Draft Genome Analysis of Antimicrobial *Streptomyces* Isolated from Himalayan Lichen^S

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There have been several studies regarding lichen-associated bacteria obtained from diverse environments. Our screening process identified 49 bacterial species in two lichens from the Himalayas: 17 species of *Actinobacteria*, 19 species of *Firmicutes*, and 13 species of *Proteobacteria*. We discovered five types of strong antimicrobial agent-producing bacteria. Although some strains exhibited weak antimicrobial activity, NP088, NP131, NP132, NP134, and NP160 exhibited strong antimicrobial activity against all multidrug-resistant strains. Polyketide synthase (PKS) fingerprinting revealed results for 69 of 148 strains; these had similar genes, such as fatty acid-related PKS, adenylation domain genes, *PfaA*, and *PksD*. Although the association between antimicrobial activity and the PKS fingerprinting results is poorly resolved, NP160 had six types of PKS fingerprinting genes, as well as strong antimicrobial activity. Therefore, we sequenced the draft genome of strain NP160, and predicted its secondary metabolism using antiSMASH version 4.2. NP160 had 46 clusters and was predicted to produce similar secondary metabolites with similarities of 5–100%. Although NP160 had 100% similarity with the alkylresorcinol biosynthetic gene cluster, our results showed low similarity with existing members of this biosynthetic gene cluster, and most have not yet been revealed. In conclusion, we expect that lichen-associated bacteria from the Himalayas can produce new secondary metabolites, and we found several secondary metabolite-related biosynthetic gene clusters to support this hypothesis.

Keywords: Antimicrobial activity, draft genome sequencing, fingerprinting, Himalayan lichen-associated bacteria, polyketide synthase, secondary metabolites

Introduction

Approximately 13,500 species of lichen are estimated to inhabit 8% of the earth's surface in various environments, including low temperature, drought, and darkness [1]. To survive in such environments, lichens produce unique secondary metabolite-related compounds that have antimicrobial, antitumoral, immunostimulating, and/or antiviral activities. In addition, they contribute to symbiosis of the mycobiont (fungal partner), photobiont (photosynthetic partner, usually a green algae or cyanobacterium), and

non-photosynthetic partner (bacterium). Before 2005, research regarding lichens was focused on lichenicolous fungus [2] and cyanobacteria [3]; the word 'lichen-associated bacteria' began to appear in the literature, due to the work by Alexandra et al., beginning in 2011 [4]. It has recently been reported that millions of lichen-associated bacteria greatly influence the survival of lichen through various roles such as resistance stress factor, detoxification of metabolites, support for photosynthesis, and nutrient supply [5]. In 2018, Delphine et al. reported using genome analysis to identify a new metabolite from the alpha-

proteobacterium strain MOLA1416, a marine type of lichen-associated *Proteobacteria* [6]. Recent studies of lichen-associated bacteria have been performed to confirm activity and select cultivable bacteria for in-depth genome analysis.

Most research studies regarding lichen-associated bacteria are based on the hypothesis that such bacteria will produce one or more unusual secondary metabolites in the symbiotic relationship. The biosynthesis of secondary metabolites, analyzed by biosynthetic gene cluster, is already well known; this includes multidomain enzymes such as polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) [7–10]. Although PKS and NRPS comprise most of the metabolite class, knowledge regarding the biological activities of PKS and NRPS genes remains insufficient. Lichen-associated bacteria have been analyzed from diverse environments, including thermal spots [5], sub-Arctic [11], and Arctic [12]. Thus far, 465 species of lichen have been identified in Nepal [13]; nevertheless, there has been no study of the diversity of lichen-associated bacteria or their PKS and NRPS genes. Notably, lichen flora, market potential, and various activities (antioxidant, antimicrobial, and toxicity) were reported regarding lichens [14, 15]. Other lichen-associated bacteria have been reported as examples of bacterial diversity [16], as sources of novel metabolites [6], and as samples for genome analysis [17].

We undertook an extensive study regarding the antimicrobial potential of lichen-associated bacteria. We aimed to identify lichen-associated bacteria from Himalayan lichens and to analyze the genomes of selected bacteria that showed antimicrobial activities with secondary metabolite-related biosynthetic gene clusters. Based on identification of PKS and NRPS genes using fingerprinting polymerase chain reaction (PCR), we propose the use of lichen-associated bacteria to produce antimicrobial agents. This study also includes draft genome analysis of lichen-associated bacteria to understand the biosynthesis of secondary metabolites.

