-amino-4-deoxychorismate lyase, which is suggested to catalyze the conversion from 4-amino-4-deoxychorismate to PABA (Zhang G.G. et al., 2012). Remarkably, all genes in the amicetin gene cluster of *S. vinaceusdrappus* and the amicetin-like gene cluster of SHP22-7 are organized in one direction, suggesting a unidirectional transcription. In this context, it would make sense that regulatory activation targets the promoter of the first gene of the unidirectional BGC. In order to

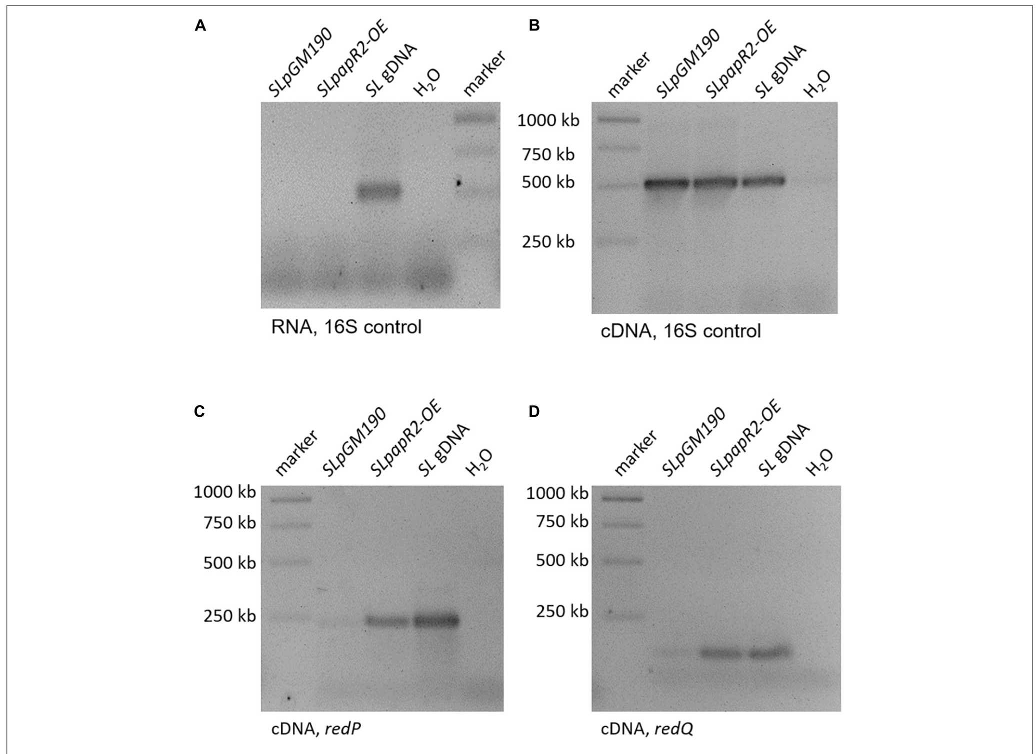

FIGURE 3 | Transcriptional analysis of samples from *SLpGM190* and *SLpapR2-OE* after 168 h. gDNA of *S. lividans* was used as internal positive control (SL gDNA) and water as internal negative control. RT-PCR analysis with RNA samples as template and 16S-specific primers in order to test the absence of DNA **(A)**. RT-PCR analysis with cDNA samples as template and 16S-specific primers in order to test for successful cDNA synthesis **(B)**. RT-PCR analysis of the gene *redP* and in the strains SLpGM190 and SLpapR2-OE **(C)**. RT-PCR analysis of the gene *redQ* and in the strains SLpGM190 and SLpapR2-OE **(D)**.

find out if PapR2 can bind to the *pliA* promoter region, EMSAs were performed with the PapR2 protein and the *pliA* upstream region containing the PapR2 consensus sequence. However, no shifted band was detected in these assays (data not shown). Thus, it might be that the motif is not functional at all and does not constitute a SARP binding motif. It could also be that due to the less conserved PapR2 consensus sequence, the motif is not functional in such an *in vitro* assay. The presence of a SARP consensus sequence would hint for a pathway-specific regulatory gene located within the amicetin(-like) BGC. However, a SARP-type regulatory gene has not been identified in any of the amicetin(-like) clusters from SHP22-7 nor *S. vinaceusdrappus* (**Table 1**; Zhang X. et al., 2012). Overall, six SARP genes have been identified in total within the SHP22-7 genome (**Supplementary Table S2**). All of them are part of BGCs (clusters 3, 6, 10, and 15). Cluster 15 harbors three SARP genes, whereas the other BGCs each contain one SARP gene (**Table 1**). Furthermore, putative SARP binding motifs have been found within the promoter regions of the BGCs (**Table 1** and **Supplementary Table S2**). It might be possible that one of these SARP-type regulators plays a role in *trans*-activating amicetin cluster transcription. So far, regulation of amicetin biosynthesis is not understood. In *S. vinaceusdrappus* three genes [*orf*(*-3*), *orf*(*-2*), *amiP*] have been identified, which encode for putative transcriptional regulators, whereby *orf*(*-3*) and *orf*(*-2*) seem not to be part of the amicetin BGC and *amiP* codes for TetR-like transcriptional regulator, which usually function as repressors of antibiotic biosynthesis (Zhang G.G. et al., 2012). Thus far, it cannot be excluded that also *trans*-acting regulator(s) are involved in the regulation of amicetin biosynthesis.

PapR2 Activates Plicacetin Production in SHP22-7

To investigate if an amicetin(-like) antibiotic is produced by SHP22-7, we analyzed methanolic extracts of cell culture samples from SHP22-7*papR2-OE* by HPLC/MS. To analyze if production is influenced by *papR2* expression, extracts from samples of

TABLE 1 | Secondary metabolite gene clusters of *Streptomyces* sp. SHP22-7 as predicted by antiSMASH 4.0 with an indication of the presence of cluster-situated SARP genes (count), predicted SARP proteins with amino acid sequence homology to PapR2 (I = identity, S = similarity), as well as identified SARP-type motifs.

Cluster	Type of secondary metabolite gene cluster	Similarity to known cluster	Localization	Cluster-situated SARP gene	Sequence homology to PapR2 I/S	SARP consensus motif(s) identified
1	Terpene	Albaflavenone (100%)	Contig 1 49588-70841	–		–
2	T2PKS	Spore pigment (66%)	Contig 1 121312-163740	–		–
3	Other	Granaticin (8%)	Contig 2 304806-348126	+ (1)	41/52%	+
4	Melanin	Melanin (60%)	Contig 3 1-5936	–		–
5	Ectoine	Ectoine (100%)	Contig 4 119873-130271	–		–
6	NRPS	Phosphonoglycans (5%)	Contig 5 129226-170626	+ (1)	31/44%	+
7	Bacteriocin	–	Contig 7 213837-225168	–		–
8	Terpene	–	Contig 7 240589-262769	–		–
9	Other	Amicetin (75%)	Contig 10 129625-170245	–		+
10	NRPS	Calcium dependent antibiotic (47%)	Contig 10 243082-272180	+ (1)	38/56%	+
11	T3PKS	Herboxidiene (8%)	Contig 11 20911-62017	–		–
12	Siderophore	Desferrioxamine B (100%)	Contig 13 127488-139260	–		–
13	Indole	Antimycin (20%)	Contig 17 55086-76126	–		–
14	Terpene	Carotenoid (36%)	Contig 17 133951-147999	–		–
15	T2PKS-Butyrolactone	Fluostatin (26%)	Contig 19 32007-96281	+ (3)	41/59% 67/76% 40/51%	+
16	Terpene	Hopene (84%)	Contig 20 8526-35239	–		–
17	Lanthipeptide	–	Contig 21 82417-98812	–		–
18	T1PKS-NRPS	Candicidin (90%)	Contig 22 1-86799	–		–
19	Arylpolyene-NRPS	Lipopeptide (29%)	Contig 24 1-40688	–		–
20	Siderophore	Grincamycin (8%)	Contig 24 46763-57062	–		–
21	T1PKS	Oligomycin (44%)	Contig 32 4049-49007	–		–
22	OtherKS	Sanglifehrin A (13%)	Contig 42 1-35910	–		–
23	Bacteriocin	Informatipeptin (57%)	Contig 44 22689-32904	–		–
24	NRPS	Coelichelin (27%)	Contig 59 1-16803	–		–
25	Lanthipeptide	–	Contig 96 1-5597	–		–

+, cluster contains gene/motif; –, cluster does not contain gene/motif.

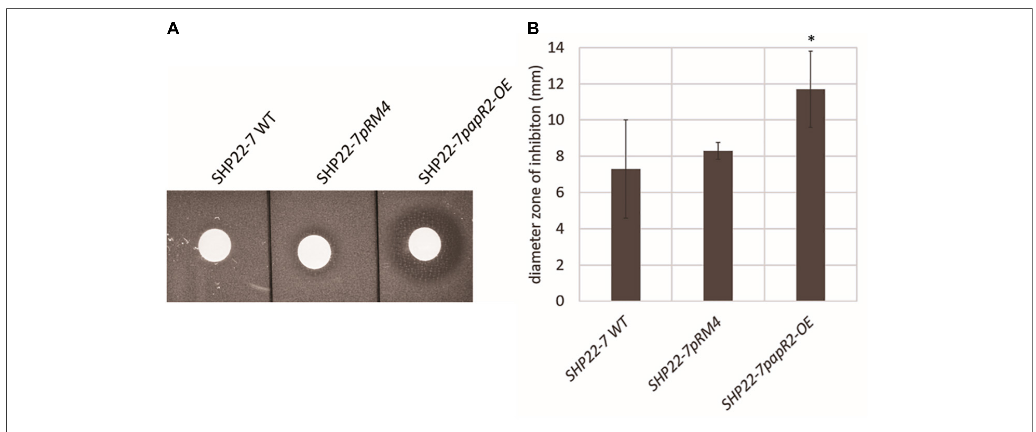

FIGURE 4 | One representative example of culture extracts from SHP22-7 WT, SHP22-7*pRM4*, and SHP22-7*papR2-OE*, respectively, against *B. subtilis.* Filter disk diffusion assay; red arrow indicates inhibition zone **(A)**. Graphical representation of the inhibition zone diameters (mm) from 10 independent biological replicates (n = 10), * indicates significance **(B)**.

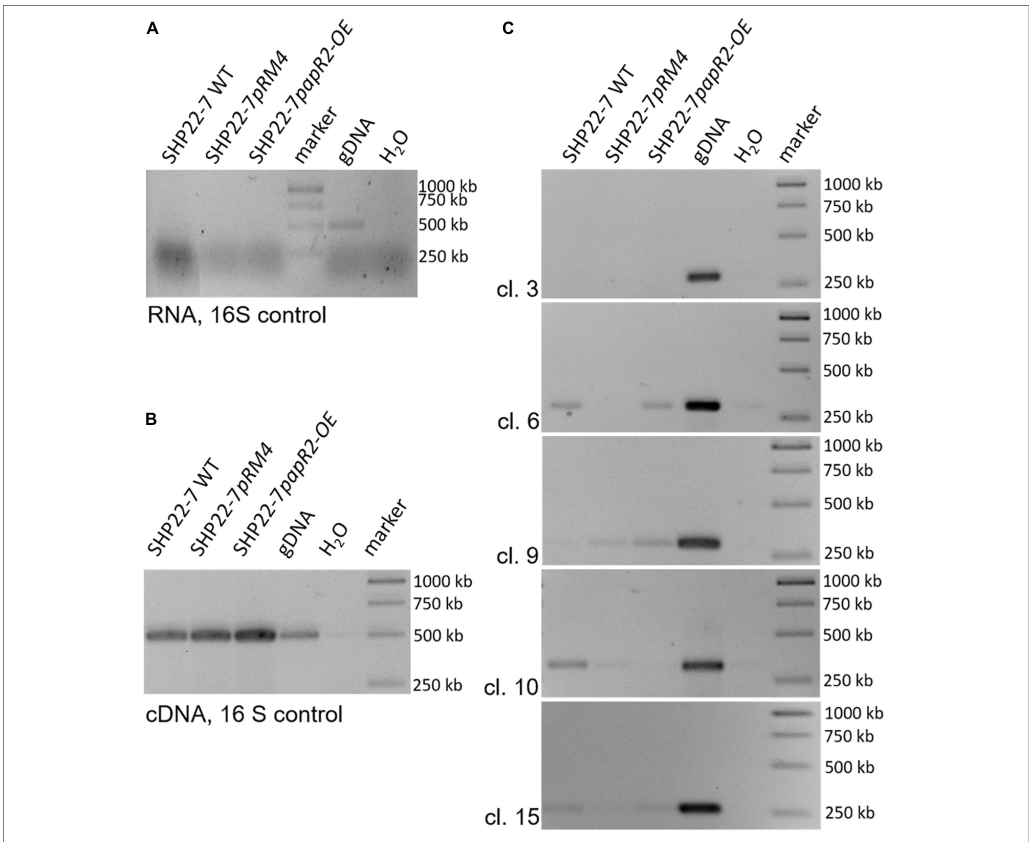

FIGURE 5 | Transcriptional analysis of the SHP22-7 strains SHP22-7 WT, SHP22-7pRM4, and SHP22-7papR2; gDNA of SHP22-7 was used as internal positive (gDNA) and water as internal negative control. The data are representative for four independent experiments. RT-PCR analysis with RNA as template and 16S-specific primers **(A)**. RT-PCR analysis with cDNA and 16S-specific primers **(B)**. Transcriptional analysis of the SARP motif containing gene clusters (cl.) 3, 6, 9, 10, and 15 with samples from SHP22-7 WT, SHP22-7pRM4, and SHP22-7papR2 **(C)**.

