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Article

Isolation, Genomic and Metabolomic Characterization of *Streptomyces tendae* VITAKN with Quorum Sensing Inhibitory Activity from Southern India

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Abstract:
Streptomyces, being one of the most promising genera due to its ability to synthesize a variety of bioactive secondary metabolites of pharmaceutical interest, here studied in relation to its genomic and metabolomic potential. Coinciding with the increase in sequenced data, mining of bacterial genomes for biosynthetic gene clusters (BGCs) has become a routine component of natural product discovery. Herein, we describe the isolation and characterization of a *Streptomyces tendae* VITAKN with quorum sensing inhibitory activity (QSI) that was isolated from southern coastal parts of India. The nearly complete genome consists of 8,621,231bp with a GC content of 72.2%. Utilizing the BiG-SCAPE-CORASON platform, a sequence similarity network predicted from this strain was evaluated through sequence similarity analysis with the MIBiG database and existing 3,365 BGCs predicted by antiSMASH analysis of publicly available complete *Streptomyces* genomes. Crude extract analyzed on LC-HRMS/MS and Global Natural Product Social Molecular Networking (GNPS) online workflow using dereplication resulted in the identification of cyclic dipeptides (2, 5-diketopiperazines, DKPs) in the extract, which are known to possess QSI activity. Our results highlight the potential use of genomic mining coupled with LC-HRMS/MS and bionformatic tools (GNPS) as a potent approach for metabolome studies in discovering novel QSI lead compounds. This study also provides the biosynthetic diversity of these BGCs and an assessment of the predicted chemical space yet to be discovered.

Keywords: natural product; actinobacteria; quorum sensing inhibition (QSI); biosynthetic gene clusters (BGCs); global natural product social networking (GNPS); cyclic dipeptides (2,5-diketopiperazines, DKPs); LC-HRMS

1. Introduction

Natural products of microbial origin have gained considerable attention because of their biological of their diversity and as the chemical compounds by them produced, as secondary metabolites represent the source for almost 35% of all present drugs [1,2]. Our current use of antibiotics poses great challenges among medical practitioners and researchers due to the development and spread of antibiotic–resistant pathogens. Moreover, the low success rates of antibiotic drug discovery programs worldwide that resulted in today's' eminent lack of new antibiotic drug leads [3]. An increased understanding on bacterial pathogenesis mechanisms and of intercellular microbial communication has revealed potential alternative strategies to treat bacteria-mediated diseases. A crucial aspect for the establishment and maintenance of a microbial population is based on cell density which triggers the genetic regulation, coordinating the physiologies of the different cell types through cell–cell communication, known as quorum sensing (QS) [4]. Different types of QS communication systems rely on diverse small, secreted signaling molecules known as autoinducers (AIs). In many cases, the responses elicited by AIs contribute directly to pathogenesis through the synchronized production of virulence determinants, such as toxins, proteases, and other immune-evasive factors [5]. Additionally, QS can contribute to behaviors that enable bacteria to resist antimicrobial compounds such as biofilm development. If the signal communication that coordinates these pathogenic behaviors was blocked, it is theorized that bacteria would lose their ability to mount an organized assault on the host and thus would be less able to form organized community structures that promote resistance to antibiotics [6,7]. Hence, an alternative natural product from prolific source could be employed to discover new drug leads.