Materials and Methods

Isolation of Lichen-Associated Bacteria

Two lichens from the Himalayas (Fig. S1 and Table S1) were collected (27.718805, 85.322705), categorized, and identified by morphological analysis. To isolate associated bacteria from lichens, a modified version of the method of Kim *et al.* [18] was used. The thallus segments of the lichens were spread with sterilized scissors, and 2.0 ml of 0.85% NaCl wash solution was added. The lichens were vortexed for 1 min and the supernatant was discarded; this step was repeated twice. Then, 100- μ l aliquots

of the washed, broken lichen solution were spread on replicate plates containing MY media (3.0 g malt extract, 3.0 g yeast extract, 10.0 g peptone, 10.0 g dextrose, and 20.0 g agar, pH 7.2), R2A media (0.5 g proteose peptone, 0.5 g casamino acid, 0.5 g yeast extract, 0.5 g dextrose, 0.5 g soluble starch, 0.3 g sodium pyruvate, 0.3 g dipotassium phosphate, and 0.05 g magnesium sulfate, pH 7.2), or Bennett's media (10.0 g glucose, 1.0 g yeast extract, 2.0 g Bacto-peptone, and 1.0 g beef extract, pH 7.2); the plates were incubated at different temperatures (8, 15, 25, and 37°C) for 3 to 30 days.

16S rRNA Gene Sequence and Phylogenetic Analysis

All isolated bacteria were identified by 16S rRNA gene sequence analysis. For 16S rRNA analysis, the genomic DNA of each strain was extracted from a 100- μ l culture sample from a 15–30 day pure culture of a single colony and centrifuged for 10 min at 10,000 $\times$ g; extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen, Inc., USA). The 16S rRNA was amplified with two universal primers: 518F (5'-CCAGCAGCCGCGTAATACG-3') and 800R (5'-TACCAGGGTATCTAATCC-3'). For difficult to analyze sequences, other primers were used: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). Sequencing was performed by Genotech Ltd. (Korea); the results were compared with the GenBank database using BLAST to identify the most similar sequences. Closest relative strains were also identified using EzBioCloud (ChunLab, Inc., Korea), and the species of the isolates were predicted. The sequences were pairwise aligned, and phylogenetic analysis was performed using a modified version of the method described by Kimura *et al.* [19]. A phylogenetic tree was inferred using the neighbor-joining method in MEGA X [20].

Extraction of Isolated Strains and Disk Diffusion Assay

For the antimicrobial assay, each of the 49 lichen-associated bacteria isolates was cultured in 200 ml liquid medium in conditions identical to those in which it was isolated. A double volume of analytical-grade ethyl acetate (Daejung, Korea) was added into the grown culture fluid, and shaken using a funnel at room temperature for 2 h for extraction. The solvent layer was concentrated using a rotary evaporator (EYELA A-1000S, Tokyo Rikakikai Co., Japan), and each extract was dissolved in 2.0 ml ethyl acetate. The antimicrobial activities of all extracts obtained from the 49 isolated strains were investigated using 16 multidrug-resistant microorganisms, including anaerobic and aerobic bacteria, by the disc diffusion method. Anaerobic bacteria were *Staphylococcus aureus* (KCTC1927), *Streptococcus mutans* (KCTC3065), *Streptococcus sanguinis* (KCTC3284), *Streptococcus sobrinus* (KCTC3308), *Streptococcus cricetid* (KCTC3640), *Streptococcus ratti* (KCTC3655), *Aggregatibacter actinomycetemcomitans* (KCTC3698), *Streptococcus anginosus* (KCTC3983), *Actinomyces viscosus* (KCTC5531), and *Actinomyces israelii* (KCTC9054); aerobic bacteria were *Bacillus subtilis* (KCTC1918), *Staphylococcus aureus* (KCTC1928), *Micrococcus luteus* (KCTC1915), *Escherichia coli* (KCTC2441), *Pseudomonas aeruginosa*

(KCTC1637), and *Enterobacter cloacae* (KCTC1685). The bacteria were purchased from the Korea Collection for Type Culture and Korea Research Institute of Bioscience and Biotechnology (Korea). All microorganisms were grown in Luria-Bertani (MBcell Ltd., Korea) or tryptic soy (Becton Dickinson Co., USA) or brain-heart infusion broth (Becton Dickinson Co.) under the appropriate conditions. The paper disk diffusion test was performed in accordance with the method of Bauer *et al.*, with some modifications [21]. The growth of each of the 16 multidrug-resistant microorganisms was standardized to 0.5 McFarland and spread with a swab. Each extract was added to a paper disk (6-mm diameter, Advantec Co., Japan) and the disks were transferred onto plates inoculated with the multidrug-resistant bacterial strains. Disks containing ethyl acetate were used as negative controls. All inoculated culture plates were incubated at 37°C, and the inhibition zones of bacterial growth were measured approximately 9 h later. All analyses were performed in triplicate, and the experimental values are reported as means $\pm$ standard deviation.