SHP22-7*pRM4* and SHP22-7 WT served as references. For these analyses we used the second culture samples obtained from SHP22-7*papR2-OE*, SHP22-7*pRM4*, and SHP22-7 WT, respectively (see above). Based on comparisons with an in-house substance database, HPLC analysis revealed the presence of amicetin [retention time (RT) 4.0 min], plicacetin (RT 4.8 min), and a plicacetin isomer (RT 5.6 min) in all three samples (**Figure 7A**). The identity of the compounds was verified by MS/MS analysis (amicetin *m/z* 617.1 [M-H]; plicacetin *m/z* 516.1 [M-H]; plicacetin isomer *m/z* 516.1 [M-H]) (**Figure 7B**; for HRMS data see **Supplementary Figures S3**, **S4**). Comparisons between the HPLC spectra from samples of SHP22-7*papR2-OE*, SHP22-7*pRM4*, and SHP22-7 WT displayed that peak intensities were especially increased for plicacetin (mAU 605) and the plicacetin isomer (mAU 259) in samples of SHP22-7*papR2-OE* compared to samples of SHP22-7pRM4 [plicacetin (mAU 120), plicacetin isomer (mAU 97)] and SHP22-7 WT [plicacetin (mAU 66), plicacetin isomer (mAU 57)] (**Figure 7C**). Thus, it could be shown that *papR2* expression in SHP22-7 activates explicitly plicacetin biosynthesis, whereas amicetin biosynthesis seems not to be affected. Overall, with this result it could be confirmed that PapR2 induced cluster 9 transcription, which resulted in an increased plicacetin production. Thus, it can be concluded that the increased bioactivity of SHP22-7*papR2-OE* samples against *B. subtilis* arises from the increased production of the nucleoside antibiotic plicacetin. However, since no direct interaction of the PapR2 regulator with the amicetin promoter region could be shown by EMSA analysis, it cannot be deduced if the activation effect is a direct or an indirect one. The SARP consensus motif harbors the central TCA triad, which is also present in the Pho

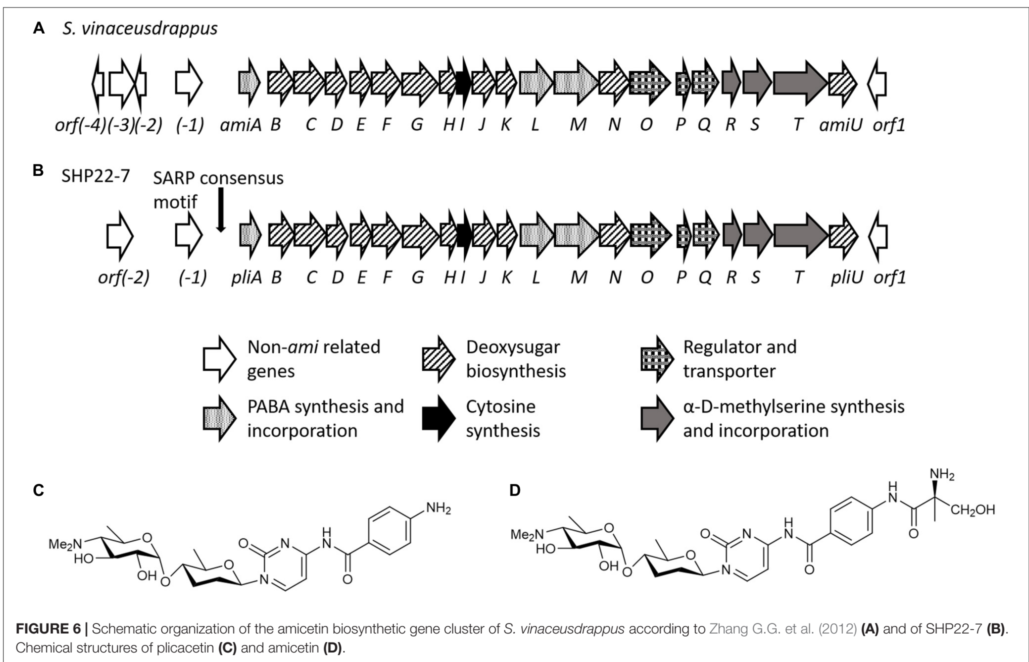

FIGURE 6 | Schematic organization of the amicetin biosynthetic gene cluster of *S. vinaceusdrappus* according to Zhang G.G. et al. (2012) **(A)** and of SHP22-7 **(B)**. Chemical structures of plicacetin **(C)** and amicetin **(D)**.

box (GTTCACC), resembling the target site of the phosphate control two-component system PhoP/PhoR (Martin and Liras, 2020). This sequence region is also known to be bound by the large size SARP regulator AfsR (Martin and Liras, 2020). Additionally, the central TCA triad can be present in binding motifs recognized by the nitrogen regulator GlnR or the DmdR1 (Flores and Martín, 2004; Martin and Liras, 2020). Cross-talk between different transcriptional regulators via the interaction of the same binding sites have been shown before (Martín et al., 2011; Martin and Liras, 2020). Thus, it is also possible that transcriptional activation of the amicetin BGC is an effect from multiple regulatory interactions.

SARP-Type Regulatory Genes Are Widespread in BGCs From Diverse Actinobacteria

Of the top 10 genera containing SARP-type regulators in the antiSMASH database 2 (Blin et al., 2019), 9 belong to the phylum Actinobacteria. Broken up by genus, 98% of *Streptomyces* (611/625), 81% of *Nocardia* (78/96), 100% of *Salinispora* (72/72), 100% of *Micromonospora* (62/62), and 97% of *Amycolatopsis* (38/39) genomes harbor SARP-type regulatory genes, showing their prevalence in filamentous Actinobacteria. On the other hand, only 42% of *Mycobacterium* (115/276) species have SARP-type regulators. Outside of Actinobacteria, mainly Proteobacteria have hits for the SMCOG1041 profile, but mostly lack hits for the HTH motif domain or the BTAD (transcriptional activator domain) [e.g., 6% (79/1236) of *Pseudomonas* hit the smCoG profile, but none of them contain a BTAD match]. Thus, SMCOG1041 profiles from Proteobacteria may not represent typical SARP-type regulators.

Of the total of 6525 proteins containing a hit against any of the four SARP-related PFAM domains, 3289 (50%) are from the order *Streptomycetales* (**Figure 8**). These again break down into 47% "small" SARPs (only containing the HTH and BTAD domains) and 36% "large" SARPs (also containing the NB-ARC and/or TPR domains). The remaining proteins miss the HTH and/or BTAD domains, likely an artifact of bad sequencing data in published draft genomes. The overrepresentation of SARP-type regulators in *Streptomycetales* may also be explained by the higher abundance of available genomes in the database. *Pseudonocardiales* cover 17% of the SARP-type proteins (1080/6525). 31% (330/1080) are "small" SARPs and 39% (421/1080) "large" SARPs. In *Micromonosporales*, accounting for 13% (822/6526) of the dataset, 30% (244/822) are "small" SARPs, and 40% (330/822) are "large" SARPs (**Figure 8**). Interestingly, *Micromonosporales* is the only order to contain a significant amount (22%, 184/882) of SARP-type proteins only containing a hit against BTAD without hitting the HTH domain, suggesting a different subfamily of transcriptional activator. Furthermore, SARP genes were found to be present in various different types of BGCs with a prominent abundance in NRPS

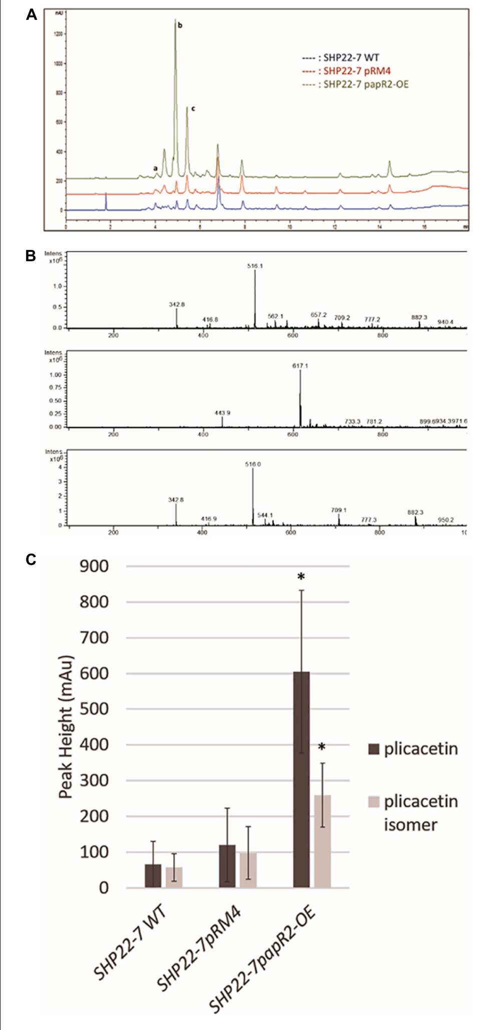

FIGURE 7 | Plicacetin and plicacetin isomer production in the strains SHP22-7 WT, SHP22-7*pRM4*, and SHP22-7*papR2-OE*, respectively. HPLC profile of all three strains. Peaks in the HPLC spectrum representing amicetin are marked with a, plicacetin marked with b, and plicacetin isomer marked with c **(A)**. Mass spectra of plicacetin **(Upper)**, amicetin **(Middle)**, and a plicacetin isomer **(Lower)** **(B)** isolated from SHP22-7 WT. Graphical representation of production levels of plicacetin and plicacetin isomer in each strain. $n = 10$; * means significance **(C)**.

and PKS gene clusters (**Figure 9**), which however might also be associated with the higher frequency of these cluster types in the database.

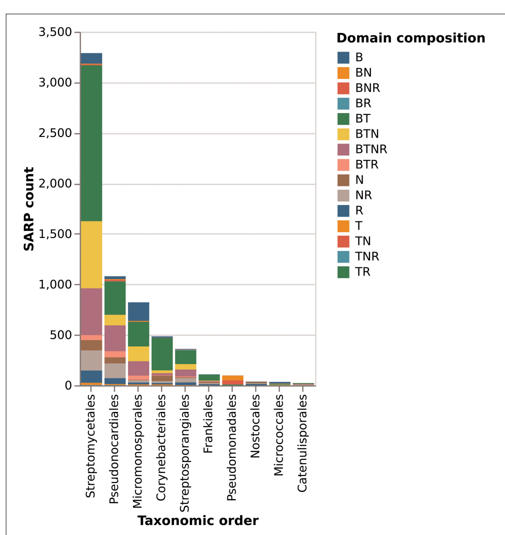

FIGURE 8 | Domain composition of SARP-like proteins in the 10 orders with most protein hits. Letters in the legend denote which domains are present. B is the BTAD domain, T is the HTH-motif DNA binding domain, N is the NB-ARC domain of unknown function, and R is the tetratricopeptide repeat domain.

DISCUSSION

Regulation is the screw plug to unlock the biosynthetic potential of natural compound producers. Here, we showed that heterologous expression of the SARP-type regulatory gene *papR2* in the foreign host *S. lividans* leads to transcriptional activation of the silent Red BGC. Since SARP regulators show a comparable protein architecture and bind to similar recognition sequences at the DNA, they can substitute for their regulatory functions as exemplified for PapR2 and RedD. This is also underpinned by a previous study, where it has been shown that overexpression of the SARP gene *vlmI* from the valanimycin producer *Streptomyces viridifaciens* in a *redD* mutant of *S. coelicolor* M512 restores Red production, demonstrating that *vlmI* can complement a *redD* mutation (Garg and Parry, 2010). Besides having gained indications for such kind of cross-regulation, our data also contribute to a better understanding of the regulation of Red biosynthesis in general. So far, there have been only bioinformatic predictions on potential regulatory binding regions within the Red BGC (Iqbal et al., 2012). Here, we provide first experimental evidence by EMSA studies and (q)RT-PCR experiments for the SARP-binding capability to promoter regions (*PredP*, *PredQ*) of the Red BGC. These data clearly show that *redP* and *redQ* are direct targets of a SARP-driven regulation during Red biosynthesis.

In the literature SARPs often are designated as "pathway-specific" transcriptional activators, which means that they control

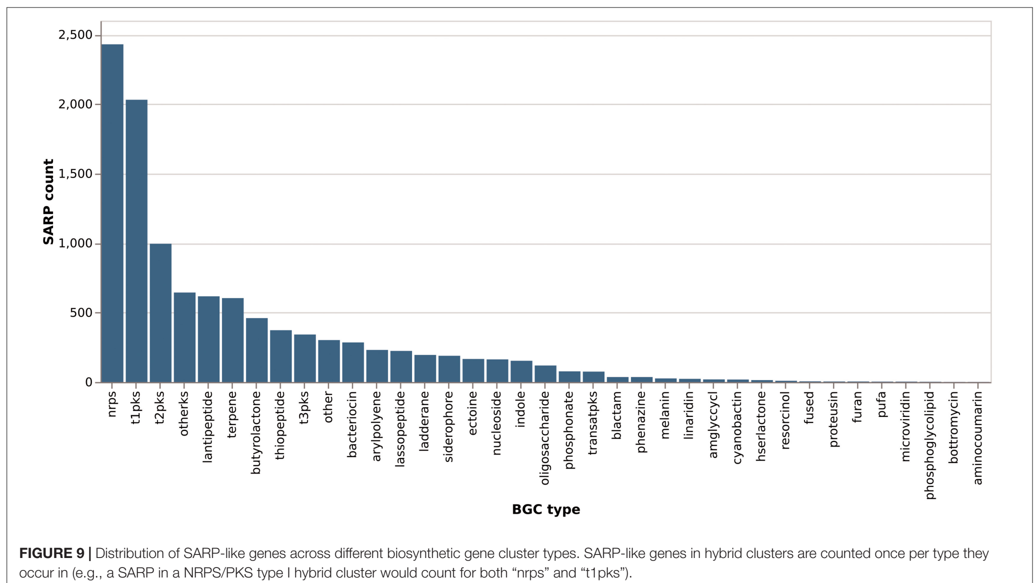

FIGURE 9 | Distribution of SARP-like genes across different biosynthetic gene cluster types. SARP-like genes in hybrid clusters are counted once per type they occur in (e.g., a SARP in a NRPS/PKS type I hybrid cluster would count for both "nrps" and "t1pks").