Initial screening and discovery for novel lead molecules is usually performed at the level of crude extract screening followed by the activity-guided fractionation and purification [8]. However, this approach suffers drawback due to large sample complexity resulting in additive or multiplicative effects of the compounds present in the extract together with potential rediscovery of already known compounds or its analogues. This can be partially overcome by pre-fractionation of the crude extract improving the detection capability coupled with advanced metabolomics approaches. One such example is the use of fast and extremely accurate methods such as high-performance liquid chromatography connected to high resolution tandem mass spectrometry (HPLC-HRMS/MS) in the field of natural products to develop high-throughput discovery pipelines [9,10]. Another drawback is the presence of cryptic genes disabling the detection of desired bioactive lead molecules using the conventional way. This can be overcome by using genome sequencing to unveil cryptic biosynthetic gene clusters (BGCs). Based on antiSMASH analysis, it is now apparent that many bacteria with large genomes, particularly actinomycetes have the coding capacity to produce large number of secondary metabolites [11]. Marine actinomycetes are the most economically and biotechnologically valuable prokaryotes responsible for production of about half the discovered bioactive secondary metabolites notably antibiotics, antitumor agents, immunosuppressive agents and enzymes [12]. To continue our effort in the isolation of new bioactive molecule, it is crucial that new groups of actinomycetes from unexplored places or under

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exploited conditions be pursued as sources of novel bioactive secondary metabolites. Despite extensive exploration of actinomycetes for their antimicrobial products in the past, the search for molecules having unique therapeutic properties continues to be an active area of research. Herein we report the isolation of *Streptomyces tendae* VITAKN with quorum sensing inhibitory activity (QSI) from southern coastal areas of India, together with its almost complete (18 scaffolds, 100% completeness, 0% heterogeneity) draft genome sequence.

2. Materials and Methods

2.1 Isolation of actinomycetes

The marine soil samples were aseptically collected from the Rameswaram coast (9.2876° N, 79.3129° E), Tamil Nadu, India. The soil sample was collected at a depth of 10-15 cm aseptically in sterile polythene bags and transported to the laboratory. The pre-treatment of the soil sample was done in a hot air oven at 700 °C for 30 min [13]. The dried soil sample was serially diluted upto 10^{-6} dilution and plated on Actinomycetes Isolation Agar (AIA) (Sodium caseinate - 2.0 gL^{-1}, L-Asparagine - 0.1 gL^{-1}, Sodium propionate - 4.0 gL^{-1}, Dipotassium phosphate - 0.5 gL^{-1}, Magnesium sulphate - 0.1 gL^{-1}, Ferrous sulphate - 0.001 gL^{-1}, Agar - 15 gL^{-1}, adjusted to a final pH of 8.1 ± 0.2) and Starch Casein Agar (SCA) (Starch - 10 gL^{-1}, Casein powder 1 gL^{-1}; Sea water 37 gL^{-1}, Agar - 15 g L^{-1};adjusted to a final pH of 7.2 ± 0.2) (HiMedia Laboratories, Mumbai, India).. The plates were then incubated at room temperature for 7-14 days. Isolated actinomycetes were sequentially sub-cultured on AIA plates until pure culture was obtained. Pure culture was maintained at 28 °C until future use.

2.2 Morphological and Cultural characteristics of potential strain

The potential isolate with moderate to strong activity was identified further using various cultural characteristics including the growth optimization parameters on different media with (3% *w/v*) sea salt; ISP1 (Tryptophan Yeast Extract Broth), ISP2 (Yeast Malt Agar), ISP3 (Oat Meal Agar), ISP4 (Inorganic Salt Starch Agar), ISP5 (Glycerol Asparagine Agar Base), ISP6 (Peptone Yeast Extract Iron HiVeg Agar), ISP7 (Tyrosine Agar Base), ISP9 (Carbon Utilisation Agar), AIA, SCA (Starch Casein Agar) and NA (Nutrient Agar), temperature (30°C, 40°C and 50°C) and pH (4, 6, 7, 9 and 12). Genus level identification of the potent isolate was carried out based on aerial and substrate mycelium, reverse side pigmentation and spore chain morphology following the Bergey's Manual of Determinative Bacteriology. The arrangement of the spores in the mycelium was observed by cover slip method under light microscope as well as by scanning electron microscope. The potential isolate growth was also evaluated on different carbon supplements (1% glucose, fructose, maltose, mannitol and starch) using ISP9 medium [14].