Fingerprinting of PKS and NRPS Genes

In the fingerprinting experiment, eight sets of degenerate primers (Table 1) targeting genes encoding the ketoacyl synthase (KS) domains of type I PKS and type II PKS (*i.e.*, PKS-1 and PKS-2) and the adenylation domain of NRPS were used to screen the biosynthetic potentials of the isolates [9, 10, 21–24]. PCR was performed as follows: the 20 μ l reaction sample contained 10 μ l Noble premix (Noble Bio Inc., Korea), 4 μ l total DNA, 4 μ l distilled

water, and 1 μ l of each primer. Amplification comprised denaturation at 95°C for 1 min, annealing at 50–68°C for 1 min, and extension at 72°C for 2 min. The sizes of the PCR products were 600–1,000 bp for PKS and NRPS genes; these were purified by electrophoresis in a 1% (wt/vol) agarose gel. Purified PCR products were ligated into pMD20-T vectors (Takara Inc., Japan), and transformed into competent *E. coli* XLI-blue. Positive recombinants were screened on 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), isopropyl- β -D-thiogalactopyranoside, and ampicillin indicator plates by color-based recombinant selection. Plasmid purification was performed using an isolation kit (GeneAll Co., Korea) and sequence analysis was carried out by Genotech Ltd. The BLASTX algorithm with default parameters was used to identify database entries related to the isolated sequences. A phylogenetic tree was generated based on the PKS and NRPS fingerprinting results; it was constructed using multiple sequence alignment tools, the Mega package, and the neighbor-joining method.

Draft Genome Sequencing of NP160

We selected lichen-associated bacteria with the highest probability to be used as a new antimicrobial agent. Genomic DNA was extracted from *Streptomyces* sp. NP160 using a QIAamp DNA Mini Kit (Qiagen, Inc.), and the quantity and purity were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) [25]. Sequencing was performed using an Illumina MiSeq system with a 300 bp paired-end library. *De novo* assembly of genome sequences was performed using Celera Assembler software (Ver. 8.3) with Illumina short reads. Prior to assembly,

Table 1. Primers used for fingerprinting of PKS and NRPS genes.

Type	Primer name	Target gene	Size (bp)	Annealing (°C)*	Reference
Type I PKS	K1F	PKS-I ketoacyl synthase (KS) domains and methyl malonyl transferase domains/PKS-I, KS-AT fragments	TSAAGTCSAACATCGGBCA CGCAGGTTSCGTACCAGTA	65	[10]
	M6R				
	PKS-I-A	KS domains	GCSATGGAYCCSCARCRCGSVT GTSCCGTSCCRTGSSCYTCSAC	700	[21]
	PKS-I-B				
	KSMAB KSMBR	Beta-KS domain	TSGCSATGGACCCSCAGCAG CCSGTSCCGTSGGCCTCSAC	~700	[22]
Type II PKS	KSI1f KSI2r	Beta-ketosynthase domains	GCI ATGGAYCCICARCARMGIVT GTICIGTICCRTGISCYTCIAC	700	[9]
	540F 1100R				
	KS α KS β	Partial KS genes of Type II PKS	GGITGCACSTCIGGIM TSGAC CCGATSGCICCSAGIGAGTG	68	[23]
	KS α KS β	PKS-II, KS α and KS β domains/ designed to target conserved sequences	TSGRCTACRCAACGGSCACGG TACSAGTCSWTCGCTGGTTC	600–700	[24]
NRPS	A3F A7R	NRPS adenylation domains/alignments of ketosynthase, acyltransferase and adenylation sequences	GCSTACSYSATSTACACSTCSGG SASGTCVCCSGTSCGGTAS	65	[24]
	MTF2 MTR				
		Adenylation A domain of NRPS	GCNGGYGGYGCNTAYGTNCC CCNCGDATYTTNACYTG	1,000	[24]

*Annealing temperature was modified based on the results of preliminary experiments for this study.

Illumina reads were trimmed using FASTX-Toolkit software (http://hannonlab.cshl.edu/fastx_toolkit) with parameters -t 20, -I 70, and -Q 33; a paired sequence was then selected from trimmed Illumina reads. Coding DNA sequences (CDSs) were predicted and annotated using the National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline and the Rapid Annotation using the Sub-system Technology server [26]. The numbers and types of secondary metabolites present in microbial genomes were determined using antiSMASH software (version 4.2) [27]. For the analysis of antiSMASH results, each gene was predicted using BLASTX and compared with known gene clusters.