the expression of an individual BCG. However, SARPs indeed can have activating effects on the biosynthesis of different substances. One example occurring in nature is provided by the SARP-type regulator CcaR from *S. clavuligerus*, which activates the cephamycin gene cluster, where *ccaR* is part of, as well as the adjacent clavulanic acid gene cluster (Pérez-Llarena et al., 1997). Thus, the term "pathway-specific" is not accurate for SARPs and should better be replaced by "cluster-situated" as suggested previously (Huang et al., 2005; Liu et al., 2013). Our results show that SARPs have the potential to control different BGCs, when present in different producer organisms: The SARP regulator PapR2 activates the transcription of the corresponding pristinamycin gene cluster in *S. pristinaespiralis* (Mast et al., 2015) but it also affects different antibiotic BGCs, such as Red and plicacetin, when expressed in foreign strains, such as *S. lividans* or SHP22-7, respectively. Especially, in SHP22-7 *papR2* expression significantly improved plicacetin production and as a result also led to an improved production of a so far not further characterized plicacetin derivative. This derivative is dissimilar from the plicacetin isomer since it shows a different retention time in HPLC analysis (~6.5 min) (**Figure 9**) and a smaller mass of 481 *m*/*z* (data not shown). If PapR2-driven plicacetin gene cluster activation is a direct or an indirect regulatory effect is unclear at the moment since no direct interaction of the PapR2 regulator with the plicacetin gene cluster could be demonstrated. Furthermore, no SARP gene is present in the plicacetin gene cluster. However, SARP genes have been found in four other SHP22-7 BGCs, which would allow the possibility that one of these regulators may act as the natural activator of amicetin/plicacetin biosynthesis. A similar untargeted effect has been observed when the PAS-LuxR-type regulator PimM from *S. natalensis* was expressed in *S. clavuligerus*, which led to an improved production of cephamycin, clavulanic acid, and tunicamycins (Martínez-Burgo et al., 2019). For tunicamycin production the regulatory effect upon *pimM* expression is unclear. *pimM* expression improved tunicamycin production without affecting tunicamycin gene cluster transcription. Besides, no pathway-specific activator has been identified within the tunicamycin BGC. In this study, the authors speculated that PimM may exert its effect on tunicamycin production, e.g., due to a positive influence on precursor supply (Martínez-Burgo et al., 2019). Furthermore, they found that PimM from *S. natalensis* shows some similarity to a PimM-like regulator, encoded by a gene of the *S. clavuligerus* genome (Martínez-Burgo et al., 2019). Thus, also in these analyses the non-native PimM regulator may have occupied the regulatory role of a homologous natural regulator and along this path provoked antibiotic production. Interspecies cross-regulation has also been shown for the LuxR-family type (LAL) regulator PikD. In *Streptomyces. venezuelae* PikD regulates the expression of pikromycin. The heterologous expression of the *pikD* homologous genes *rapH* and *fkbN* from *Streptomyces hygroscopicus* in *S. venezuelae* increased the production of the antibiotic pikromycin. LAL-family regulators resemble SARP-type regulators in the ATP-binding motif at the N-terminus and in the DNA-binding motif to some extent, which in both regulator types consists of an HTH-motif (Mo and Yoon, 2016).

The fact that SARP genes are widely distributed among actinomycetes, where they occur in many different types of secondary metabolite gene clusters makes them good candidates

to be used as engineering tools for the activation of BCGs. Especially in *Streptomyces* they are predominant occurring with a ∼100% abundance. Interestingly, there might be a phylogenetic grouping of different SARP regulator types from different actinobacterial genera. If so this would raise the question if there is a co-evolution of certain regulator genes with their corresponding gene clusters. Besides that, the statistical analysis revealed that there is a different abundance of different types of SARP regulators, belonging either to the small SARPs (<400 amino acids) with only HTH and BTAD domains (e.g., ActII-ORF4 or RedD) or the large SARPs, which contain an additional NTPase domain and/or a conserved C-terminal TPR domain of unknown function (∼1000 residues) (Liu et al., 2013). Overall small SARP type regulators are more abundant in actinomycetes than the large ones (**Figure 8**). Besides, more experimental data are available on small SARP-guided regulations, and the unclear function of the additional domains included in the large SARPs likely causes further constraints in the function of the regulatory activity. Thus, we propose that especially representatives from the group of small SARP regulators are good candidates to be used for activation approaches. As outlined above, SARP regulators can bind to recognition sequences, which occur at various positions within the promoter of the target genes. The variety of binding sites may also reflect the diversity of SARP-type activators. Thus, it would be interesting to bioinformatically group the SARP regulators by taking into account their DNA-binding domains, which may provide a better picture of the different SARP subsets.

That SARP expression can lead to the activation of silent gene cluster expression has been shown in a recent study. Here, the aim was to activate some of the more than 20 cryptic gene clusters from *Streptomyces* sp. MSC090213JE08 (Du et al., 2016). In this study the authors combined an OSMAC approach with the expression of several native SARP genes from *Streptomyces* sp. MSC090213JE08. Thereby, four of the seven generated recombinant SARP expression strains produced nine metabolites that were hardly detected in the control strains. Expression of one of the SARP genes (SARP-7) in *Streptomyces* sp. MSC090213JE08 led to the production of the novel polyene-like substance ishigamide (Du et al., 2016). This study successfully showed the potency of a SARP-guided silent gene cluster activation. However, the drawback of this approach is that several conditions (number of different culture media) need to be tested and a set of genetic manipulations (cloning of each individual SARP gene) has to be done in order to provoke cluster activation. Further experimental setup is then linked to untargeted laborious compound purifications and analytics. Indeed, this is also the main problem of several other efforts to activate secondary metabolite synthesis in actinomycetes as outlined above. Thus, based on our gained knowledge we propose a targeted SARP-guided strategy for the activation of BGCs in actinomycetes. Our strategy involves (1) prioritization of strains with SARP genes and SARP binding motifs in the BGC. In our experimental setup we focus on SARP genes that encode for predicted proteins with high similarity to PapR2 (>55% amino acid sequence similarity), as well as the occurrence of a PapR2 consensus motif within the promoter region(s) of the SARP-gene containing BGCs, (2) introduction of a SARP-expression construct [in our approach this means heterologous expression of the PapR2 regulator with the help of the pGM190/papR2 and/or pRM4/papR2 expression construct(s)], (3) comparative biological and chemical analyses of SARP-activated expression samples with samples from non-manipulated strains. In our study we focus on PapR2 as the activator brick, however, of course any other type of SARP regulator with a known consensus sequence might be used as the basis for such an activation approach. For sure there might be limitations in such an activation strategy, e.g., transcriptional activation may fail due to SARP-specificity reasons or a lack of a broader set of well-characterized SARP regulators to be tested as candidate elicitors. However, here we disclose a screening idea or a kind of dragnet investigation, which does not aim to cover all possible SARP-regulated clusters but highlights the most probable ones to be activated upon SARP expression. Our strategy has two main advantages: (1) In contrast to completely unspecific cluster activation efforts, such as addition of general elicitors, co-culture approaches or the OSMAC strategy, our approach is more targeted, as it focuses only on a defined set of BGCs, namely those ones that are bioinformatically predicted to be under SARP control. Applying combinatory bioinformatics, such as antiSMASH and PatScan allows to directly identify the associated BGC. In addition, the detection of the activated compound is more straightforward if the outline of the structure can be deduced from the cluster sequence. (2) In contrast to activation strategies that are absolutely specific for the respective BGC, e.g., heterologous expression of the BGC, introduction of artificial promoters in front of the BGC, or the manipulation of cluster-situated regulators, no major effort to manipulate the genome is necessary with our procedure since it only involves cloning of one SARP gene-containing expression construct into the respective strain(s) of interest. These major benefits make the activation of BGCs by SARPs a promising strategy to be applied on putative antibiotic producers.

DATA AVAILABILITY STATEMENT

The complete genome sequence of *S. lividans* T7 has been deposited at DDBJ/ENA/GenBank under the accession number ACEY00000000. The main genome scaffold sequence of SHP22-7 has been deposited at DDBJ/ENA/GenBank under the accession number QXMM00000000. Raw sequencing data are available under SRA accession number PRJNA489221.

AUTHOR CONTRIBUTIONS

YM generated the strains *SLpGM190* and *SLpapR2-OE*, carried out the bioinformatic analyses, and designed, supervised, and coordinated the study. KB performed the statistical analyses. JK created the vector pRM4/papR2 while the vector was inserted in SHP22-7 by IH. IH tested the bioactivity. JK performed all qualitative and quantitative transcriptional analyses. AK and IH performed the HPLC analyses. YM, JK, IH, and KB wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00225/full#supplementary-material

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The reviewer JA declared a past co-authorship with one of the authors AK to the handling Editor.

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Article

Mining Indonesian Microbial Biodiversity for Novel Natural Compounds by a Combined Genome Mining and Molecular Networking Approach

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Abstract: Indonesia is one of the most biodiverse countries in the world and a promising resource for novel natural compound producers. Actinomycetes produce about two thirds of all clinically used antibiotics. Thus, exploiting Indonesia's microbial diversity for actinomycetes may lead to the discovery of novel antibiotics. A total of 422 actinomycete strains were isolated from three different unique areas in Indonesia and tested for their antimicrobial activity. Nine potent bioactive strains were prioritized for further drug screening approaches. The nine strains were cultivated in different solid and liquid media, and a combination of genome mining analysis and mass spectrometry (MS)-based molecular networking was employed to identify potential novel compounds. By correlating secondary metabolite gene cluster data with MS-based molecular networking results, we identified several gene cluster-encoded biosynthetic products from the nine strains, including naphthyridinomycin, amicetin, echinomycin, tirandamycin, antimycin, and desferrioxamine B. Moreover, 16 putative ion clusters and numerous gene clusters were detected that could not be associated with any known compound, indicating that the strains can produce novel secondary metabolites. Our results demonstrate that sampling of actinomycetes from unique and biodiversity-rich habitats, such as Indonesia, along with a combination of gene cluster networking and molecular networking approaches, accelerates natural product identification.

Keywords: Indonesia; biodiversity; novel antibiotics; drug screening; bioactivity; gene cluster networking; GNPS

1. Introduction

It is now 80 years ago that Selman Waksman and Boyd Woodruff discovered actinomycin from *Actinomyces (Streptomyces) antibioticus*, which was the first antibiotic that was isolated from an actinomycete [1]. Since then, actinomycetes have been widely used as sources for drug discovery and development [2]. Most antibiotics and other useful natural products applied in human medicine, veterinary, and agriculture are derived from these filamentous bacteria [3,4]. Within the family of Actinomycetales, *Streptomyces* is the most prominent genus in respect to the production of bioactive secondary metabolites since it is the origin of more than 50% of all clinically useful antibiotics [5]. Successfully, the intensive screening campaigns of soil-derived streptomycetes yielded many currently recognized drugs, such as the antibacterial substance streptomycin, the antifungal metabolite nystatin, and the anticancer compound doxorubicin during the golden era of antibiotics [6,7]. However, in the last few decades, discovering and developing new drugs from these soil microorganisms has declined immensely, while the need for new drugs to overcome multidrug resistance has become greater than ever [8]. Nowadays, one of the major problems in antibiotic screening programs, in particular with streptomycetes, is the high rediscovery rate of already-known antibacterial compounds through the classical bioactivity-guided paradigms [3].

Sampling actinomycetes from conventional environments such as soils often leads to the rediscovery of known species producing already-known antibiotics [9]. Thus, gaining access to unusual unique habitats with the pursuit to isolate new strains as sources of novel bioactive compounds represents a current barrier in drug discovery research [9]. In recent years, the bioprospection of underexplored niches such as extreme or marine environments has become an efficient approach to find novel *Streptomyces* species that might produce novel compounds [10,11]. *S. asenjonii* strain KNN 42.f, isolated from a desert soil sample, is one example of a novel *Streptomyces* species from an extreme habitat, which produces the three new bioactive compounds asenjonamides A–C [12]. Another example displays the marine *S. zhaozhouensis* CA-185989 that produces three new bioactive polycyclic tetramic acid macrolactams [13]. *Micromonospora* sp. as turbinimicin producer represents a further example of prolific marine bacteria that can deliver new antifungal compounds [14]. These are only a few examples demonstrating that unusual or aquatic territories can be promising avenues as new natural products reservoirs.

Indonesia is the world's largest archipelagic country, spanning into three time zones, covering more than 17,000 islands, with 88,495,000 hectares of tropical forest, 86,700 square kilometers of coral reefs, and 24,300 square kilometers of mangrove areas [15,16]. It has the second-highest level of terrestrial biodiversity globally after Brazil [17], while being ranked as first if marine diversity is taken into account [16,17]. With the given species-rich flora and fauna besides endemic and ecologically adapted species, mega biodiversity of microbial species is gratifyingly represented across various unique habitats [18–20], such as acidic hot springs [21], peatland forests [22], the Thousand Islands reef complex [23], Enggano Island [24], fish species [25], and leaves of traditional medicinal plants [26]. Thus, since unique Indonesian niches are expected to deliver untapped potential actinomycetal strains that may produce novel bioactive secondary metabolites, different locations were targeted for the sampling of actinomycetes in this study.

The latest analyses of genome sequence data from actinomycetes revealed a remarkable discrepancy between the genetic potential of the secondary metabolism, known to be encoded by biosynthetic gene clusters (BGCs), and the actual natural compound production capacity of such isolates, upon their growth under standard laboratory conditions. This is attributed to the fact that numerous BGCs are not expressed under conventional lab

parameters and occur as so-called "silent" or "cryptic" BGCs [27]. The activation of these silent clusters allows one to unlock the chemical diversity of the tested organisms and enables the discovery of new molecules for medical and biotechnological purposes [28]. Thus, several efforts, e.g., involving genetic and cultivation methods, are employed to activate the expression of silent gene clusters [29]. One cultivation-based approach to exploit the metabolic capacity of the natural compound producers is the "one strain many compounds (OSMAC)" strategy [3,28,30]. Such a strategy simply relies on the variation of media compositions as a basis to test for different natural compound production profiles since global changes in the specialized metabolic pathways can occur under variable cultivation conditions [31]. The OSMAC concept represents a well-established model that was suggested nearly two decades ago; however, it still leads to the discovery of new chemotypes, such as the novel aromatic polyketide lugdunomycin from *Streptomyces* sp. QL37 [32] or an eudesmane sesquiterpenoid and a new homolog of the Virginiae Butanolides (VB-E) from from *Lentzea violacea* strain AS 08 [33]. Along the lines of the OSMAC concept, an elicitor screening approach has recently been suggested, which intends to mimic natural trigger molecules that can induce the biosynthesis of formerly unknown metabolites. This format has been conducted in a high-throughput approach and was coupled with MALDI-MS analysis. In the case of *S. ghanaensis*, this strategy led to the discovery of the antibiotically active depsipeptide cinnapetide [34].