2.3 Crude extracts preparation and quorum sensing inhibitory (QSI) activity

Pure culture of the strain was further inoculated in small scale fermentation AM3 medium (Soluble starch 15 g/L, soybean powder 5 g/L, peptone bacteriological 15 g/L, glycerol 15 g/L, $CaCO_3$ 2 g/L, 3% sea salt, pH 7.4). After one week of incubation cell free supernatant was extracted thrice using ethyl acetate and dried in vacuum to obtain the crude extract. Crude extract was serially diluted to obtain 12.5-0.0487 mg/ml in 20% DMSO in water. QSI activity were tested using *E. coli* pSB401 and *E. coli* pSB1075, luminescence based reporters and were quantified on a TriStar Multimode Microplate reader (Berthold Technologies GmbH & Co. KG, Germany) following [15,16]. Plasmid pSB401 and pSB1075 contains *luxI/R* and *lasI/R* gene promoter respectively regulating

luxCDABE expression, and responds well to exogenously provided AHLs [15,17]. The inoculum of the reporter strains (OD600 0.01, final concentration) were prepared from an overnight culture and added (50 µL) to each well of 96 well plates. The inoculum was supplemented with N-(3-oxo-hexanoyl)-l-homoserine lactone (3-oxo-C6-HSL, 1 µM final concentration) and N-(3-oxo-dodecanoyl)-l-homoserine lactone (3-oxo-C12-HSL, 2 µM final concentration) to stimulate QS of pSB401 and pSB1075 biosensors, respectively. The bioluminescence was recorded every 30 min for 7 hours at 30°C. The production of bioluminescence in the graphs is given as the relative light units (RLU), obtained at 4 h. A parallel experiment was run to determine non inhibitory concentration for the crude extract dilutions against both the biosensor without the exogenously addition of its cognate HSL.

2.4 Genomic characterization

Strain AKN, were grown on ISP No. 1 medium for 4 days (Tryptone yeast extract broth) (HiMedia, Mumbai, India) to obtain the mycelium for DNA isolation. Later, the genomic DNA was isolated using High pure PCR template Preparation Kit, Roche. DNA libraries were prepared using the TruSeq DNA PCR free kit. Metagenomic shotgun libraries were sequenced on an Illumina platform (100-bp paired-end reads) in Macrogen, Korea, yielding a total of 6.6G bp of sequence. Sequences were trimmed using Trimmomatic version 0.36 (minlen 100, slidingwindow 4:20) (**8**), and read quality was assessed using FastQC version 0.11.5 [18] . Prior to *de novo* assembly using SPADES version 3.13.0 (parameters: -k 21,33,55,77,91 --careful --only-assembler --cov-cutoff auto) [19]. Scaffolds ≥2 kb was used for further analysis. Completeness and contamination were estimated with checkM version 1.0.7 [20] based on 460 markers and using taxonomy workflow for *Streptomycetaceae*. The near-full-length 16S rRNA gene sequence was obtained as followed; rRNA sequences were first sorted from the raw shotgun readsusingSortMeRNA (v. 2.1b) [21] and SILVA 132as reference database. Second, the obtained reads were assembled with rnaSPAdes [22] using default parameters. SILVA 132 non-redundant databases was downloaded from the SILVA official web site (http://www.arb-silva.de). For the phylogenetic analysis, sequences of the closest described type strains were obtained from EzTaxon [23]. Open reading frames (ORFs) for AKN genome were identified and annotated with the RASTtk algorithm [24,25]. The sequences were compared with those available in Genbank using BLASTn [26].

To further assess the biosynthetic capacity of this strain, antiSMASH v4.1 [27] was used to identify the BGCs encoded in its genome, along with other publicly available 113 complete *Streptomyces* RefSeq genomes obtained from the National Center for Biotechnology Information. BGCs were then clustered into groups based on sequence similarity using BiG-SCAPE [28] using default parameters, including singletons, and a distance cut-off score of 0.3. MIBiG database v1.4 [29] was also applied to the networks to assign BGCs that putatively produce known compounds. Generated networks were visualized through Cytoscape software [30].

