Rubber Degrading Strains of Microtetraspora and Dactylosporangium

Ann Anni Basik

Universiti Sains Malaysia

Nanthini Jayaram

International University of Malaya-Wales

Chia Tiong Yeo

Sarawak Biodiversity Centre

Kumar Sudesh (✉ ksudesh@usm.my)

Universiti Sains Malaysia

Research Article

Keywords: Biodegradation, Dactylosporangium, Latex clearing protein, Microtetraspora, operon

Posted Date: April 14th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-402279/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Rubber Degrading Strains of *Microtetraspora* and *Dactylosporangium*

Ann Anni Basik^{a,b}, Jayaram Nanthini^c, Tiong Chia Yeo^b, Kumar Sudesh^{a*}

^aSchool of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia

^bSarawak Biodiversity Centre, Km. 20 Jalan Borneo Heights, Semengoh, 93250 Kuching, Sarawak, Malaysia

^cInternational University of Malaya-Wales, 50480 Kuala Lumpur, Malaysia

*Corresponding author:

K. Sudesh,

Ecobiomaterial Research Laboratory, School of Biological Sciences, Universiti Sains

Malaysia, 11800 Penang, Malaysia

Tel: +60-4-6534367; Fax: +60-4-6565125

Email: ksudesh@usm.my

Abstract

Latex clearing protein (*lcp*) found in Actinobacterial strains is reportedly critical for the initial oxidative cleavage of poly(*cis*-1,4-isoprene), the major polymeric unit of rubber. In this study, we screened 940 Actinobacterial strains isolated from various locations in Sarawak on NR latex agar and identified 18 strains from 5 genera that produced clearing zones and contained *lcp* genes. We report here the first *lcp* genes from *Microtetraspora* sp. AC03309 (*lcp* 1 and *lcp*2) and *Dactylosporangium* sp. AC04546 (*lcp*1, *lcp*2, *lcp*3), together with their operon structure. Complete 16S rDNA gene sequence revealed that *Dactylosporangium* sp. AC04546 is 99% identical to *Dactylosporangium sucinum* RY35-23 whereas *Microtetraspora* sp. AC03309 is 98% identical to *Microtetraspora glauca* IFO14761. Morphological changes and the spectrophotometric detection of aldehyde and keto groups in rubber samples incubated with the strains confirm the strains' ability to degrade rubber-based products.

Keywords

Biodegradation, *Dactylosporangium*, Latex clearing protein, *Microtetraspora*, operon

1.0 Introduction

Sarawak is recognised internationally as one of the world's 25 biological hot spots, with rich biodiversity, distinctive ecosystems, and extraordinary biological elements ¹. The Sarawak Biodiversity Centre (SBC) (<https://www.sbc.org.my/>) has been investigating and documenting the diversity of species in the State, and since 2005 and have collected more than 13,000 Actinobacteria strains, isolated from 137 various locations across Sarawak. Our interest in novel strains of Actinobacteria stems from studies showing that these bacterial strains degrade a large variety of organic materials, have high catabolic capacity, and are resilient in the face of unfavourable environmental conditions ². Moreover, the same species of Actinobacteria growing in different locations or ecological niches are known to produce environmentally specific metabolites ³. Using microbes such as Actinobacteria that have evolved genes encoding catabolic enzymes functionally capable of degrading modified rubber are a potential solution for end-of-life management of rubber products ⁴. In addition to this, most rubber degrading strains discovered to date belongs to the phylum, Actinobacteria and are rarely found among Gram-negative bacteria and fungi ⁵. While Sarawak with its wide range of biodiversity therefore provide unique microenvironments that contributes to the novelty of Actinobacterial strains.

There is great interest in developing environmentally sustainable methods for removing rubber wastes globally. Natural rubber (NR) is highly modified through compounding and vulcanisation in industrial processes and is used in more than 50,000 products today ^{6,7}. Natural and synthetic isoprene rubber, primarily composed of highly unsaturated hydrocarbon poly(*cis*-1,4-isoprene), are widely used. Difficulties in reusing rubber materials due to their resistance to thermal and chemical degradation (REF) results in majority of them being discarded in landfills. Robust, economical, and easily scalable methods for degrading rubber products into minimally hazardous compounds that ideally can be reused are badly needed.