Results and Discussion

Identification of Lichen-Associated Bacteria and Phylogenetic Analysis

We isolated 185 lichen-associated bacteria from the rarely studied Himalayan region. Based on their color and morphology when grown on media, we selected 49 isolates for further analysis (Table S2). The isolated bacteria ratios were balanced (Fig. 1), which contrasts with the findings of prior studies in which most such bacteria were classified as *Alphaproteobacteria* [28]. Forty-nine of the isolates underwent phylogenetic analysis. Based on BLAST analysis of 16S rRNA gene sequences, we found that the isolates were divided into three dominant groups: 17 species of *Actinobacteria*, 19 species of *Firmicutes*, and 13 species of *Proteobacteria* (Fig. 1A). The 17 species of *Actinobacteria* were classified as *Curtobacterium* spp., *Leifsonia* spp., *Streptomyces* spp., and *Rhodococcus* spp. Furthermore, a phylogenetic tree constructed using the neighbor-joining method enabled us to identify isolates at the genus level with over 98% identity (Fig. 1B). According to Suzuki *et al.*, there have been some studies of lichen-associated bacteria from various lichens, and secondary metabolites of *Amycolatopsis* have been reported [29].

Antimicrobial Properties of Lichen-Associated Bacteria

To evaluate the antimicrobial potentials of the isolated bacteria, a paper disk diffusion test was performed. In this test, our extracts showed similar antimicrobial strengths, with zone of inhibition diameters of 6–20 mm against both anaerobic and aerobic bacteria. As shown in Table 2, most strains had no antimicrobial activity or narrow antimicrobial activity; however, 12 isolates showed inhibitory antimicrobial activity against both Gram-negative and Gram-positive bacteria. In particular, strains NP088, NP131, NP132, NP134, and NP160 had the strongest inhibitory activity, which suggested that they produced antimicrobial agents.

These isolates were classified as *Bacillus*, *Methylobacterium*, and *Streptomyces*. Strain NP160 showed the most potent antimicrobial activity, with a zone of inhibition diameter of 22.89 ± 1.07 mm against *Streptococcus anginosus*, which causes pyogenic liver abscess. In our antimicrobial activity assays, each bacterial species exhibited the strongest antimicrobial resistance known.

Lichen-associated bacteria have been isolated from a variety of lichen. Each bacterium plays a role in symbiosis and has potential for biotechnological applications; these primarily include nitrogen fixation and phosphate solubilization, which can promote plant growth [30]. Only Kim *et al.* reported the antimicrobial activity of lichen-associated bacteria; these organisms showed zone of inhibition diameters of 8–12 mm [18]. Our isolates showed stronger inhibitory antimicrobial activity and were predicted to produce antimicrobial agents. We presume that each lichen-associated bacterial species has a unique role within the lichen. Therefore, we further assessed the antimicrobial activity and corresponding biosynthetic analysis using fingerprinting of PKS and NRPS genes.

Fingerprinting of PKS and NRPS Genes

Many intriguing secondary metabolites are biosynthesized by the PKS, NRPS, and PKS-NRPS hybrid systems. To assess the biosynthetic potentials of lichen-associated bacteria regarding PKS and NRPS systems, we evaluated each of the 49 isolated strains by amplifying PKS (type I and type II) and NRPS genes with eight sets of diverse primers. The results revealed 148 PCR products. Only the products of our desired size were refined and analyzed, and the results were obtained for 69 samples. We confirmed the identities of PKS and NRPS genes by BLASTX comparison with those in the GenBank database. Most PKS genes showed high similarities of 40–100%. We identified 19 acyltransferase domain genes, nine adenylation-related domain genes, five beta-ketoacyl domain genes, 13 fatty acid-related PKS genes and four hybrid PKS-NRPS genes (Table S3). PKS fingerprinting results revealed that most strains had more than one PKS-NRPS-related gene. Most fingerprinting genes were involved in bacillaene biosynthesis (*e.g.*, *PksC*, *PksF*, *PksG*, *PksH*, *PksI*, *PksJ*, *PksL*, *PksM*, *PksN*, and *PksR*) [31]. Strain NP160 had the greatest number of PKS and NRPS genes without any bands indicative of nonspecific amplification (Fig. S2); it also demonstrated the strongest antimicrobial activity. Fingerprinting showed that NP160 had six types of PKS- and NRPS-related genes. Although the primers were constructed on the basis of PKS and NRPS domains, it was difficult to determine whether

Fig. 1. Classification of lichen-associated bacteria from the Himalayas. (A) Phylogenetic tree of lichen-associated bacteria from *Usnea* sp. and *Ramalina* sp. (B) Phylogenetic tree of *Actinomycetes* including BlastN results. Neighbor-joining phylogenetic tree analysis of 16S rRNA of lichen-associated bacteria.

enzyme function was consistent among the isolates. Therefore, we aimed to analyze the draft genome in detail; we selected strain NP160, which was predicted to produce several secondary products.

Fig. 1. Continued.