2.5 HPLC-HRMS/MS analysis and Metabolites identification using GNPS dereplication

Crude extract was analyzed using high-resolution ESI mass spectrometry experiments (LC-HRMS and LC-HRMS/MS) using a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Spa, Rodano, Italy) coupled to an Agilent model 1100 LC system (Agilent Technologies, Cernusco sul Naviglio, Italy) equipped with a solvent reservoir, an in-line degasser, a

binary pump and a refrigerated autosampler [31]. The spectra were recorded by infusion into the ESI source using MeOH as the solvent. A 5 µm Kinetex C18 column (50 × 2.1 mm), maintained at 25 °C, was operated using a gradient elution of H_2O and MeOH both with 0.1% formic acid, running at 200 µL/min. The gradient program was as follows: 10% MeOH for 3 min, 10%–90% MeOH over 30 min, 90% MeOH for 3 min. All the mass spectra were recorded in the positive-ion mode. MS parameters were a spray voltage of 5 kV, a capillary temperature of 230 °C, a sheath gas rate of 12 units N_2 (ca. 120 mL/min), and an auxiliary gas rate of 5 units N_2 (ca. 50 mL/min). Data were collected in the data-dependent acquisition mode, in which the five most intense ions of a full-scan mass spectrum were subjected to high resolution tandem mass spectrometry (HRMS/MS) analysis. HRMS/MS scans were obtained for selected ions with CID fragmentation, isolation width of 2.0, normalized collision energy of 35, Activation Q of 0.250, and activation time of 30 ms. A molecular network was created with the Feature-Based Molecular Networking (FBMN) workflow [32] on GNPS (https://gnps.ucsd.edu, [33,34]). The mass spectrometry data were first processed with MZMINE2 [35] and the results were exported to GNPS for FBMN analysis. The data was filtered by removing all MS/MS fragment ions within +/- 17 Da of the precursor m/z. MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the +/- 50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 2 Da and the MS/MS fragment ion tolerance to 0.02 Da. A molecular network was then created where edges were filtered to have a cosine score above 0.7 and more than 6 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. The spectra in the network were then searched against GNPS spectral libraries [33,36]. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks. The DEREPLICATOR was used to annotate MS/MS spectra [37]. The molecular networks were visualized using Cytoscape software [30].

3. Results and discussion

The isolate AKN had white powdery, raised colonies with small to medium sized colonies when cultured on AIA plates. The substrate mycelium was pale white in color. The isolate was Gram- positive (Figure 1). The spores had smooth surface when examined under Scanning electron microscope (1000X magnification). The spores of the isolate were oblong in shape and were arranged in chains (Figure 1). The Scanning electron microscopic image of the isolate depicts that the isolate belongs to the *Streptomyces* sp. The isolate AKN was further characterized by the methods recommended by International *Streptomyces* Project. The growth of the isolate on different media is given in the Table S1. The isolate AKN grew abundantly on ISP 1, ISP 2, ISP 4 and ISP 7 respectively. In ISP 3, ISP 5, ISP 6, nutrient agar and starch Casein Agar (SCA) the growth of the isolate was observed to be good to moderate. The isolate AKN showed positive for Methyl red test and Citrate utilization test. It showed negative for Indole and Voges Proskauer's test. It showed an alkaline butt with alkaline slant in triple sugar iron test. The isolate AKN was non-motile and negative for mannitol test. The isolate was also found positive for lipase, oxidase, catalase and urease and negative for amylase respectively (Table S2). The cultural condition for the growth of the isolate was optimized. The isolate grew very well up to 4% NaCl concentration, moderate growth in 6% NaCl

and no growth was observed in 10% NaCl concentration. The isolate also grew excellent in the pH 7 and moderate growth was observed in pH 6 and 9 respectively. Based on the temperature, the isolate had very good growth between 28-30°C. It grew moderately at 40°C and no growth at 50°C.