Besides the variable trials to elicit the BGCs via pleiotropic approaches, a mass spectrometry dereplication step is frequently included in the current screening programs to address the formerly stated challenge of the high rediscovery rate prior to the tedious screening, isolation, and purification processes [35–37]. The utility of such a platform is to pinpoint known compounds in the initial phase of the discovery pipeline and leverage the process of finding new drugs. Integrated genomic and metabolomic mining methods have proven as an efficient dereplication strategy for compound identification in recent years [38–41]. While genome mining involves the identification of putative BGCs based on the genome sequences of the natural compound producers [42,43] using in silico bioinformatics tools such as antiSMASH [44], metabolome mining encompasses sorting out the chemical compounds in extracts of natural compound producers via their mass fragmentation patterns. Counting on the fact that metabolites with a similar chemical architecture tend to generate similar mass fragmentation patterns in mass spectrometry (MS) analysis, the implementation of the computational platform Global Natural Product Social (GNPS) to group the structurally related entities, often derive from a common biosynthetic origin, as a connected set of a molecular family cluster is an overgrowing necessity [45]. Such a platform iteratively proves its effectiveness to arrange seamlessly large numbers of samples enabling dereplication and tentative structural identification and/or classification [46]. The combinatorial employment of both computational tools side by side empowers the rapid identification of new substances, which can be highlighted by discovering the antibacterial substance thiomarinol from *Pseudoalteromonas luteoviolacea* [38] and microviridin 1777, a chymotrypsin inhibitor from *M. aeruginosa* EAWAG 127a [47].

Taken all together with the promises that highly biodiverse habitats can offer in synergy with an effective and practical mining technique, this study aimed to characterize the secondary metabolomes of selected actinomycetes isolated from three different locations within Indonesia. A collection of 422 actinomycetes from Lombok, Bali, and Enggano Islands were sampled and preliminary filtered with different bioactivity tests, where nine actinomycetes with the most bioactive potential were nominated for a hybrid genome mining and molecular networking approach in order to assess their biosynthetic capacity for the production of novel natural compounds.

2. Results and Discussion

2.1. Isolation and Characterization of Indonesian Actinomycetes

To isolate actinomycetes, soil samples were collected from two specific habitats (terrestrial and marine) in three different geographic areas of Indonesia using standard isolation

protocols [48–52]. Enggano Island was chosen as a sampling location for terrestrial habitats since it is a pristine island with many endemic species and high biodiversity [53,54], whereas Bali and Lombok Island were selected as sampling sites for marine habitats resulting in 422 strains in total (Table 1). Among all sampling locations, the Enggano Island soil samples contributed to the highest number of actinomycetes isolates (56.2%), followed by sediment samples from Lombok (37.2%) and Bali island (6.6%).

Table 1. Indonesian strains, isolation method, source of isolation (compare Figure 1), and most closely related species (%) based on 16S rRNA gene sequence phylogenetic analysis with EzTaxon.

Strain (*Streptomyces* sp.)	Isolation Method	Source of Isolation	Most Closely Related Species Based on 16S rDNA Analysis
SHP 22-7	phenol	Soil under a ketapang tree (*Terminalia catappa*) from Desa Meok (B1), Enggano Island	*Streptomyces rochei* NBRC 12908^T (99.59%)
SHP 20-4	phenol	Soil under a kina tree (*Cinchona* sp.), Desa Banjarsari (B2), Enggano Island	*Streptomyces hydrogenans* NBRC 12908^T (99.68%)
SHP 2-1	phenol	Soil under a hiyeb tree (*Artocarpus elastica*) near Bak Blau water spring, Desa Meok (B3), Enggano Island	*Streptomyces griseoluteus* NBRC 13375^T (98.96%)
DHE 17-7	dry heat	Soil under a ficus tree (*Ficus* sp.), Desa Boboyo (B4), Enggano Island	*Streptomyces lannensis* TA4-8^T (99.78%)
DHE 12-3	dry heat	Soil under a cempedak tree (*Artocarpus integer*), Desa Boboyo (B4), Enggano Island	*Streptomyces coerulescens* ISP 51446^T (98.87%)
DHE 7-1	dry heat	Soil under a terok tree (*Artocarpus elastica*), desa Boboyo (B4), Enggano Island	*Streptomyces adustus* WH-9^T (99.59%)
DHE 6-7	dry heat	Soil under forest snake fruit tree (*Salacca* sp.), Desa Malakoni (B5), Enggano Island	*Streptomyces parvulus* NBRC 13193^T (98.55%)
DHE 5-1	dry heat	Soil under a banana tree (*Musa* sp.), Desa Banjar sari (B2), Enggano Island	*Streptomyces parvulus* NBRC 13193^T (99.79%)
BSE 7-9	NBRC medium 802	Mangrove sediment near plant rhizosphere, Kuta (C1), Bali Island	*Streptomyces bellus* ISP 5185^T (99.06%)
BSE 7F	NBRC medium 802	Mangrove sediment near plant rhizosphere, Kuta (C1), Bali Island	*Streptomyces matensis* NBRC 12889^T (99.72%)
I3	humic acid-vitamin + chlorine 1%	Mangrove sediment from Pantai Tanjung Kelor, Sekotong (D2), West Lombok Island	*Streptomyces longispororuber* NBRC 13488^T (99.23%)
I4	humic acid-vitamin + chlorine 1%	Mangrove sediment from Pantai Tanjung Kelor, Sekotong (D2), West Lombok Island	*Streptomyces griseoincarnatus* LMG 19316^T (99.89%)
I5	humic acid-vitamin + chlorine 1%	Mangrove sediment from Pantai Tanjung Kelor, Sekotong (D2), West Lombok Island	*Streptomyces viridodiasticus* NBRC13106^T (99.31%)
I6	humic acid-vitamin + chlorine 1%	Mangrove sediment from Pantai Tanjung Kelor, Sekotong (D2), West Lombok Island	*Streptomyces spongiicola* HNM0071^T (99.78%)
I8	humic acid-vitamin	Sea sands from Pantai Koeta (D3), Lombok Island	*Streptomyces smyrnaeus* SM3501^T (98.44%)
I9	humic acid-vitamin	Sea sands from Pantai Koeta (D3), Lombok Island	*Streptomyces gancidicus* NBRC 15412^T (98.82%)

Within the frame of a preliminary bioactivity screening, all 422 isolates were evaluated for their antimicrobial activities in agar plug diffusion bioassays against selected Gram-positive (*Bacillus subtilis*, *Micrococcus luteus*, and *Staphylococcus carnosus*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas fluorescens*). The 16 most potent isolates were selected based on their antimicrobial activity against the tested organisms, indicated by the largest inhibition zones around the agar plug. All 16 isolates showed bioactivity against the Gram-positive test organism *B. subtilis* (Figure 2A), and nine exerted further activity against Gram-negative test strains (Figure 2B), while only four strains (BSE 7–9, BSE 7F, I3, and I6) displayed potency against both (Figure 2).

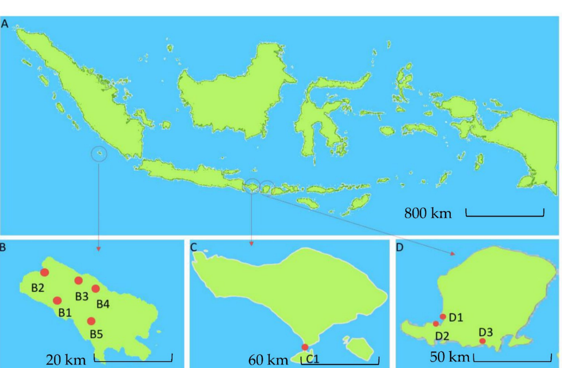

Figure 1. Map of Indonesia showing three geographical regions (**A**). Sampling site location in Enggano Island (**B**), Bali Island (**C**), and Lombok Island (**D**). Red dot shows the sampling locations at Enggano Island, B1: Desa Meok; B2: Desa Banjar Sari; B3: Bak Blau Waterspring, Desa Meok; B4: Desa Boboyo; B5: Desa Malakoni; at Bali Island C1 for Kuta; and Lombok Island D1: Pantai Cemara, Lembar; D2: Pantai Tanjung Kelor, Sekotong; D3: Pantai Koeta.

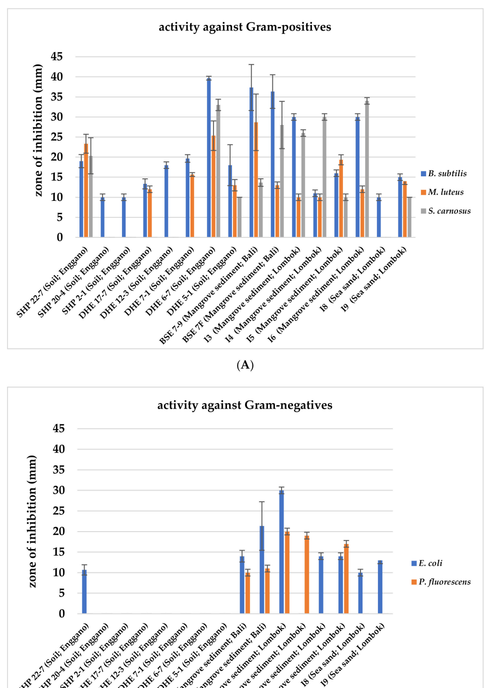

Figure 2. Antimicrobial bioassays with 16 Indonesian actinomycetes strain samples against Gram-positive (**A**) and Gram-negative test strains (**B**). Inhibition zone diameters of agar plug test assays are given in mm. Agar plugs were used after ten days of growth of the respective actinomycetes strains. Data shown are as the result of three independent biological replicates.

To investigate the phylogenetic relationship of the 16 bioactive actinomycetal isolates, 16S rRNA gene sequence analyses were performed. For this purpose, the genomic DNA was isolated from each and was used as a template in a PCR approach with 16S rRNA gene-specific primers. The resulting 16S rRNA gene amplifications were sequenced, and the 16S rRNA gene sequences were compared using the EzTaxon database (www.ezbiocloud.net/, accessed on 28 May 2018) to determine the phylotype of the strains [55]. EzTaxon analysis revealed that all isolates belong to the genus *Streptomyces* with similarity values amongst the various predicted related species ranging from 98.44–99.89% (Table 1).

Subsequently, nine strains were prioritized based on their bioactivity profile and taxonomic position. Strains SHP 22-7, BSE 7-9, BSE 7F, I3, I4, I5, and I6 were selected since they showed antibacterial activity against Gram-positive and Gram-negative bacteria (Figure 2A,B). DHE 17-7 and DHE 7-1 were selected as they exerted bioactivity against at least two different Gram-positive test strains. DHE 6-7 and DHE 5-1, which showed bioactivity against all Gram-positive test strains, were not chosen for further analysis because both strains showed a close phylogenetic relationship to *Streptomyces parvulus* (Table 1), which is a known producer of the polypeptide antibiotic actinomycin D [56]. In an initial attempt with HPLC-MS analysis of the methanolic extracts of culture samples from DHE 6-7 and DHE 5-1, actinomycin D was detected as a product (Figure S1), ruling out both strains from further investigations.

2.2. Phylogenomic Analysis of Nine Prioritized Indonesian Streptomyces Strains

To obtain a better understanding of the phylogenetic relationship about the prioritized nine *Streptomyces* strains, a phylogenetic analysis based on their full-length genomes sequences was performed. For this purpose and the genome mining studies mentioned below, the genomic DNA was isolated from each sample and sequenced by using the Pacific Biosciences RS II (PacBioRSII) platform [57–59]. The resultant genome sequences ranged in sizes between 7.05 Mbp (*Streptomyces* sp. I6) and 8.36 Mbp (DHE 17-7) and GC contents between 72.08% (DHE 7-1) and 72.47% (*Streptomyces* sp. I6) (Table S1), which share comparable values reported for *Streptomyces* species (genome sizes of 6-12 Mb [60] and GC contents of 72–73% [61,62]).

In order to run a whole-genome phylogenetic analysis, the genome sequences were submitted to the Type (Strain) Genome Server (TYGS) (https://tygs.dsmz.de, accessed on 13 December 2019) [63], which allows a phylogenetic analysis based on full-length genome sequences and compares genomic data with the database genomes. The resulting phylogenetic information is more authentic than those obtained from 16S rDNA- or multi-locus sequence analysis (MLSA)-based classifications, which only use small sequence fragments as a basis for sequence comparisons [63]. The TYGS analysis provides information on the similarity of a strain to its nearest related type strain, derived from the digital DNA-DNA hybridization (dDDH) values calculated by the genome-to-genome distance calculator (GGDC) 2.1 (http://ggdc.dsmz.de, accessed on 13 December 2019) [64]. TYGS phylogenomic analysis revealed that all nine isolates belong to the genus *Streptomyces*. The dDDH values between the nine Indonesian strains and their closest relatives ranged between 31.4% (*Streptomyces* sp. I4) and 51.5% (*Streptomyces* sp. I6) (using GGDC distance formula *d4*) (Table 2), which is below the threshold of 70% used for species delineation [65,66], proposing a novel collection of *Streptomyces* species.

Table 2. Data from pairwise comparisons between genome sequences from nine Indonesian strains and their closest related strains based on dDDH analysis. "Query strain" refers to analyzed strain, and "subject strain" refers to most closely related Indonesian strain sample. Degree of relatedness is given as dDDH distance formula *d4* as previously described by Meyer-Kolthoff et al. [66].