Draft Genome Analysis of *Streptomyces* sp. NP160

In previous studies, some lichen-associated bacteria were shown to produce secondary metabolites, and corresponding genomic analyses were performed [32]. Researchers have found much of interest in the genomes and secondary metabolites of lichen-associated bacteria. Among the 49 lichen-associated bacteria, we found many isolates that

could produce various substances. Although many researchers have reported on lichen-associated bacteria, this study describes secondary metabolite substance-producing strains and fatty acid synthesis-related PKS genes. Strain NP160 had robust expression of six types of PKS and NRPS genes, as well as strong antimicrobial activity. PKS fingerprinting methodology cannot readily be

Table 2. Antimicrobial activity per bacterial genus.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
NP007	-	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+
NP008	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
NP010	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP011	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP012	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+
NP016	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
NP020	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-
NP021	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP030	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+
NP038	+	-	+	+	+	+	+	+	-	-	-	+	-	-	-	-
NP043	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+
NP049	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP050	-	+	-	-	+	+	-	+	+	+	-	-	-	+	-	-
NP054	++	+	+	++	+	+	+	+	+	++	+	-	+	+	+	+
NP062	+	-	+	+	+	+	+	+	-	-	-	+	+	+	-	+
NP063	-	-	+	+	-	+	+	++	+	-	+	+	+	+	+	+
NP069	-	-	+	+	-	-	-	+	-	-	-	-	-	-	+	-
NP071	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-
NP072	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+
NP073	+	+	+	+	+	+	+	-	++	-	+	+	+	+	+	++
NP074	+	-	+	+	-	-	+	+	-	-	-	+	-	-	-	+
NP077	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-
NP078	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
NP086	+	-	+	+	-	-	+	+	+	+	-	-	+	-	-	-
NP088	+++	+++	-	+++	+++	-	+++	+++	+++	+++	+++	+++	+++	-	+++	+++
NP090	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP091	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP092	+++	-	-	++	+	-	-	-	-	-	-	-	-	-	-	+
NP093	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+
NP094	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP097	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP108	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP115	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP121	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
NP125	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP127	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	+
NP131	+++	+++	+++	++	+	+	-	-	-	-	-	-	-	-	-	-
NP132	+++	++++	+++	+++	++++	++++	++++	++++	+++	++++	-	++++	-	++++	-	++++
NP134	+++	++	-	++	+	+	-	-	-	-	-	-	-	-	-	-
NP139	-	+	+	+	+	+	-	-	+	-	-	+	-	+	-	+
NP141	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP142	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP143	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP157	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2. Continued.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
NP160	++	+	+	+	+	+	++++	++++	+++	-	-	++++	-	++++	-	++++
NP161	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NP167	++	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
NP175	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-	-
NP183	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-

*1, *Bacillus subtilis*; 2, *Staphylococcus aureus*; 3, *Micrococcus luteus*; 4, *Escherichia coli*; 5, *Pseudomonas aeruginosa*; 6, *Enterobacter cloacae*; 7, *Staphylococcus aureus*; 8, *Streptococcus mutans*; 9, *Streptococcus sanguinis*; 10, *Streptococcus sobrinus*; 11, *Streptococcus criceti*; 12, *Streptococcus rattii*; 13, *Aggregatibacter actinomycetemcomitans*; 14, *Streptococcus anginosus*; 15, *Actinomyces viscosus*; 16, *Actinomyces israelii*.

*, negative; +, > 6 mm; ++, > 8 mm; +++, > 10 mm; and +++, > 20 mm.

used to classify gene clusters of antimicrobial agents; therefore, we assembled a draft genome of strain NP160 to identify biosynthetic genes related to secondary metabolite

gene clusters. The draft genome sequence had a size of 4,774,418 bp with a G+C content of 50.01%, and generated a total of 2,455,256 reads. Predicted gene sequences were translated and searched against the NCBI non-redundant, Clusters of Orthologous Groups, and Kyoto Encyclopedia of Genes and Genomes databases. A total of 4,319 CDSs were predicted; the coding region comprised 89.1% of the genome. In addition, 3 rRNAs and 44 tRNAs were predicted, and genome assembly resulted in 186 contigs, as shown in Fig. 2. Based on the draft genome results, strain NP160 was classified as *Streptomyces mauvecolor*. Thus far, this species has only one strain; the anti-fungal activity of *S. mauvecolor* BU16 was previously described [33]. Importantly, our analysis demonstrated biosynthesis of secondary metabolites in a unique bacterial strain, *S. sp.* NP160.

Predicted Biosynthetic Gene Cluster for Secondary Metabolites in *S. sp.* NP160

S. sp. NP160 was assessed for the presence of secondary metabolite gene clusters using antiSMASH, based on draft genome information. The results showed that *S. sp.* NP160 had 46 secondary metabolite gene clusters; 19 of these gene clusters were similar to previously described secondary metabolite biosynthetic gene clusters, including sioxanthin, meiligmycin, lomaivitcin, divergolide, lasalocid, incedinine, GE81112, 5'-hydroxystreptomycin, furaquinocin, ravidomycin, calicheamicin, paromomycin, surfactin, teicoplanin, chlortetracycline, chlorizidine A, and alkylresorcinol. These gene clusters demonstrated diverse similarities of 5–100%, as shown in Table 3. There were several types of clusters, such as terpene, saccharide, fatty acid, and PKS type III; these contained PKS-related clusters (e.g., divergolide, lasalocid, and meridamycin), but showed low similarity with well-known gene clusters. Therefore, we chose to predict four types of clusters that putatively

Fig. 2. Genome information of NP160.