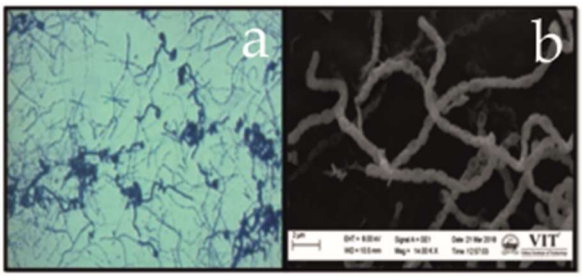

Figure 1. Image of strain VITAKN a) Gram's staining on light microscope and b) Scanning electron microscopy.

Crude extract was evaluated for determination of its non-inhibitory concentration (NIC) against *E. coli* pSB401 (pSB401) *an E. coli* pSB1075 (pSB1075) the two strains used for testing quorum sensing inhibitory activity. Determination of NIC is important to rule out any possible growth inhibition artifacts during quorum quenching assays. The growth-inhibitory activities were tested at concentrations between 0.0487-12.5 mg/mL. No growth inhibition compared with the control (solvent only) was observed. Therefore, this concentration was used for further evaluation of QSI activity. Cruse extracts inhibited luminescence by 20% at 0.78125 mg/mL (for pSB401) and 1.56 mg/mL (for pSB1075). These data suggested the presence of some QSI active molecule in the extract and hence required further investigation.

AKN genome assembly yielded 20 scaffolds with an N_{50} value of 788,548 bp, the largest contig having 1,671,513bp. The genome consists of 8,621,231 bp with a GC content of 72.2 %with 100% estimated completeness, 0.06% contamination and 0% heterogenity. AKN genome contains 7,898 predicted and 69 RNA genes (67 tRNA genes). 16S rRNA gene was assembled separately (see Materials and Methods). Based on EzTaxon analyses of extracted 16SrRNA gene (1564bp), the strain showed 99.8% similarity to *Streptomyces tendae* ATCC 19812 and hence strain AKN was designated as *Streptomyces tendae* VITAKN. antiSMASH analysis identified 33 clusters, mostly encoding for PKS and hybrid-PKS (12) and NRPS (7) BGCs, both of which are well known for producing structurally diverse compounds displaying various bioactivity. Detected BGCs belonging to other classes includes five terpenes, two siderophores, two bacteriocins and one of each of the following: melanin, ectoine, lanthipeptide, indole, and an unassigned cluster (Figure 2). Also, 9 clusters poses sequence similarity more than 75% to known BGCs. Interestingly, two BGCs with 100% similarity, SapB (a lantibiotic-like peptide) and coelichelin (siderophore) both known to be produced by *Streptomyces coelicolor*. SapB is a morphogenetic peptide that is important for aerial mycelium formation by the filamentous bacterium *Streptomyces coelicolor* [38]. Another BGC encoding for coelibactin, a NRPS synthesized peptide with predicted zincophore activity has been implicated in antibiotic regulation in *Streptomyces coelicolor* A3 (2) [39]. Manual curation of antiSMASH data inspired us to mine the genome to explore and exploit BGCs diversity at larger extend. These BGCs can be readily identified in a genome sequence as they are usually clustered together on the chromosome. The novelty and uniqueness of the BGCs predicted from this strain was evaluated through sequence similarity analysis with the MIBiG database and existing 3,365 BGCs predicted by antiSMASH analysis of publicly available complete *Streptomyces* genomes respectively. The genus *Streptomyces* is one of the