Query Strain	Subject Strain	dDDH (d_4, in %)
I3	I4	99.6
BSE 7-9	BSE 7F	95.7
DHE 17-7	SHP 22-7	86.7
I4	I5	82.6
I3	I5	82.5
BSE 7F	I5	78.4
BSE 7-9	I5	78.4
BSE 7-9	I4	77.2
BSE 7F	I4	77.2
BSE 7F	I3	77
BSE 7-9	I3	77
I6	*Streptomyces spongiicola* HNM0071	51.5
SHP 22-7	*Streptomyces luteus* TRM 45540	43.6
DHE 17-7	*Streptomyces luteus* TRM 45540	40.3
DHE 7-1	*Streptomyces bungoensis* DSM 41781	32.3
I3	*Streptomyces capillispiralis* DSM 41695	31.5
BSE 7-9	*Streptomyces capillispiralis* DSM 41695	31.5
I5	*Streptomyces capillispiralis* DSM 41695	31.5
I4	*Streptomyces capillispiralis* DSM 41695	31.4
BSE 7F	*Streptomyces capillispiralis* DSM 41695	31.4

According to the TYGS phylogenomic tree, the terrestrial Enggano Island strains SHP 22-7 and DHE 17-7 belong to the same clade (clade A) (Figure 3) and most likely resemble the same type of species with a dDDH value of 86.7% (Table 2). Both bacteria are found to be closely related to *S. luteus* TRM 45540, isolated from a soil sample from China [67]. All mangrove isolates originating from sediments of Lombok Island (*Streptomyces* sp. I3, I4, and I5) and Bali Island (BSE 7F, and BSE 7-9) were allied in clade B, suggesting a correlative connection (Figure 3). Additionally, the dDDH analysis showed that BSE 7F is closely related to BSE 7-9 with a value of 95.7% and thus most likely represent the same subspecies (Table 2), while *Streptomyces* sp. I3 and I4 probably represent the same species having a dDDH score of almost 100% (Table 2). The nearest related type strain of all five mangrove strains is *S. capillispiralis* DSM 41695 isolated from a Sweden soil sample [68].

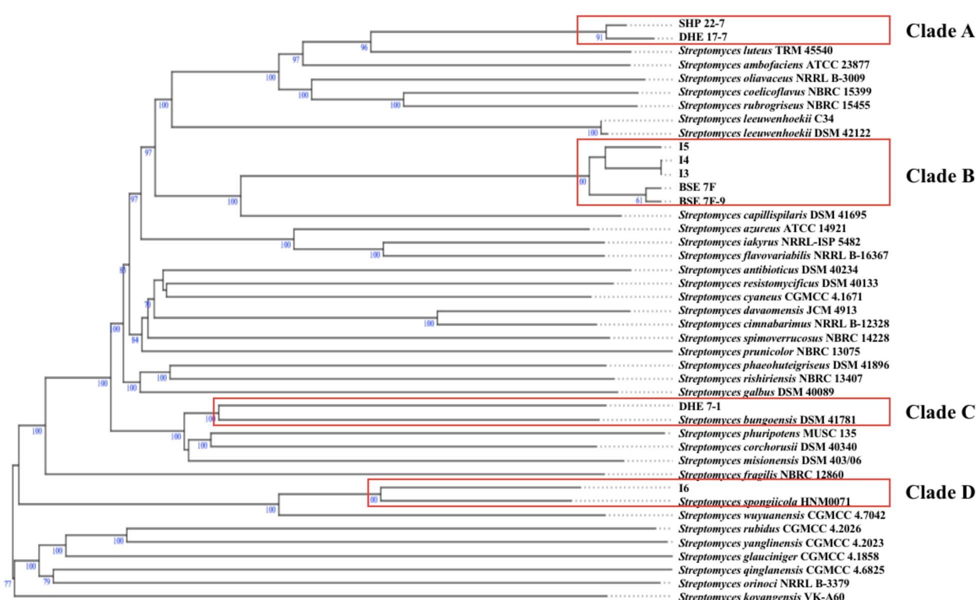

Figure 3. Whole-genome sequence tree generated with the TYGS web server for nine Indonesian *Streptomyces* isolates (highlighted by red boxes) and closely related type strains. Tree inferred with FastME from GBDP distances was determined from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d_5. The numbers above branches indicate GBDP pseudobootstrap support values > 60% from 100 replications, with an average branch support of 84.4%. The tree was rooted at the midpoint.

By contrast, the soil sample DHE 7-1 and mangrove *Streptomyces* sp. I6 were found to group separately in distinct clades (clade C and D, respectively) (Figure 3). The soil *S. bungoensis* DSM 41781 collected in Japan [69] shares a dDDH value of 32.3% as the closest related strain to DHE 7-1 (Table 2), while the nearest related neighbor of *Streptomyces* sp. I6 is *S. spongiicola* HNM0071, isolated from a marine sponge collected from China [70] with a dDDH value of 51.5% (Table 2). Additional information on the specific polyphasic characteristics of the representative type strains from each clade can be found in the Supplementary Material. Altogether, 16S rRNA gene-based phylogenetic and phylogenomic studies revealed that all nine prioritized isolates belong to the genus *Streptomyces* and, based on dDDH analysis, represent novel species (Figure 3).

2.3. Genetic Potential for Secondary Metabolite Biosynthesis of Nine Indonesian Streptomyces Strains

To infer the genetic potential of the strains for the biosynthesis of secondary metabolites, the genomes were analyzed bioinformatically using the web tool antiSMASH version 5.0 (https://antismash.secondarymetabolites.org, accessed on 13 November 2019) [44]. The antiSMASH analysis yielded a sum of 206 potential BGCs for the nine isolates (Table 3) with the lowest BGC count of 17 for strain *Streptomyces* sp. I3 and the highest number of 30 BGCs for strain DHE 17-7 (Table 3). On average, this makes 23 BGCs per strain, which is lower than the average value of 40 BGCs reported for *Streptomyces* genomes [71]. However, the lower BGC count is most likely a result of the underlying PacBio genome sequences, which generally yield less contigs than other sequencing technologies, resulting in less interrupted BGCs and thus less BGC counts in antiSMASH analyses. The genome of DHE 17-7 exhibited a slight correlation between genome size (8.4 Mbp) and the observable number of BGCs

(30 BGCs) (Table 3 and Table S1). Several of the identified BGCs from the nine Indonesian isolates showed a high similarity (>60%) to already-known BGCs (Figure 4), e.g., all strains harbored BGCs encoding compounds that are commonly produced by streptomycetes, such as desferrioxamine, which is a vital siderophore for the growth and development [72], hopene, as a substance of the cytoplasmic membrane modulating membrane fluidity and stability [73], and a spore pigment for protection against UV radiation [74]. This result is consistent with previous observations, where these BGCs have been reported for most analyzed *Streptomyces* genomes [71]. Ectoine and geosmin BGCs were found in all Indonesian isolates except for *Streptomyces* sp. I6 (Figure 4). Moreover, albaflavenone BGCs were uncovered in all strains, excluding *Streptomyces* sp. I6 and DHE 7-1. Interestingly, in the genomes of the mangrove-derived isolates BSE 7F, BSE 7-9, I3, I4, and I5, two ectoine BGC were identified, suggesting that the additional ectoine BGCs may play a role in the adaptation of these organisms to the osmotic stress of such high-salinity environments.

Table 3. List of Indonesian actinomycetes strains with number and type of BGCs as predicted by antiSMASH analysis.

Strain	Total BGCs	PKS	NRPS	Hybrid BGC	Terpene	RiPP	Siderophore	Others
DHE 17-7	30	6	7	-	6	3	3	5
DHE 7-1	27	6	6	3	5	-	3	4
SHP 22-7	25	5	6	1	4	1	2	6
I4	19	3	2	-	4	4	2	5
I3	17	4	2	1	4	2	2	3
I5	19	3	2	1	4	4	2	3
BSE 7F	23	3	1	4	5	4	2	4
BSE 7-9	22	4	2	3	4	1	2	6
I6	24	3	6	1	2	1	2	9

Figure 4. Presence (grey color) and absence (white color) of BGCs in nine Indonesian strains as predicted by antiSMASH analysis with similarity above 60%.

Aborycin and alkylresorcinol gene clusters were discovered in the five mangrove *Streptomyces* strains, whereas amicetin, candicidin, coelichelin, and fluostatin M-Q BGCs were only detected for the soil-based isolates SHP 22-7 and DHE 17-7. Candicidin, as an example of a fungizide [75], is most likely produced by terrestrial streptomycetes in order to defend themselves against local fungal competitors. Coelichelin is a further spotted siderophore which might be necessary for the soil-living streptomycetes to sequester poorly soluble environmental Fe^{3+} [76], which is quite scarce and highly contested by other microorganisms in soils. The discovery of the same BGC composition in strains derived

from the same habitat, such as soil or mangroves, is probably attributed to the fact that each biosynthetic product has its specific biochemical relevance in the respective environment. Of the nine strains, only *Streptomyces* sp. I6 harbored a staurosporine, scabichelin, echinomycin, flaviolin, and tirandamycin BGC. Likewise, DHE 7-1 together with *Streptomyces* sp. I6 were the only representatives comprising an isorenieratene BGC among the nine strains (Figure 4). Both strains, I6 and DHE 7-1, were found to be phylogenetically distant from the other strains (Figure 3), outlining that phylogenetically related isolates tend to have similar biosynthetic elements known as BGCs shaped by the environmental conditions. A similar finding has already been made by Meij et al., who reported that ecological conditions play an important role in controlling the formation of secondary metabolites in actinomycetes [77].

To glean a more detailed picture about the BGC distribution amongst the strains, the genome sequences from the nine strains have been analyzed using the BiG-SCAPE software (https://bigscape-corason.secondarymetabolites.org/, accessed on 13 November 2019) [78]. BiG-SCAPE allows fast computation and visual exploration of BGC similarities by grouping BGCs into gene cluster families (GCF) based on their sequences and Pfam protein families similarities [79]. Comparing all shared BGCs within the nine Indonesian strains with BiG-SCAPE allows visualization of the more common BGCs (large nodes) and the less frequent ones (doubletons, (singletons are not shown) (Figure 5). With this approach, we visualized the occurrence of eight GCFs with a similarity of less than 60% similarity to known BGCs as predicted by antiSMASH. Ectoine-butyrolactone-NRPS-T1PKS GCF, which has similarities with polyoxypeptin (48%) or aurantimycin A (51%), was distributed among strains I3, I4, I5 and BSE 7F (Figure 5, Tables S6, S7, S9 and S10). A type III polyketide (T3PKS) GCF was shared amongst the strains DHE 17-7, SHP 22-7, and DHE 7-1, which showed 7–8% BGC similarity to the herboxidiene BGC (Figure 5, Tables S4, S5 and S11). In the strains *Streptomyces* sp. I3 and I4 of clade B, we found the others-type I polyketide (otherks-T1PKS), which showed 48–55% BGC similarity to the nataxazole BGC, and an aminoglycoside/aminocyclitol (amglyccycl) BGC type, which led to 2% similarity to the BGC of cetoniacytone A (Figure 5, Tables S6 and S7). We identified two unique GCFs in the strains BSE 7F and BSE 7-9 of clade B, namely a transAT-PKS GCF, which showed 54–58% similarity to the weishanmycin and phenazine BGC types, and did not show any similarity to any BGC in the antiSMASH database (Figure 5, Tables S9 and S10). Moreover, we detected two GCFs of an indole, which showed 23–33% BGC similarity to the 5-isoprenylindole-3-carboxylate β-D-glycosyl ester BGC and other BGC type, which do not belong to any BGCs in the antiSMASH database for the phylogenetically related species of SHP 22-7 and DHE 17-7 of clade A (Figure 5, Tables S4 and S5). Altogether, the Big-SCAPE analysis revealed eight unique GCFs, which could not be associated with known BGCs and may have the potential to encode for new substances. Furthermore, the obtained data disclosed that phylogenetically related strains derived from a similar environmental habitat tend to share similar BGC composition profiles. Inferred from this observation, one can conclude that it is worth it to make an effort to sample actinomycetes from unique environmental habitats, since this may lead to the isolation of phylogenetically unique species, which have a higher potential of producing novel natural compounds, as also previously described by Hug et al. [9].

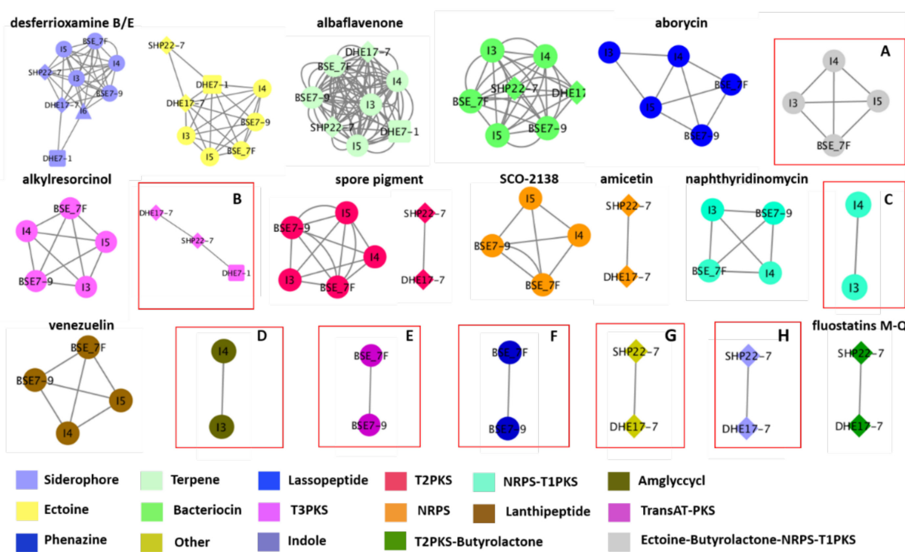

Figure 5. Similarity network of the predicted biosynthetic gene clusters (BGCs) of the nine Indonesian *Streptomyces* strains. Shared similar BGCs are indicated by a connected line. Each node represents a specific BGC type (labeled with different colors). The shape node represents the same species, i.e., clade A (SHP 22-7 and DHE 17-7) indicated with diamond, clade B (I4, I5, BSE 7F, and BSE 7-9) shown with ellipse, clade C (DHE 7-1) with a cube, and clade D (I6) indicated with a triangle. BGCs with similarities less than 60% are highlighted by red boxes: (**A**) Ectoine-butyrolactone-NRPS-T1PKS; (**B**) T3PKS; (**C**) Otherks-T1PKS, (**D**) Amglyccyc; (**E**) TransAT-PKS; (**F**) Phenazine; (**G**) Other; and (**H**) Indole.