(A) Circular representation of *Streptomyces sp.* NP160 draft genome. The map was created using CGview Comparison tools. (B) Characteristics of NP160 genome.

Table 3. Summary of NP160 antiSMASH results (v 4.2).

Cluster	Type	From	To	Most similar known cluster (similarity, %)	MBiG BGC-ID
Cluster 1	Terpene	7223	28248	Sioxanthin biosynthetic gene cluster (60)	BGC0001087_c4
Cluster 2	Cf_putative	45854	67462	Meilingmycin biosynthetic gene cluster (2)	BGC0000093_c1
Cluster 3	Cf_putative	107150	117184	Lomaiviticin biosynthetic gene cluster (3)	BGC0000241_c1
Cluster 4	Cf_putative	99211	113801	Divergolide biosynthetic gene cluster (6)	BGC0001119_c1
Cluster 5	Cf_putative	116246	137055	Lasalocid biosynthetic gene cluster (3)	BGC0000087_c1
Cluster 6	Cf_putative	163699	170292	Incednine biosynthetic gene cluster (2)	BGC0000078_c1
Cluster 7	Cf_putative	2860	15687	GE81112 biosynthetic gene cluster (7)	BGC0000360_c1
Cluster 8	Cf_saccharide	304	48382	5'-Hydroxystreptomycin biosynthetic gene cluster (13)	BGC0000690_c1
Cluster 9	Cf_fatty_acid-Cf_saccharide	70760	102725	Furaquinocin A biosynthetic gene cluster (8)	BGC0001078_c1
Cluster 10	Cf_putative	24494	35734	Meilingmycin biosynthetic gene cluster (2)	BGC0000093_c1
Cluster 11	Cf_saccharide	81255	107170	Ravidomycin biosynthetic gene cluster (5)	BGC0000263_c1
Cluster 12	Cf_putative	125432	138595	Meridamycin biosynthetic gene cluster (5)	BGC0001011_c1
Cluster 12	Cf_saccharide	1	32710	Calicheamicin biosynthetic gene cluster (4)	BGC0000033_c1
Cluster 13	Cf_putative	31926	36355	Paromomycin biosynthetic gene cluster (5)	BGC0000712_c1
Cluster 14	Cf_putative	15433	26486	Meridamycin biosynthetic gene cluster (5)	BGC0001011_c1
Cluster 15	Cf_putative	110365	121107	Surfactin biosynthetic gene cluster (8)	BGC0000433_c1
Cluster 16	Cf_putative	27142	62873	Teicoplanin biosynthetic gene cluster (3)	BGC0000440_c1
Cluster 17	Cf_saccharide	85115	120159	Chlortetracycline biosynthetic gene cluster (5)	BGC0000209_c1
Cluster 18	Cf_fatty_acid	142859	163881	Chlorizidine A biosynthetic gene cluster (7)	BGC0001172_c1
Cluster 19	T3pks-Cf_saccharide	18544	79268	Alkylresorcinol biosynthetic gene cluster (100)	BGC0000282_c1

*cf, possible cluster.

encoded known pathways for the production of sioxanthin, furaquinocin A, chlorizidine A, and alkylresorcinol. Although most clusters displayed low levels of similarity with well-known clusters, cluster 46 of *S. sp.* NP160 had 100% similarity with the alkylresorcinol biosynthetic gene cluster (Fig. 3). Alkylresorcinol is classified as a resorcinolic lipid, which can help prevent cells from becoming cancerous. Nearly all gene clusters had PKS-related genes, such as acyl carrier protein and ketoacyl transferase; cluster 2, which had a terpene gene cluster, was an exception. Based on these results, our PKS fingerprinting data matched genome analysis data in that both showed several genes related to PKS, such as acyltransferase and acyl carrier protein (Table S3 and Fig. 3).