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most chemically exploited genera for discovering novel drugs since the discovery of penicillin and streptomycin. Further, the establishment of full genome sequence of two well studied natural product producing strains *Streptomyces coelicolor* [40] and *Streptomyces avermitilis* [41], led the scientist to notice the unexplored potential hidden in bacterial genomes [42]. A *Streptomyces* genome contains on average about 30 secondary metabolite gene clusters, but the limitation of detection analytically inspired us to explore the strains much more in depth and suggested that even well studied strains contain the genetic potential to synthesize many more compounds than detected analytically. One of the largest collections, MIBiG containing manually curated BGCs provided us a highly curated reference dataset (~1920 BGCs). Out of all the BGCs curated, 636 BGCs have been entered from genus *Streptomyces* alone followed by *Aspergillus* (88 BGCs) and *Psudomonas* (68 BGCs) [43]. This survey assesses the potential to discover novel metabolites from isolated *S. tendae* VITAKN and depicts unexplored biosynthetic space. Out of 33 BGCs observed, only nine BGCs displayed similarity with known BGCs, and only 17 BGCs associated with other BGCs extracted from existing *Streptomyces* genomes (Figure 3), warranting further exploration of the biosynthetic potential of this strain for producing novel bioactive secondary metabolites.

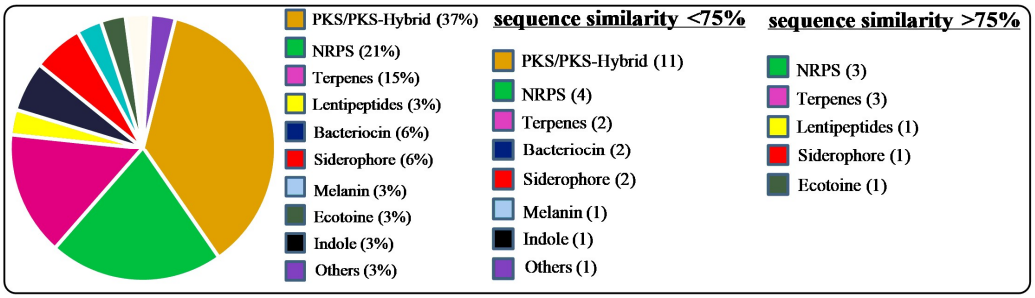

Figure 2. Total 32 BGCs with or without homology to MIBiG clusters identified using antiSMASH for *Streptomyces tendae* VITAKN**,** out of them 9 clusters poses sequence similarity more than 75%.

The chemical structures of their products can be predicted to a certain extent, based on the analysis and biosynthetic logic of the enzymes encoded in a BGC and their similarity to known counterparts [44]. Whereas, as mentioned before its not very common and easy that these BGCs product compounds to be detected analytically, still we analyzed crude extract of *S. tendae* VITAKN for metabolites by LC-HRMS and LC-HRMS/MS. This was planned to determine whether the extract features some unique chemical diversity and also how does it match with the predicted BGCs product. To create molecular network, the LC-MS/MS data were analyzed with publicly available data sets accessed via the GNPS-MassIVE database. To date, >93 million MS/MS spectra from various instruments (including Orbitrap, Ion Trap, qTOF, and FT-ICR) have been searched at GNPS, yielding putative dereplication matches of 7.7 million spectra to 15,477 compounds [33,45]. The usage of this platform yielded the formation of the molecular network containing 327 parent ions (nodes) (Figure S1).