2.4. Optimal Cultivation Conditions for Compound Production of Nine Indonesian Streptomyces Strains

In order to infer the biosynthetic capacity of the prioritized nine isolates in a bioactivity context, various media following the OSMAC strategy were screened to define the optimal production conditions [30,31]. For this purpose, SHP 22-7, DHE 17-7, DHE 7-1, BSE 7-9, BSE 7F, I3, I4, I5, and I6 were each grown in twelve different liquid cultivation media (SGG, YM, OM, R5, MS, TSG, NL19, NL300, NL330, NL500, NL550, and NL800 (Table S2)), and culture samples were harvested at different time points (48, 72, 96, and 168 h). Cell cultures were extracted with ethyl acetate, concentrated in vacuo, and then re-dissolved in methanol. Methanolic extracts were tested in bioassays against a selected panel of pathogenic strains *B. subtilis, M. luteus, S. carnosus, E. coli,* and *P. fluorescens*. Samples with the largest inhibition zones in bioassay tests were defined as the ones grown under optimal cultivation conditions. For each Indonesian *Streptomyces* strain, the optimal production conditions have been defined for cultivation in liquid media (Table S3). In addition, it is hypothesized that filamentous actinomycetes as soil organisms grow and develop better on solid nutrient substrates and that a well-grown healthy culture produces more diverse secondary metabolites [80]. Thus, to extend the probability of finding new substances by exploring the biosynthetic potential of the nine strains for secondary metabolite production, we recruited an antibiotic extraction also from solid media. For this purpose, each isolate was spread on agar plates consisting of the respective abovementioned media and incubated for 7–10 days at 28 °C until spores formed. Grown agar samples were squeezed out and concentrated. The aqueous phase of the solid medium extract was used for bioassays and further chemical analysis.

For *Streptomyces* sp. I3 and I4, the same cultivation parameters were found to be optimal. Both strains showed a promising potency upon their growth in liquid NL550

medium for 72 h on solid MS medium (Table S3). Such similar production behavior might be ascribed to their most possible likelihood to represent the same species as suggested above. Furthermore, we found that most of the nine isolates (*Streptomyces* sp. I3, I4, I5, and I6) produced best on solid MS medium (Table S3). In general, MS is a suitable medium for streptomycetes regarding spore isolation [81]. This would support the hypothesis that strains produce better, when they show healthy growth and development.

2.5. Identification of Natural Compounds from Nine Indonesian Actinomycetes

To putatively identify the specialized bioactive substances which are produced by the nine isolates under the various conditions, the culture extract samples were submitted to high-resolution mass spectrometry (HRMS) coupled with the GNPS platform. For this purpose, the obtained extracts from the optimal medium in liquid and solid were firstly fractionated by solid-phase extraction (SPE) and then qualitatively profiled against their main crudes and media controls using HPLC. Subsequently, the prioritized profiles and/or SPE fractions that mainly cover the whole metabolomes with fewer media components were chosen for further metabolomics mass identification through HRMS/MS. The acquired tandem-MS mass spectra from the positive mode were recruited to build a feature-based molecular network, while the negative ionization was consulted, if needed, during the annotation step to further validate the feature identities [45,82]. The dereplication of the known compounds, chemical analogues, and potential novel chemical structures was carried out either by matching their MS/MS spectra against the literature if available, GNPS spectral libraries [45] and/or assisted by manual in silico annotation via Sirius+CSI: FingerID 4.0.1 integrated with Antibase and Pubchem databases [83,84] (see Material and Methods).

Among the numerous identified secondary metabolites from the nine isolates, antimycins cluster were swiftly retrieved through the identical similarity of their MS/MS spectra to the publicly shared ones of GNPS libraries (Figures S2–S6). Tracking down such features in liquid BSE 7F fractions, particularly the one eluted with 100% MeOH in negative mode, expanded this set with further known members (Figures S5 and S6). In alignment with the formerly described positional and stereogenic isomers of the antimycin family entities, the extracted ion chromatograms (EICs) unambiguously displayed such an isomeric behavior under both modes (Figures S2, S4 and S5) [85–87]. In a similar fashion to antimycins, a different cluster comprising ferrioxamines was deciphered with the aid of GNPS spectral libraries. Ferroxamine D1, 656.2830 Da in size as $C_{27}H_{48}N_6O_9$ [88,89], was displayed as the primary ion linked with an additional unknown analogue, 627.3303 Da as $C_{26}H_{51}FeN_8O_6$ (Figures S7–S9). Despite the fact of observing these two features under only solid cultivation parameters across different isolates (*Streptomyces* sp. I3, I4, I6, BSE 7F, DHE 17-7, and SHP 22-7) with variable concentrations, two extra unknown amphiphilic trihydroxamate-containing siderophores were also grouped (Figures S7 and S10). Interestingly, BSE 7-9 was the sole producer of such amphiphilic entities under exclusive liquid conditions. Moreover, two additional unknown ferrioxamines were retrieved as unique features singly produced by the DHE 17-7 isolate (Figure S11).

Analogously, staurosporine, with two further congeners, was dereplicated from the I6 sample assisted by shared spectral repositories (Figure S12). Manual annotation of a pair of singletons, 1137.45 as $[M+H]^+$ and 560.22 as $[M + 2H]^{2+}$ from the I6 extract, uniquely grown under solid conditions, could decipher echinoserine and depsiechinoserine, respectively (Figures S13–S15) [90,91]. Although the two features were supposed to group together considering their skeletons, the MS/MS spectra of their triggered singly and doubly pseudomolecular ions were different enough not to serve such a purpose resulting in scattered self-looped nodes (Figures S13 and S14). Furthermore, traces of the structurally related echinomycin [92] were also observed within *Streptomyces* sp. I6 extracts, expanding in this way the molecular compound family (Figure S15). Likewise, a tirandamycins cluster was disclosed in *Streptomyces* sp. I6 extracts upon liquid cultivation depicting the known tirandamycin A in connectivity with further related chemotypes (Figure S16). In parallel,

the observed UV absorbance of the annotated mass ion at m/z 418.18 as tirandamycin A was in alignment with its reported characteristic value [93,94], additionally confirming the identity of the dereplicated feature (Figure S17). Notably, the anticipated molecular formula of the grouped ions of the tirandamycin cluster, besides their degrees of unsaturation, was also reflected by their observed UV absorbances, which differed from the characteristic known one (Figure S17).

An additional constellation of ions mainly derived from isolates BSE 7-9 and I5 was uncovered through manual annotation as naphthyridinomycins cluster (Figure S18). The in silico annotation considering the molecular formula prediction and their MS^2 spectra deconvoluted naphthyridinomycin-A, aclidinomycin A, and bioxalomycin-β2 besides several unknown related products (Figures S19–S22) [95–97]. Similarly, the manual interrogation of an exclusive group of ions derived from DHE 17-7 led to the putative dereplication of ECO-501, a PKS product so far only reported from *Amycolatopsis orientalis* ATCC 43491 [98] (Figures S23–S26). Interestingly, the putative annotation of such a feature was in complete alignment regarding the observed UV absorbance and the formerly reported MS/MS spectra (Figures S27–S29). Moreover, amicetin and cytosaminomycins as structurally related entities were uncovered from SHP 22-7 samples as a big group of ions (Figures S27–S29), encompassing a wide scope of structural modifications as expected according to previous reports in addition to a putatively new set of congeners (Figure S30) [99–101].

The compound naphthyridinomycin was detected in several culture extract samples from strains of mangrove origin, such as *Streptomyces* sp. I3, I4, I5, BSE 7F, and BSE 7-9 (Figure 6, Table 4), while amicetin was detected as a biosynthetic product from the isolates SHP 22-7 and DHE 17-7 obtained from soil samples of Enggano Island (Figure 6, Table 4). Furthermore, we observed that *Streptomyces* sp. I6 produces echinomycin (Figure 6, Table 4), a substance also reported as the biosynthetic product from the closely related type strain *Streptomyces spongiicola* HNM0071, which was originally derived from a marine sponge [102]. These results underline our assumption that phylogenetically related strains are likely to produce similar compounds as a response to their natural-habitat environmental conditions. Specifically, the isolates *Streptomyces* sp. I3 and I4 have been found to most likely represent the same species derived from a similar habitat as indicated by the dDDH value of almost 100% and the high overall similarities of BGC composition and secondary metabolite production profile of both strains (see above). In this context, it should be mentioned that current antibiotic research often addresses the problem of dereplication of known compounds during drug-screening approaches [103–105]. However, what should also be taken into account is the fact that there is also an issue of dereplication of producer strains as observed in the current study. Thus, it is worth it to put effort into phylogenetic profiling at the beginning of the screening strategy in order to sort out known producer strains.

Interestingly, the ferrioxamine molecular family was only detected for samples of strains grown on solid media (Tables 4 and 5). In addition to the abovementioned metabolites, the solid media uniquely delivered a putative new molecular family consisting of likely three peptides with *m/z* 598.2834 $[M + 2H]^{2+}$, 662.8048 $[M + 2H]^{2+}$, and 727.3259 $[M + 2H]^{2+}$, for which no known substance could be associated. These compounds were detected in samples of strains I3, I5, and BSE 7F (Figure S31, Table 5), highlighting that cultivation conditions have a substantial effect on the chemical profiles. A further example of rendering the impact of the adopted cultivation method was represented with an additional cluster of unknown features from SHP 22-7 isolate, designated compound group I, which were exclusively produced under nonliquid fermentation (Figure S32).

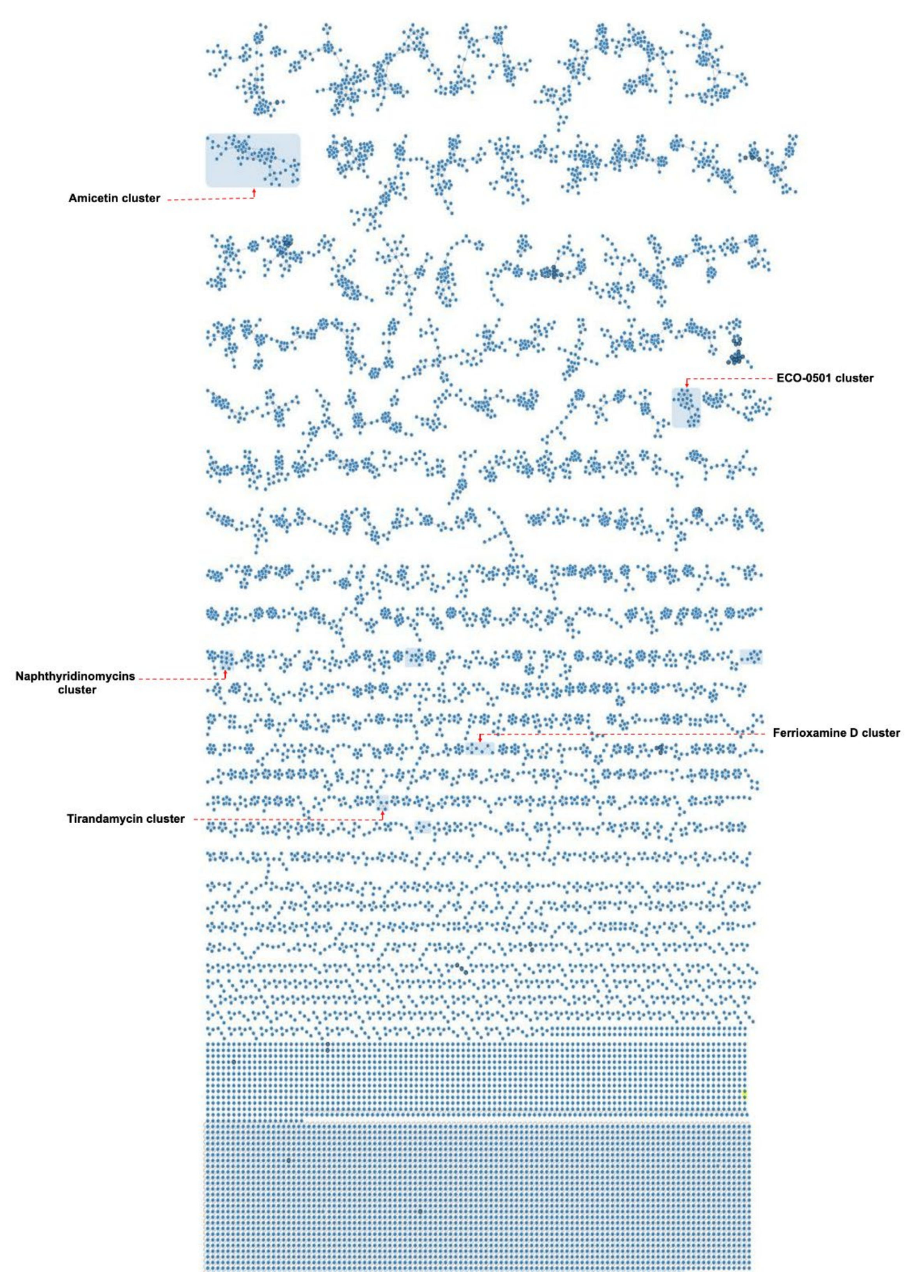

Figure 6. Molecular networking of extract and fraction samples from nine Indonesian *Streptomyces* strains. Molecular families containing a known substance are highlighted by blue boxes.

Table 4. Correlation between known compounds and BGC distribution in the nine Indonesian strains. A checkmark (√) indicates identified BGC in the studied strain, a question mark (?) indicates that BGC is not identified in the studied strain, and a minus sign (-) indicates the compound is not present in the medium.

Ion Cluster Name (Ion Formula)	*m*/*z* Measured	Adduct	Main Producer and Media Type		BGC Identified
			Solid	Liquid	
Ferrioxamine D1 ($C_{27}H_{48}N_6O_9$)	656.2830	$[M - 2H + Fe]^+$	SHP 22-7; I3; I4; I6	-	√
Naphthyridinomycin A ($C_{21}H_{28}N_3O_6$)	418.1980	$[M + H]^+$	I3; I4; I5	BSE 7F; BSE 7-9; I5	√
Amicetin ($C_{29}H_{43}N_6O_9$)	619.3100	$[M + H]^+$	-	SHP 22-7; DHE 17-7	√
Antimycin A2 ($C_{27}H_{39}N_2O_9$)	535.2659	$[M + H]^+$	-	BSE 7F	√
ECO-0501 ($C_{46}H_{69}N_4O_{10}$)	837.5022	$[M + H]^+$	-	DHE 17-7	?
Echinoserine ($C_{51}H_{69}N_{12}O_{14}S_2$)	1137.4504	$[M + H]^+$	I6	-	√
Echinomycin ($C_{51}H_{65}N_{12}O_{12}S_2$)	1101.4279	$[M + H]^+$	I6	-	√
Tirandamycin A ($C_{18}H_{25}O_6$)	337.1650	$[M + H]^+$	I6	-	√
Staurosporine ($C_{28}H_{27}N_4O_3$)	467.2070	$[M + H]^+$	I6		√

Table 5. Overview of analogs and putative new compounds identified for the nine Indonesian *Streptomyces* strains. A minus sign (-) indicates that the compound is not present in the medium.