In conclusion, most lichens produce unique secondary metabolites and are known to contain multiple chemical constituents; these include mono-substituted phenyl rings, terpenes, fatty acids, and polysaccharides, with antitumor, antimicrobial, anti-inflammatory, antioxidant, and antithrombosis activities. In particular, lichens of *Ramalina* sp. have been reported to exhibit antioxidant activities due to the presence of salazinic acid and usnic acid (Table S1). Although most bioactive compounds in lichens are produced

by fungi, lichen-associated bacteria also produce bioactive compounds. *Streptomyces* species can perform biosynthesis of secondary metabolites [34]. It was recently published that lichen-associated bacteria can produce a phthalazinone derivative [29]. Therefore, we hypothesized that our strain could produce secondary metabolites or other unique metabolites. Based on antimicrobial activity and PKS fingerprinting results, we selected a strain for draft genome analysis, and identified putative secondary metabolite-related biosynthetic gene clusters. *S. sp.* NP160 had 30 unknown gene clusters that were predicted to have antimicrobial activity. The biosynthetic gene cluster results showed low similarity with those of known clusters; only two *S. sp.* NP160 biosynthetic gene clusters showed similarity > 60% with known clusters. We expect that new secondary metabolites can be isolated from *S. sp.* NP160. In addition, strain NP160 was predicted to contain meilingmycin and alkylresorcinol, which have been reported to exhibit antimicrobial activity. Therefore, we presume that NP160 can produce an alkylresorcinol derivative with antimicrobial activity. In the present study, we assessed antimicrobial activity and performed PKS fingerprinting of cultivable lichen-associated bacteria from the Himalayas to identify

Fig. 3. Biosynthetic gene clusters predicted from *Streptomyces* sp. NP160.

potential new antimicrobial agents. Our results can aid in the investigation of novel antimicrobial agents and provide a basis for genome analysis of lichen-associated bacteria; moreover, the results increase the available knowledge regarding the relationship between lichens and bacteria.

Nucleotide Sequence Accession Numbers

The draft genome information of *Streptomyces* sp. NP160 was deposited in GenBank under the accession number VDMJ000000000.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

- White PA, Oliveira RC, Oliveira AP, Serafini MR, Araújo AA, Gelain DP, *et al.* 2014. Antioxidant activity and mechanisms of action of natural compounds, isolated from lichens: a systematic review. *Molecules* **19**: 14496-14527.
- He H, Bigelis R, Yang HY, Chang LP, Singh MP. 2005. Lichenicolins A and B, new bisnaphthopyrones from an unidentified lichenicolous fungus, strain LL-RB0668. *J. Antibiot. Tokyo* **58**: 731-736.
- Oksanen I, Jokela J, Fewer DP, Wahlsten M, Rikkinen J, Sivonen K. 2004. Discovery of rare and highly toxic microcystins from lichen-associated cyanobacterium *Nostoc* sp. strain IO-102-I. *Appl. Environ. Microbiol.* **70**: 5756-5763.
- Mushegian AA, Peterson CN, Baker CC, Pringle A. 2011. Bacterial diversity across individual lichens. *Appl. Environ. Microbiol.* **77**: 4249-4252.
- Parrot D, Legrave N, Delmail D, Grube M, Suzuki M, Tomasi S. 2016. Review - lichen-associated bacteria as a hot spot of chemodiversity: focus on unciamycin, a promising compound for future medicinal applications. *Planta Med.* **82**: 1143-1152.
- Parrot D, Intertaglia L, Jehan P, Grube M, Suzuki MT, Tomasi S. 2018. Chemical analysis of the alphaproteobacterium strain MOLA1416 associated with the marine lichen *Lichina pygmaea*. *Phytochemistry* **145**: 57-67.
- Bertrand RL, Sorensen JL. 2018. A comprehensive catalogue of polyketide synthase gene clusters in lichenizing fungi. *J. Ind. Microbiol. Biotechnol.* **45**: 1067-1081.
- Miyana A, Kudo F, Eguchi T. 2018. Protein-protein interactions in polyketide synthase-nonribosomal peptide synthetase hybrid assembly lines. *Nat. Prod. Rep.* **35**: 1185-1209.
- Zhang W, Zhang F, Li Z, Miao X, Meng Q, Zhang X. 2009. Investigation of bacteria with polyketide synthase genes and antimicrobial activity isolated from South China Sea sponges. *J. Appl. Microbiol.* **107**: 567-575.
- Ayuso-Sacido A, Genilloud O. 2005. New PCR primers for the screening of NRPS and PKS-I systems in actinomycetes: detection and distribution of these biosynthetic gene sequences in major taxonomic groups. *Microb. Ecol.* **49**: 10-24.
- Sigurbjörnsdóttir MA, Vilhelmsson O. 2016. Selective isolation of potentially phosphate-mobilizing, biosurfactant-producing and biodegradative bacteria associated with a sub-Artic, terricolous lichen, *Peltigera membranacea*. *FEMS Microbiol. Ecol.* **92**(6): fiw090.