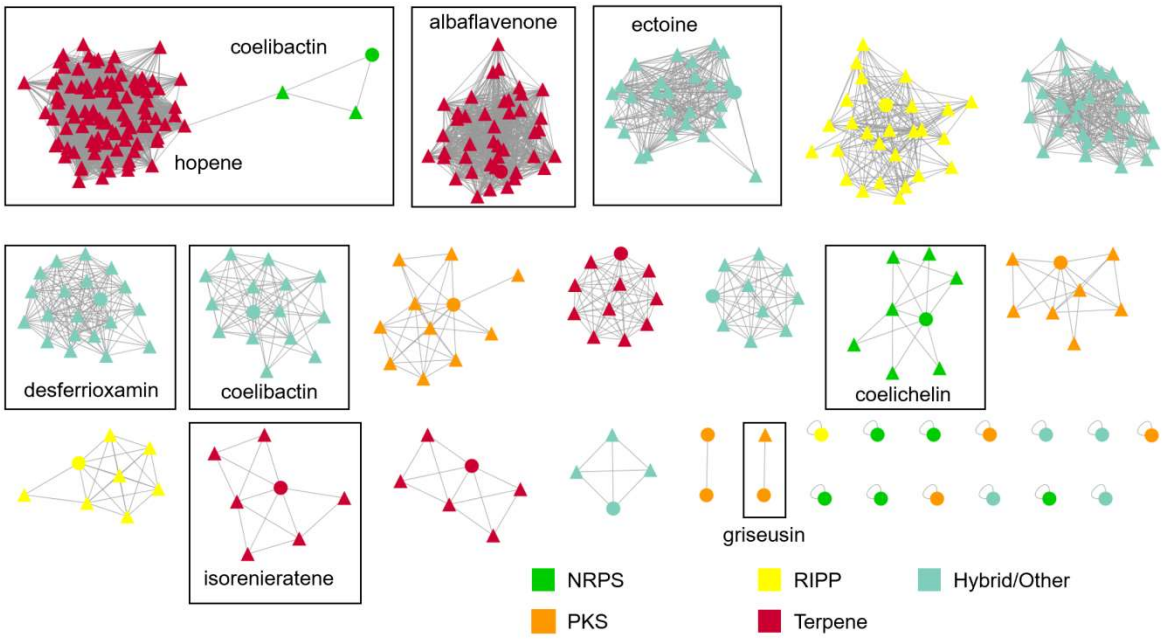

Figure 3. Sequence similarity network of the 33 BGCs detected in *S. tendae* VITAKN compared against BGCs in the MIBiG database and other *Streptomyces* strains. Circular nodes represent BGCs from this strain, and triangular nodes represent MIBiG and other *Streptomyces* BGCs. Boxed clusters represent clusters containing nodes associated with a MIBiG BGC. Colors were schemed according to different BGC family annotation.

These metabolites formed twenty-four clusters and one hundred eighty six self loop nodes. High throughput molecular networking in our study showed putative presence of cyclic dipeptides (2,5-diketopiperazines, DKPs) in cluster 4. The four cyclic peptides identified using the database in our present study; are cyclo-(Leu-Phe), cyclo-(L-Leu-L-Pro), cyclo-(L-Pro-L-Val) and cyclo-(L-Trp-L-Pro) (Figure 4). These DKPs have been previously reported to activate or inhibit LuxR-type proteins in AHL biosensor strains, albeit at significantly higher concentrations than native lactones [46]. These finding further emphasizes to continue our effort on isolating and purifying these molecules and identify the exact activity of these molecules and also identify other molecules present in the clusters which can be possibly a new analogue of these DKPs. Also, we were not able to identify any other metabolites, which were putatively predicted using the BGCs pathway. This strain may produce large number of yet unknown compounds one of which may be the relevant for the QSI activity observed apart from detected DKPs in the extract. Furthermore, the use DEREPLICATOR as part of library search for *in silico* identification of both peptidic and non-peptidic natural products including polyketides, lipids, terpenes, benzenoids, alkaloids, flavonoids, etc., is an advanced tool to identify the known among unknown [9,10].