Ion Cluster Description	*m*/*z* Measured	Adduct	Main Producer and Media Type	
			Solid	Liquid
Ferrioxamine analogs	627.3303	$[M - 2H + Fe]^+$	I3; I4; I6; DHE 17-7	-
	788.3753	$[M - 2H + Fe]^+$	BSE 7-9	-
	840.4060	$[M - 2H + Fe]^+$	-	BSE 7-9
	640.2520	$[M - 2H + Fe]^+$	-	DHE 17-7
	654.2685	$[M - 2H + Fe]^+$	DHE 17-7	-
Putative new peptides	598.2834	$[M + 2H]^{2+}$	I3, I5, BSE 7F	-
	662.8048	$[M + 2H]^{2+}$	I3, I5, BSE 7F	-
	727.3259	$[M + 2H]^{2+}$	I3, I5, BSE 7F	-
Putative new compound group I	821.3349	$[M + H]^+$	SHP 22-7	-
	734.3031	$[M + H]^+$	SHP 22-7	-
	679.2430	$[M + H]^+$	SHP 22-7	-
	647.2710	$[M + H]^+$	SHP 22-7	-
Putative new compound group II	435.2774	$[M + 2H]^{2+}$	-	DHE 17-7
	442.2857	$[M + 2H]^{2+}$	-	DHE 17-7
	449.2934	$[M + 2H]^{2+}$	-	DHE 17-7
	474.2833	$[M + 2H]^{2+}$	-	DHE 17-7

Within the same context, strain DHE 17-7 also offered several putative new compounds (compound group II) which were detected when grown in a liquid medium and presented themselves only as a set of doubly charged entities (Figure S33) (Table 5). Thus, in regard to drug-discovery efforts, strain DHE17-7 is the most promising strain to be investigated further. The potent biosynthetic capacity is also reflected by the genetically encoded biosynthetic potential since DHE17-7 has a total of 30 BGCs, which is the largest BGC set compared to the other Indonesian strains (Table 3). In summary, 16 potential novel compounds (Table 5) have been identified as biosynthetic products from the Indonesian strains, which could not be associated with any known compound and thus demonstrate the value of new strains for drug-discovery research.

Furthermore, we observed a correlation between growth conditions and compound production. It is known that sources of complex nitrogen such as soybean meal and corn steep liquor can increase ferrioxamine production in streptomycetes [106,107]. Interestingly, ferrioxamine B/D and its analogs has been mainly identified for strains grown on solid media, such as MS agar (*Streptomyces* sp. I3, I4, I6) and NL300 agar (SHP 22-7) (Tables 4 and S3), which contain soy flour and cotton seed powder, respectively (Table S2). We could detect ferrioxamines only in samples obtained from strains grown on solid medium. This might be because in liquid media iron (Fe^{3+}) is more evenly distributed compared to solid media. Thus, cells grown on solid media might be faced with local iron depletion conditions, which lead to induction of ferrioxamine biosynthesis [76]. In addition to ferrioxamine and its analogs, several known and unknown compounds were only discovered in samples from strains grown on solid medium, i.e., the three known compound echinomycin, staurosporine, and tirandamycin for *Streptomyces* sp. I6, and three putative new peptides for *Streptomyces* sp. I3; I5; BSE 7F, as well as the putative new compound group I for *S.* sp. SHP 22-7 (Tables 4 and 5). Apart from that, we also found some unknown and known compounds in strains grown in liquid media only, such as amicetin (SHP 22-7 and DHE 17-7), antimycin and its analogs (BSE 7F), ECO-0501 (DHE 17-7), or the putative new compound group II for strains *Streptomyces* sp. DHE 17-7 (Tables 4 and 5). This indicates that cultivation conditions significantly affect the formation of substances. Therefore, both the liquid and solid cultivation approach are feasible for increasing the probability of discovering new compounds.

2.6. Identification of Potential BGCs Responsible for Compound Production in the Nine Indonesian Streptomyces Strains

To identify the BGCs responsible for compound production in the nine Indonesian *Streptomyces* strains, we aimed to link the compound production profile and BGC composition by correlating the BGCs data with the MS-based molecular networking results. As described above, strains SHP 22-7, I3, I4, and I6 produce desferrioxamine B/D when grown on solid media (Table 4). We observed that the corresponding BGCs associated with desferrioxamine B/D biosynthesis were present in all of the four strains. Furthermore, we were able to assign the BGC responsible for the biosynthesis of naphthyridinomycin in the strains BSE 7F, BSE 7-9, I3, I4, and I5 (Table 4). Additional BGCs could be assigned to the compound formations of amicetin in SHP 22-7 and DHE 17-7, antimycin in BSE 7F, echinomycin, staurosporine, and tirandamycin A in I6 (Table 4). Furthermore, we could not identify the BGC encoding the biosynthesis of ECO-0501 in strain DHE 17-7 based on the antiSMASH output. A potential candidate gene cluster could be cluster region 24, which is a predicted type I PKS BGC that shows some similarity (<55%) to BGCs encoding structurally related macrolactam natural products, such as vicenistatin, sceliphrolactam, and streptovaricin (Figure S34).

In addition to the metabolites mentioned earlier, we also discovered a group of new peptides, which were detected in samples of strains *Streptomyces* sp. I3, I5, and BSE 7F grown on solid media (Table 5). Notably, for all three strains a bacteriocin BGC could be detected (Tables S6, S8 and S9), which showed 42–57% similarity to the informatipeptin BGC. Alternatively, all three strains also share a combined NRPS/ectoine/butyrolactone/other/T1PKS gene cluster (Tables S6, S8 and S9), and it is also conceivable that the peptide group

might be encoded from this region. A similar cluster was found on regions 21 and 22 for the phylogenetically related strain BSE 7-9, for which, however, no respective compound was detected (Table S10). Moreover, we found the putative new compound group IIwith masses ranging from 435–474 Da, produced by strain DHE 17-7 when grown in liquid medium (Table 5). Five BGCs are present in DHE 17-7 (region 10, 16, 17, 22, and 28), which do not show any similarity to known BGCs in the antiSMASH database and nine BGCs (region 3, 4, 6, 11, 13, 19, 24, 25, and 26) have similarities of less than 50%. Thus, the so far unknown metabolites might be encoded by some of the unique BGCs from DHE 17-7 (Table S4). The same applies for the putative new compound group I detected in strain SHP 22-7. Its genome comprises 13 BGCs with similarities of less than 50% and therefore represents all putative candidates. Similar observations have been made in comparable studies, where it has been shown that BGCs encoding ectoine, desferrioxamine, spore pigment, and bacteriocin production are very abundant in actinobacterial natural compound producers; however, each strain still possesses numerous BGCs that code for potential yet unknown substances [108–110]. That Indonesian habitats can serve as a promising reservoir for antibiotic active substances has already been highlighted in several previous screening studies [111–115]. Especially Indonesian actinomycetes have been reported as producer strains of new secondary metabolites, as for example shown for the Indonesian *Streptomyces* sp. strains ICBB8230, ICBB8309, and ICBB8415, which produced new angucyclinones [116,117], *Streptomyces* sp. ICBB8198, producing new phenazine derivatives [118], and *Streptomyces* sp. ICBB9297, which produced new elaiophylin macrolides [119]. Furthermore, Indonesian non-*Streptomyces* strains, as for example *Micrococcus* sp. ICBB8177 and *Amycolatopsis* sp. ICBB8242, have also been reported to produce novel compounds, as for example the limazepines or succinylated apoptolidins, respectively [120,121]. Thus, Indonesian habitats can indeed be considered a promising source for new bioactive natural products.

Altogether, the combined GNPS and cluster networking approach disclosed several potentially novel compounds from the Indonesian strains *Streptomyces* sp. I3, I4, I5, I6, BSE 7F, BSE 7-9, and DHE 17-7—some of which could be assigned to potential encoding BGCs, and some are expected to be encoded by unique BGCs. The new Indonesian isolates thus represent a valuable resource for further drug research and development approaches. We conclude that the combined phylogenomic, GNPS, and cluster-networking approach is an efficient strategy to prioritize phylogenetically unique producer strains and focus on potentially novel compounds encoded by special BGCs.

3. Materials and Methods

3.1. Sample Collection and Treatment

Soil samples were collected from Enggano Island (5°22′57.0792″ S, 102°13′28.2792″ E), Indonesia, in December 2015 (Figure 1B). Marine samples were collected from marine sediments from Bali Island (8°43′5.5″ S, 115°10′7.8″ E), Indonesia, in May 2014 (Figure 1C), and Lombok Island West Nusa Tenggara (8°24′17.133″ S, 116°15′57.228″ E), Indonesia, in May 2017 (Figure 1D). Soil and sediment samples were taken aseptically from 10 cm depth of soil samples and the center of sediment in mangrove and tidal area. Soil and sediment samples were transferred into sterile 50 mL conical tubes and placed on ice and then stored at 4 °C until further treatment.

3.2. Isolation of Actinomycetes

Isolation and enumeration of actinomycetes were done using a serial dilution of Humic Acid-Vitamin (HV) medium [48] and/or NBRC No. 802 Medium [49] by using the direct method [50], the dry heat method [51], and the phenol method [51]. In the direct method, an air-dried soil sample or marine sediment was ground in a mortar and heated in a hot-air oven at 110 °C for 30 min. One gram of the heated samples was transferred to 10 mL of sterile water and mixed for 2 min, then diluted with sterile water to 10^{-1}, 10^{-2}, and 10^{-3} times. In total, 200 µL of each dilution was inoculated on isolation medium

agar of HV [48] or NBRC No. 802 Medium [49] with or without the addition of 1% NaCl. The inoculated plates were incubated for 2–4 weeks at 28 °C. The colonies showing the *Streptomyces* morphological characteristics were selected and streaked on fresh plates of the modified *Streptomyces* International Project 2 (ISP2 ≙ YM) agar [52]. The cultures were resuspended in sterile 0.9% (*w*/*v*) saline supplemented with 15% (*v*/*v*) glycerol and stored at −80 °C. This dry-heat method [51] was used to isolate heat-tolerant actinomycetes spores. In the dry-heat method, the soil or sediment samples were incubated at 100 °C for 40 min and then cooled to 28 °C in a desiccator. The samples were distributed on HV medium agar plates with a spatula tip and incubated at 28 °C for 2–3 weeks. The phenol method was used to select for spores, which survive in the presence of phenol. In total, 1 mL of 10^{-1} dilution of one gram of oven-dried soil or marine sample was transferred to 9 mL of sterile 5 mM-phosphate buffer (pH 7.0) containing phenol at a final concentration of 1.5%. The sample was then heated and diluted in serial dilution (10^{-1}, 10^{-2}, 10^{-3}). Next, 100 or 200 µL of each dilution was spread over the surface of HV medium agar plates and incubated for 2–4 weeks at 28 °C.

3.3. Antimicrobial Bioassays

The preliminary screening of actinomycetal strains for antimicrobial activity was performed using the agar plug diffusion method (see Supplementary for test plate preparation). Gram-positive (*B. subtilis* ATCC6051, *M. luteus*, and *S. carnosus* TM300) and Gram-negative bacteria (*E. coli* K12 W3110 and *P. fluorescens*) were chosen as test organisms. The isolates were spread evenly over the agar plate surface of soya flour mannitol medium (MS) (mannitol 20 g, soy flour (full fat) 20 g, agar 16 g in 1 L of distilled water) [80] and incubated for 10 days at 28 °C. Agar discs of the 10 days inoculum were cut aseptically with a cork borer (9 mm diameter) and placed on the bioassay test plate. Bioassays to determine optimal cultivation conditions in the liquid culture were examined using a disc diffusion assay against the test Gram-positive (*B. subtilis* ATCC6051, *M. luteus*, and *S. carnosus* TM300) and Gram-negative bacteria (*E. coli* K12 W3110 and *P. fluorescens*). In total, 10 µL methanolic extract obtained from liquid cultures of the actinomycetal strains was pipetted on a filter disc (6 mm) and then placed on the respective test plates. In addition, 5 µL kanamycin (50 µg/mL) was used as positive control and 10 µl methanol as a negative control.

The bioassay plates were incubated overnight at 37 °C for *B. subtilis*, *E. coli*, and *S. carnosus* and at 28 °C for *M. luteus* and *P. fluorescens* to allow for the test organisms' growth. The antimicrobial activity of the isolates was assessed by measuring the diameter of the inhibition zone (mm) around the agar plug or the discs. All bioassay tests were carried out as three independent biological replicates.

3.4. Isolation of Genomic DNA and 16S rDNA Phylogenetic Analysis

For isolation of genomic DNA, the producer strains were grown for two days in 50 mL of R5 medium at 30 °C [81]. The genomic DNA was extracted and purified with the Nucleospin® Tissue kit from Macherey-Nagel (catalog number 740952.50) following the standard protocol from the manufacturer. The DNA was applied as a PCR template for 16S rRNA gene amplification using polymerase chain reaction (PCR). Primers used for PCR were 27Fbac (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492Runi (5′-TACGGTTACCTTAC GACTT-3′). The PCR amplicons were subcloned into the cloning vector pDrive (Qiagen) using basic DNA manipulation procedures as previously described by Sambrook et al. [122]. The respective 16S rDNA fragments were sequenced at MWG Eurofins (Ebersberg, Germany) with primers 27Fbac. The 16S rDNA sequence data were analyzed using the EzTaxon database (https://www.ezbiocloud.net, accessed on 28 May 2018).