Rubber degrading enzymes, known as rubber oxygenases, produced by microbes are a key discovery for biological rubber degradation. There are 2 groups of rubber degrading bacteria: (i) strains that produce clear zones on NR latex agar and (ii) strains that require direct contact with rubber substrates for subsequent degradation ⁸⁻¹⁰. In both cases, enzymes produced by the bacteria extracellularly cleave rubber polymers to mixtures of low molecular products (C₂₀, C₂₅, C₃₀, and higher oligo-isoprenoids as end products) that can be taken up by the bacteria ¹¹.

Our present survey of the SBC's collection of Actinobacteria is geared towards discovering novel rubber degrading Actinobacteria as degraded products obtained from NR or synthetic polyisoprene can differ in their molecular weight, and number of isoprene units,

depending on the bacterial strain used ¹². We are thus interested in characterizing the latex clearing protein (*lcp*) genes in strains that can degrade rubber, as these may have evolved differently in each genus and modulated the strains' functional activity ¹³. Little is known about the breakdown mechanism, microorganisms and enzymes involved in rubber degradation. To augment existing information and evaluate the diverse activities in these group of bacteria, the discovery and characterization of new rubber degraders and *lcp* genes is necessary.

2.0 Results

• Screening for NR Latex Degrading Bacteria

To identify rubber degrading strains from SBC's Microbial Natural Product Library (MNPL), random miniaturised screening using 6-well plates for bacteria producing clear zones on NR latex agar were conducted. Eighteen (18) strains were found capable of degrading NR latex, producing ~1 to 2 mm clearance surrounding the colony. By standardizing the clear zone size and day of clear zone detection, we were able to compare the activity of the strains (Fig. 1).

Figure 1. Size of clearing zone (mm) on NR latex agar in 6-well plate in relation to the day of clear zone formation for 18 rubber degrading Actinobacteria detected in this study.

• Identification of NR Latex Degrading Bacteria

All strains producing clear zones were successfully identified using through partial 16S rDNA gene having blast homology ranging from 99 to 100% (MT005091, MT005089,

MT005088, MT005098, MT005101, MT005095, MT005096, MT005104, MT005094, MT005105, MT005103, MT005100, MT005099, MT005102, MT005097, MT005093, MT005090, MT005092). Blast homology of 98.7 - 99.5% are putative novel species, 99.6 - 99.8% are putative known species and 99.9% and above are identical or closely related species¹⁴.

Clear zone forming strains were distributed among 5 genera: *Microtetraspora* sp. (7 strains), *Micromonospora* sp. (6 strains), *Streptomyces* sp. (2 strains), *Nonomuraea* sp. (2 strains) and *Dactylosporangium* sp. (1 strains). Although, 39% of clear zone formers identified in this study belong to *Microtetraspora* genus, phylogenetic distribution of 16S rRNA gene suggests that they are not identical (Fig. 2).

Figure 2. Rubber degrading strains 16S rDNA evolutionary relationships of taxa together with top 1 blast homology in NCBI database.

The evolutionary history was inferred using the Neighbor-Joining method conducted in MEGA X.

• Profiling of *Lcp* Genes in NR Latex Degrading Bacteria

Lcp genes (498 to 1099 bp) were amplified from the NR latex degrading bacteria found in our screen (MN148090, MT241322, MN148093, MN148092, MN148094, MN148095, MT252675, MN148097, MN148098, MT241320, MN148096, MT241323, MT241319, MT241318, MT241317, MT252676, MT241321, MN148089). The *lcp* genes blast homology was conducted in UniProt database and gave results showing that homology of the selected strains ranged from 81.2% to 100% identity.

- Strain of interest: *Microtetraspora* sp. AC03309 (JCM 34240) and *Dactylosporangium* sp. AC04546 (JCM 34239)

Lcp gene for both genera has not been recorded in the NCBI database, therefore we choose to further characterize these strains through: (i) morphological studies (Plate 1), (ii) their ability to utilize rubber products as the sole carbon source, and (ii) identification of genes related to rubber degradation through genome sequencing.

Microtetraspora sp. AC03309 has blue green surface and honey gold reverse colour on yeast extract-malt agar (ISP2) agar with moderate aerial mycelia. Colony is wrinkled with regular shape. It has external short spore chain (approx. 3 μ m length) with 4 spores formed on sporophore branching from aerial hyphae. Spores are slightly oval to cylindrical with smooth surface. It grows well at 30 °C on ISP2 agar.