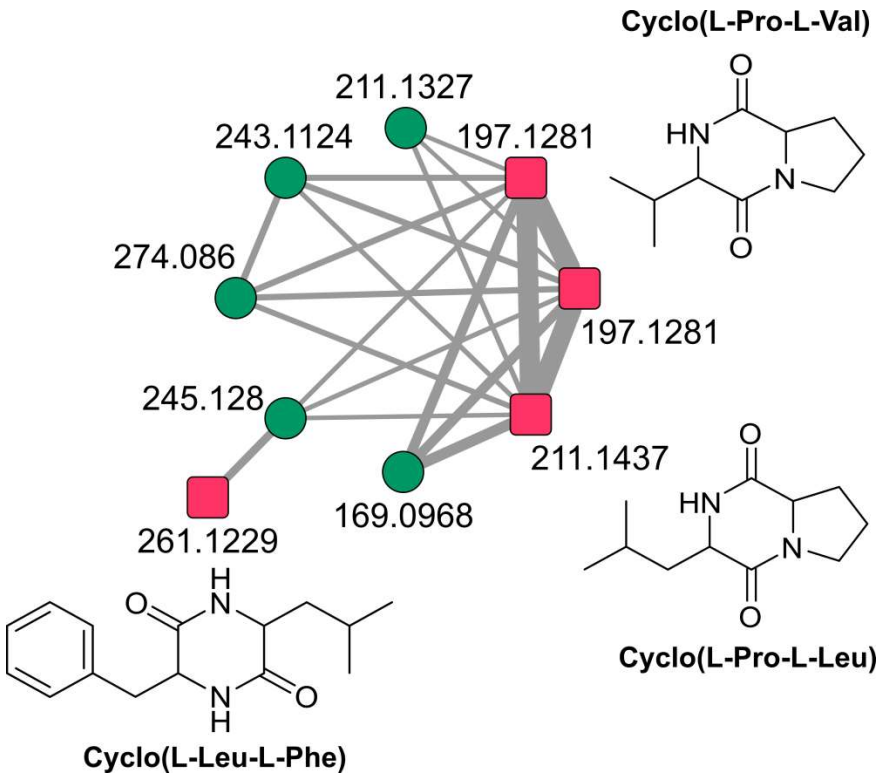

Figure 4. DKP cluster in *S. tendae* VITAKN molecular network. Each node represents m/z value of the parent ion and edge thickness is related to cosine score similarity

4. Conclusion

The continued rise in antibiotic resistance needs to be addressed by discovering an alternative way for tackling the infection. Quorum sensing inhibitory activity (QSI) is one of many strategies to start with, whereas at the same time, it should also be noted to explore the potential of isolating a strain from a unexplored place. Assessment of *Streptomyces tendae* VITAKN biosynthetic gene cluster and comparing it with all the available genome data from *Streptomyces* genus can provide insight to direct responsible discovery efforts. The present study also makes a use of chemical fingerprinting and molecular networking tool to identify the presence of metabolites in the crude extract. However, the vast discrepancies between BGCs with and without sequence similarity warranting further exploration of the biosynthetic potential of this strain for producing novel bioactive secondary metabolites.

Data deposition and job accessibility: The mass spectrometry data were deposited on public repository (provide the deposition accession number), such as MassIVE or MetaboLights. The molecular networking job can be publicly accessed at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=2f692735adc7493c84080eb3042fadb5.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Growth pattern of isolate AKN on different media Table S2: Biochemical tests for VITAKN Figure S1: Generation of molecular network with various clusters produced by crude extract of *Streptomyces tendae* VITAKN by GNPS above similarity score threshold. Nodes highlighted in coloured boxes represent parent ions.

Author's Contributions: All authors have read and agree to the published version of the manuscript. Conceptualization, N.I., K.S., K.K., L.S. and V.C.; methodology, K.S., R.T., I.B., J.J. ; data curation, K.S., I.B., S.S.,

D.E., P.H., L.S., and V.C.; writing—original draft preparation, K.S., N.I., I.B., J.J., R.T., L.S., and V.C.; writing—review and editing, K.S., I.B., R.T., K.K., P.H., L.S., and V.C.; supervision, K.S., L.S., V.C.,.; project administration, K.S., K.K., L.S., and V.C.; funding acquisition, K.S., P.H. and V.C.

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