3.5. Phylogenomic and Genome Mining Analysis

For phylogenomic and genome mining studies, full-genome sequence data have been obtained as reported previously [57–59]. Genomic DNA was isolated to construct a 10–20 kb paired-end library for sequencing by Macrogen (Seoul, South Korea) with the Pacific

Biosciences RS II technology (Pacbio). The genome was assembled using Hierarchical Genome Assembly (HGAP) V.3. and annotated with Prokka version 1.12b and the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). The phylogenomic analysis of the nine selected strains was carried out with the Type (Strain) Genome Server (TYGS), a free bioinformatics tool (https://tygs.dsmz.de/, accessed on 13 December 2019) for whole-genome-based taxonomic analysis [63]. The identification of potential biosynthesis gene clusters (BGCs) was accomplished by analyzing the genome sequences with antiSMASH version 5.0 [44]. The antiSMASH results were further analyzed using the BiG-SCAPE platform [78] to cluster the predicted BGCs into gene cluster families (GCFs) based on their sequences and Pfam protein family similarities [79]. BiG-SCAPE was conducted on global mode with default parameters [78], with the exception of the raw distance cutoff and the "–mix"parameter. Raw distance cutoff was set to 0.4 to ensure that even clusters with a pairwise distance higher than 0.3 (the default) were included in the output. The resulting network of BiG-SCAPE was visualized with Cytoscape version 3.7.2 [82].

3.6. Cultivation Conditions for Optimal Compound Production of Nine Indonesian Strains

To determine optimal cultivation conditions in liquid culture, the nine Indonesian actinomycetes strains, SHP 22-7, DHE 17-7, DHE 7-1, BSE 7-9, BSE 7F, I3, I4, I5, and I6, were each cultivated in 50 mL inoculum medium (NL410) in 500-mL Erlenmeyer flasks (with steel springs) in an orbital shaker (180 rpm) at 28 °C. After 48 h, 10 mL of preculture was inoculated into 100 mL of twelve different production medium (SGG, YM, OM, R5, MS, TSG, NL19, NL300, NL330, NL500, NL550, and NL800 (Table S2) and cultivated for 48–168 h. Cell culture samples were harvested at different time points (48, 72, 96, and 168 h). In addition, 5 mL of each cell culture sample was extracted with the same volume of ethyl acetate (EtOAc) for 30 min at room temperature. The EtOAc was dried in a rotary evaporator and suspended in a total volume of 0.75 mL methanol. The methanolic extracts were used for bioassay experiments. The culture extract samples, which yielded the largest zone of inhibition in the bioassays against the test organisms, were used for further compound identification analysis. To determine optimal cultivation conditions on solid culture, the nine Indonesian strains were each spread on 100 mL agar plates consisting of the respective abovementioned cultivation media and then incubated for 7–10 days at 28 °C until spore formation was visible on agar plates. The overgrown agar was then used for bioassay experiments and further compound identification analysis.

3.7. Sample Preparation for Chemical Identification

For chemical identification in the liquid sample, the nine Indonesian isolates were each cultivated in 50 mL of NL410 medium. After 48 h, 10 mL of the preculture was inoculated into 100 mL of optimal production medium. The 100 mL whole broth of each cell culture was extracted as described above. Then, the extracts were used for further experiment. For chemical identification from samples grown on solid medium, overgrown agar was cut into pieces and transferred to 50 mL Falcon tubes. The Falcon tubes were centrifuged at 13,000 rpm for 30 min at room temperature. The aqueous phase was concentrated to 1/5 of the original volume in the Genevac Centrifugal Evaporator EZ-2 Elite (SP Scientific). The concentrated aqueous phase was used for further chemical profiling.

The culture extract samples obtained from liquid medium extraction and the aqueous phase of the solid medium extraction were separated by solid-phase extraction (SPE) columns. The columns were washed twice with 2 mL methanol and 2 mL distilled water for activating the columns. The samples were prepared by adding 100% methanol to the culture extract samples and the aqueous phase until the samples were dissolved completely. The methanolic samples were applied onto the activated columns with a flow rate of 2 mL/min. The column was washed twice with distilled water. The column was eluted consecutively with 2 mL of 100% methanol, 50% methanol, and distilled water. Samples from the elution column were defined as fractions. The column was eluted with 100% methanol as the 100% fraction, with 50% methanol as the 50% fraction, and distilled water

as the distilled water fraction. The fractions were dried in the Genevac EZ-2 Elite (SP Scientific) and then dissolved with 0.5 mL methanol. The crude extracts and all fractions were analyzed with HPLC and high-resolution mass spectrometry (HRMS).

3.8. HPLC-HRMS/MS Analysis

The HRMS analysis was carried out on MaXis 4G instrument (Bruker Daltonics, Bremen, Germany) coupled to an Ultimate 3000 HPLC (Thermo Fisher Scientific, Bremen, Germany). HPLC-method was applied as follows: the spectrometer using a gradient (solvent A: 0.1% formic acid (FA) in H_2O, and solvent B: 0.06% formic acid in acetonitrile), a gradient of 10–100% B in 45 min, 100% B for an additional 10 min, using a flow rate of 0.3 mL/min; 5 μL injection volume and UV detector (UV/VIS) wavelength monitoring at 210, 254, 280, and 360 nm. The separation was carried out on a Nucleoshell 2.7 μm 150 × 2 mm column (Macherey-Nagel, Düren, Germany), and the range for MS acquisition was *m/z* 100–1800. A capillary voltage of 4500 V, nebulizer gas pressure (nitrogen) of 2 (1.6) bar, ion source temperature of 200 °C, the dry gas flow of 9 (7) l/min source temperature, and spectral rates of 3 Hz for MS1 and 10 Hz for MS^2 were used. For acquiring MS/MS fragmentation, the ten most intense ions per MS1 were selected for subsequent collision-induced dissociation (CID) with stepped CID energy applied. The employed parameters for tandem MS were applied as previously detailed by Garg et al. in 2015 [123]. Sodium formate was used as an internal calibrant and Hexakis (2,2-difluoroethoxy) phosphazene (Apollo Scientific Ltd., Stockport, UK) as the lock mass. Data processing was performed using Bruker Daltonics Data Analysis 4.1(Bremen, Germany).

3.9. MS/MS Molecular Networking

Mass-spectral data were analyzed using Compass Data Analysis 4.4 (Bruker Daltonik, Bremen, Germany), whereas MetaboScape 3.0 (Bruker Daltonik, Bremen, Germany) was consulted for molecular features selection. Raw data files were imported into MetaboScape 3.0 for the entire data treatment and preprocessing in which T-ReX 3D (time-aligned region complete extraction) algorithm is integrated for retention time alignment with an automatic detection to decompose fragments, isotopes, and adducts intrinsic to the same compound into one single feature. All the harvested ions were categorized as a bucket table with their corresponding retention times, measured *m/z*, molecular weights, detected ions, and their intensity within the sample. The Bucket table was prepared with an intensity threshold (1e3) for the positive measurements with a minimum peak length 3, possessing a mass range of 150–1800 Da. For detailed parameters employed for the MetaboScape analysis, see Table S13. The features list of the preprocessed retention time range was exported from MetaboScape as a single MGF file, which was in turn uploaded to the GNPS online platform where a feature-based molecular network (FBMN) was created. The precursor ion mass tolerance was set to 0.03 Da and a MS/MS fragment ion tolerance of 0.03 Da. A network was then created where edges were filtered to have a cosine score above 0.70 and more than 5 matched peaks. 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. Finally, the maximum size of a molecular family was set to 100, and the lowest-scoring edges were removed from molecular families until the molecular family size was below this threshold. Cytoscape 3.5.1 was used for molecular network visualization.

4. Conclusions

In this study, we report on the isolation of 422 actinomycetes strains from three different unique areas in Indonesia. A combined genomics and metabolomics approach was applied to nine of the most potent antibiotic producer strains, which allowed us to uncover 16 so far unknown compounds. When cultivating the strains in various liquid and solid media, we found that culture conditions significantly affected the ability to produce specific compounds. Thus, the combination of both cultivation methods, solid and liquid cultivation, is a suitable approach to tap the full biosynthetic potential of actinomycetes.

By phylogeny-associated genome mining studies, we found that phylogenetically related species tend to have a similar BGC composition. Additional metabolomics data suggested that the ability of the strains to produce certain compounds may be influenced by the environmental conditions, where the producer strains have been derived from.

Overall, the described methodology represents an efficient strategy for drug discovery and the reported unknown compounds may serve as a basis for further drug development.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/md19060316/s1, Figure S1. Actinomycin D production in DHE 6-7 (a) and DHE 5-1 (b). Peaks in HPLC representing actinomycin are marked with red colour. Mass spectra of actinomycin D detected in DHE 6-7 (c); Figure S2. Positive extracted ion chromatograms (EICs), ions cluster and predicted molecular formula of antimycins; Figure S3. GNPS spectral libraries hits of antimycins; Figure S4. Positive MS2 spectra of the detected antimycins from isolate BSE7F; Figure S5. Negative EICs and predicted molecular formulae of antimycins; Figure S6. Negative MS2 spectra of the detected antimycins from isolate BSE7F; Figure S7. Ions cluster of ferrioxamines and GNPS spectral libraries hit of ferrioxamine D1; Figure S8. Positive EICs and molecular formula prediction of ferrioxamine D1; Figure S9. Positive EICs, molecular formula prediction and MS2 of an unknown ferrioxamine; Figure S10. Positive EICs and MS1 of unknown amphiphilic ferrioxamines; Figure S11. Positive EICs and MS2 of DHE 17-7 ferrioxamines; Figure S12. Ions cluster of staurosporines and GNPS spectral libraries hit of staurosporine; Figure S13. Ions clusters of echinoserine; Figure S14. Ion singleton of depsiechinoserine, positive EICs and MS2 of depsiechinoserine; Figure S15. Positive EICs and MS1 of echinomycin; Figure S16. Ions cluster, Positive EICs and MS1 of tirandamycin A in addition to its congeners; Figure S17. UV absorbance, and MS2 of tirandamycin A in addition to its congeners; Figure S18. Ion cluster of naphthyridinomycins and their predicted molecular formula (MF); Figure S19. Positive EICs and MS2 of naphthyridinomycin and their related entities from isolate BSE7-9; Figure S20. Positive EICs and MS1 of naphthyridinomycin from isolates BSE7-9 and I5; Figure S21. Positive EICs and MS1 of aclidinomycin A from isolates BSE7-9 and I5; Figure S22. Positive EICs and MS1 of bioxalomycin-β2 from isolates BSE7-9 and I5; Figure S23. Ions cluster of ECO-0501 and its related congeners from isolate DHE 17-7; Figure S24. UV absorbance, MS2 of ECO-0501 and its related congeners from isolate DHE 17-7; Figure S25. Comparative positive MS2 of ECO-0501 from isolate DHE 17-7 and its reported version from Amycolatopsis orientalis; Figure S26. Negative MS2 of ECO-0501 from isolate DHE 17-7 and its proposed fragmentation scheme; Figure S27. Ions cluster of amicetins and its related congeners from isolate SHP 22-7; Figure 28. Comparative positive MS2 of amicetin and streptocytosin A; Figure S29. Comparative negative MS2 of amicetin and streptocytosin A; Figure S30. Comparative positive MS2 of some unknown members of amicetin molecular family; Figure S31. Comparative positive MS2 of some unknowns of likely peptides; Figure S32. Comparative positive MS2 of some unknowns from isolate SHP 22-7; Figure S33. Comparative positive MS2 of some unknowns from isolate DHE 17-7; Figure S34. Cluster similarity between the DHE 17-7 gene region 24 (query sequence) and the streptovaricin, sceliphrolactam and vicenistatin cluster; Table S1. Genome characteristics from nine Indonesian actinomycetes strain isolates; Table S2. media tested for antibiotic production in agar and liquid culture. All data refer to 1 l H2Odeion. For solid media 16 g/l agar is added, except for R5 medium 18 g/l agar is added; Table S3. List of optimal culture conditions (media, time point) and bioactivity profile of nine Indonesian strain isolates; Table S4. List of predicted BGCs of strain DHE 17-7 derived from antiSMASH analysis. The minus sign (-) indicates the BGC did not have any similarity with any BGCs in the antiSMASH database; Table S5. List of predicted BGCs of strain SHP22-7 derived from antiSMASH analysis. The minus sign (-) indicates the BGC did not have any similarity with any BGCs in the antiSMASH database; Table S6. List of predicted BGCs of strain I3 derived from antiSMASH analysis. The minus sign (-) indicates the BGC did not have any similarity with any BGCs in the antiSMASH database; Table S7. List of predicted BGCs of strain I4 derived from antiSMASH analysis. The minus sign (-) indicates the BGC did not have any similarity with any BGCs in the antiSMASH database; Table S8. List of predicted BGCs of strain I5 derived from antiSMASH analysis. The minus sign (-) indicates the BGC did not have any similarity with any BGCs in the antiSMASH database; Table S9. List of predicted BGCs of strain BSE 7F derived from antiSMASH analysis. The minus sign (-) indicates the BGC did not have any similarity with any BGCs in the antiSMASH database; Table S10. List of predicted BGCs of strain BSE 7-9 derived from antiSMASH analysis. The minus sign (-) indicates the BGC did not have any similarity with any BGCs in the antiSMASH database; Table S11. List of

predicted BGCs of strain DHE 7-1 derived from antiSMASH analysis. The minus sign (-) indicates the BGC did not have any similarity with any BGCs in the antiSMASH database; Table S12. List of predicted BGCs of strain I6 derived from antiSMASH analysis. The minus sign (-) indicates the BGC did not have any similarity with any BGCs in the antiSMASH database; Table S13. Parameters used in MetaboScape analysis;

Author Contributions: S.R. and S.A. isolated strains and performed preliminary bioassays; I.H. carried out phylogenetic analysis and antibiotic bioassays; I.H. and J.K. performed extraction of culture broths; A.K. and H.S. carried out HPLC-MS analysis, H.S. performed GNPS studies; I.H. and Y.M. performed genome-sequence-based bioinformatic analysis, A.G. performed BiG-SCAPE analysis; Y.M. and H.G. conceived the research. Y.M., W.W., H.G., P.L., W.K., and N.Z. supervised the work. I.H. wrote the original draft of paper, which was revised by Y.M., W.W., H.G., P.L., W.K., and N.Z. and approved by all authors. All authors have read and agreed to the published version of the manuscript.

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