Dactylosporangium sp. AC04546 has yellowish orange surface and reverse on ISP2 agar with moderate aerial mycelia. Colony is wrinkled with regular shape. It has oblong shaped sporangia (~1.5 μ m in length) with smooth surface, emerged directly from vegetative mycelium, arranged singly or in clusters. It is a mesophilic strain, growing well at 28 °C and up to 45 °C on ISP2 agar.

Plate 1. Strain morphology (i) ISP2 agar plate (ii) SEA agar (iii) FESEM/ SEM (iv) NR latex agar at Day 15 using 100 μ L of 1×10^6 cells/ mL (a) *Microtetraspora* sp. AC03309 and (b) *Dactylosporangium* sp. AC04546

- Identification of *lcp* orthologs in strain *Microtetraspora* sp. AC03309 and *Dactylosporangium* sp. AC04546

Genomic sequencing revealed the presence of 2 *lcp* genes located on the chromosome of *Microtetraspora* sp. AC03309 (MW659698 and MW659699) and 3 *lcp* genes located on the chromosome of *Dactylosporangium* sp. AC04546 (MW659700, MW659701, MW659702). *Lcp* genes were confirmed again through PCR.

- Operon structure of *lcp* *Microtetraspora* sp. AC03309 and *Dactylosporangium* sp. AC04546

Using SnapGene Viewer version 5.1.3.1 (www.snapgene.com), we were able to identify *lcp* genes and related rubber degrading genes of *oxiA* and *oxiB*, and TATR for both strains. *OxiA* and *oxiB* are involved in degradation of low-molecular weight rubber-degrading compounds in *Streptomyces* sp. strain K30, while TetR/AcrR-type transcriptional regulators (TATRs) is involved in the transcriptional regulation of the rubber-degrading genes¹⁵.

Figure 3. The organization and transcription of the *lcp* gene clusters in strain (a) *Microtetraspora* sp. AC03309 (b) *Dactylosporangium* sp. AC04546. Open arrows indicate the genes. Location (bp) are indicated above/ below the genes.

For strain AC03309, *lcp1*, *lcp2*, *oxiA* and *oxiB* genes are organized in the same transcriptional unit and orientation (Fig. 3a), while for strain AC04546, *lcp 1* was not located in the same transcriptional unit (Fig. 3b). Similar observation was seen in other rubber degrading containing 3 *lcp* genes, *Streptomyces* sp. strain CFMR7 and *Actinoplanes* sp. strain OR16^{15,16}. TATR gene is located next to the *lcp* genes for both *Microtetraspora* sp. AC03309 and *Dactylosporangium* sp. AC04546.

Using pairwise distance (MegaX), the amino acid sequence identities among biochemically characterized *lcps* from Actinobacteria are summarized in Table 1. *Lcp1* and *lcp3* from *Dactylosporangium* sp. AC04546 showed highest similarity (94.1% and 72.3% respectively) to *Lcp2* (plasmid) of *G. polyisoprenivorans* VH2, while *lcp2* from *Dactylosporangium* sp. AC04546 showed highest similarity (66.7%) to *Lcp3* of *Actinoplanes* sp. strain OR16. *Lcp1* and *lcp2* from *Microtetraspora* sp. AC03309 showed highest similarity (74.7% and 82.4% respectively) to *lcp3* of *Actinoplanes* sp. strain OR16.

- Utilization of Rubber Materials by *Microtetraspora* sp. AC03309 and *Dactylosporangium* sp. AC04546

When cultivated in ISP2 broth at 180 rpm, *Microtetraspora* sp. AC03309 was actively growing by Day 7 while *Dactylosporangium* sp. AC04546 was by Day 10. SEM images for rubber materials after 30 days of inoculation showed that the strain was able to grow and utilize fresh latex, latex glove, or tyre as the sole carbon source. Biodegradation of the rubber polymer begins with microbial attachment on the surface (Plate 2b to 2c, black arrow) in comparison to non-inoculated samples (Plate 2a). Once attached, the microorganism releases degrading enzymes through its mycelia, initiating the first step of rubber degradation. This can be seen through the presence of rough and cracked surfaces (white arrow) on the rubber materials (Plate 2b – 2c).