12. Sigurbjörnsdóttir MA, Heiðmarsson S, Jónsdóttir AR, Vilhelmsson O. 2014. Novel bacteria associated with Arctic seashore lichens have potential roles in nutrient scavenging. *Can. J. Microbiol.* **60**: 307-317.
13. Baniya CB, Solhøy T, Gauslaa Y, Palmer MW. 2010. The elevation gradient of lichen species richness in Nepal. *Lichenologist* **42**: 83-96.
14. Devkota S, Chaudhary RP, Werth S, Scheidegger C. 2017. Indigenous knowledge and use of lichens by the lichenophilic communities of the Nepal Himalaya. *J. Ethnobiol. Ethnomed.* **13**: 1-10.
15. Jha BN, Shrestha M, Pandey DP, Bhattarai T, Bhattarai HD, Paudel B. 2017. Investigation of antioxidant, antimicrobial and toxicity activities of lichens from high altitude regions of Nepal. *BMC Complement Altern. Med.* **17**(1): 282 doi: 10.1186/s12906-017-1797-x.
16. Bates ST, Cropsey GW, Caporaso JG, Knight R, Fierer N. 2011. Bacterial communities associated with the lichen symbiosis. *Appl. Environ. Microbiol.* **77**: 1309-1314.
17. Han SR, Yu SC, Ahn DH, Park H, Oh TJ. 2016. Complete genome sequence of *Burkholderia* sp. strain PAMC28687, a potential octopine-utilizing bacterium isolated from Antarctica lichen. *J. Biotechnol.* **226**: 16-17.
18. Kim MK, Park H, Oh TJ. 2014. Antibacterial and antioxidant capacity of polar microorganisms isolated from Arctic lichen *Ochrolechia* sp. *Pol. J. Microbiol.* **63**: 317-322.
19. Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**: 111-120.
20. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **35**: 1547-1549.
21. Bauer AW, Kirby MM, Sherris JC, Truck M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* **45**: 493-496.
22. Zhao K, Penttinen P, Guan T, Xiao J, Chen Q, Xu J, *et al.* 2011. The diversity and anti-microbial activity of endophytic actinomycetes isolated from medicinal plants in Panxi plateau, China. *Curr. Microbiol.* **62**: 182-190.
23. Gaber AA, Badr OM, Emara SA, Ibrahim AM. 2015. Antitumor activity of two *Streptomyces* extracts (Ag18 & Ag20) on Ehrlich ascites tumor in mice: in vitro and in vivo studies. *J. Biosci. Appl. Res.* **1**: 20-29.
24. Wawrik B, Kerkhof L, Zylstra GJ, Kukor JJ. 2005. Identification of unique type II polyketide synthase genes in soil. *Appl. Environ. Microbiol.* **71**: 2232-2238.
25. Han SR, Lee JH, Kang S, Park H, Oh TJ. 2016. Complete genome sequence of opine-utilizing *Variovorax* sp. strain PAMC28711 isolated from an Antarctic lichen. *J. Biotechnol.* **225**: 46-47.
26. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, *et al.* 2008. The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* **9**: 75.
27. Blin K, Wolf T, Chevrette MG, Lu X, Schwalen CJ, Kautsar SA, *et al.* 2017. antiSMASH 4.0-improvements in chemistry prediction and gene cluster boundary identification. *Nucleic Acids Res.* **45**: W36-W41.
28. Ayuso A, Clark D, González I, Salazar O, Anderson A, Genilloud O. 2005. A novel actinomycete strain de-replication approach based on the diversity of polyketide synthase and nonribosomal peptide synthetase biosynthetic pathways. *Appl. Microbiol. Biotechnol.* **67**: 795-806.
29. Zheng KX, Jiang Y, Jiang JX, Huang R, He J, Wu SH. 2019. A new phthalazinone derivative and a new isoflavonoid glycoside from lichen-associated *Amycolatopsis* sp. *Fitoterapia* **135**: 85-89.
30. Sigurbjörnsdóttir MA, Andrésson ÓS, Vilhelmsson O. 2016. Nutrient scavenging activity and antagonistic factors of non-photobiont lichen-associated bacteria: a review. *World J. Microbiol. Biotechnol.* **32**: 68.
31. Butcher RA, Schroeder FC, Fischbach MA, Straight PD, Kolter R, Walsh CT, Clardy J. 2007. The identification of bacillaene, the product of the PksX megacomplex in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* **104**: 1506-1509.
32. Schneider O, Simic N, Aachmann FL, Rückert C, Kristiansen KA, Kalinowski J, *et al.* 2018. Genome mining of *Streptomyces* sp. YIM 130001 isolated from lichen affords new thiopeptide antibiotic. *Front Microbiol.* **9**: 3139.
33. Jeon BJ, Kim JD, Han JW, Kim BS. 2016. Antifungal activity of rimocidin and a new rimocidin derivative BU16 produced by *Streptomyces mauvecolor* BU16 and their effects on pepper anthracnose. *J. Appl. Microbiol.* **120**: 1219-1228.
34. Calcott MJ, Ackerley DF, Knight A, Keyzers RA, Owen JG. 2018. Secondary metabolism in the lichen symbiosis. *Chem. Soc. Rev.* **47**: 1730-1760.