Plate 2. SEM images for (i) fresh latex (ii) latex glove (iii) tyre granules – (a) controls (b) *Dactylosporangium* sp. AC04546 and (c) *Microtetraspora* sp. AC03309 incubated for 30 days, 180 rpm, 30 °C

- ATR-FTIR Analysis of Degraded Rubber Materials

ATR-FTIR spectroscopy is a useful tool to determine the formation or disappearance of functional groups of materials that indicate degradation of the original material (Fig. 4). During rubber utilization, *lcp* catalyzes the oxidative C-C cleavage of poly(*cis*-1,4-isoprene) in NR as well as in synthetic rubber by the addition of oxygen (O₂) to the double bonds, leading to a mixture of oligonucleotide-isoprenoids with terminal keto and aldehyde groups (endo-type cleavage)^{11,17}. The cleavage products are of different lengths, ranging from C₂₀ (four isoprene units) to higher oligo-isoprenoids^{17,18}.

Figure 4. *Cis*-1,4-polyisoprene biodegradation pathway by oxygen attack at the double bond (modified from Linos et al., 2000)

Based on the IR spectra sample (see Supplementary Fig. 1 - 3), peaks due to stretching vibrations of the C-C main chain in the *cis*-1,4-unit are obvious at ~1000 cm⁻¹. The increase and broadening of IR spectra at 1,700 to 1,600 cm⁻¹ of inoculated samples indicates the formation of oligo-isoprene (aldehyde groups and conjugated ketones). The broadening of the IR spectra at ~3,200 cm⁻¹ is due to O-H stretching. Fresh latex showed the most degradation

physically, and in the IR profile followed by latex glove. Differences in the IR spectra for the tyre samples were only observed in samples incubated with *Dactylosporangium* sp. AC04546.

3.0 Discussions

We were able to quickly screen a portion of this collection by modifying the screening method using NR latex agar in 6-well plates instead of latex agar overlay technique as described by Braaz et al., (2004). We also prolonged the incubation period from 1 week to 4 weeks as some Actinobacteria strains are known to be slow growers. Using 6-well plates, we prepared NR latex agar (without overlay) in smaller volume, utilizing minimal space and less NR substrate. Separation of media by wells also avoid cross contamination especially by sporulating strains. Similar method using NR latex agar in 24-well plate was not successful in detecting the formation of clear zone. Using MSM broth added with NR latex in test tubes, 6-well plate and 24-well plate did not show changes in the turbidity of the medium. Based on the screening results, the size of clearing zone on NR latex agar differs within each genus, suggesting each species may have evolve their ability to degrade rubber differently (Fig. 1).

Lcp genes of clear zone forming strains in this study were compared to *lcp* genes available in the NCBI database (Table 2). Majority of *lcp* genes deposited in the NCBI database were from *Streptomyces* sp., but the closest blast homology to *Streptomyces* sp. AC04842 and *Streptomyces* sp. AC00383 did not contain any *lcp* gene (Fig. 2). *Streptomyces* sp. AC04842 has a larger clearing zone compared to *Streptomyces* sp. AC00383. The genomic content and functionality of *lcp* genes in *Streptomyces* sp. AC04842 will be reported in a following study.

Back in 1997, a publication indicated that *Microtetraspora* sp. strain 3880-19B (DSM 44333) isolated from soil sample collected in Malaysia, and *Dactylosporangium thailandense* DSM 43158 isolated from Thailand produced clearzone on NR latex overlay agar²⁰. However, no further studies on these strains have been reported to the best of our knowledge. Comparison between 16S rDNA gene of AC04546 and DSM 43158 showed 98% similarity, however no sequence was deposited for DSM 44333. *Lcp* genes from *Microtetraspora* sp. AC03309 and *Dactylosporangium* sp. AC04546 also did not cluster together when compared to other biochemically characterized *lcp* (Fig. 5), their amino acid sequences were also separate (21.4% to 94.1% similarity) from other known *lcp* genes (Table 1).

Figure 5. Phylogenetic tree of characterised *lcp*s and *lcp*s from *Microtetraspora* sp. AC03309 and *Dactylosporangium* AC04546.

Enzymes: LcpK30 from *Streptomyces* sp. strain K30 (AAR25849); Lcp1VH2 from *G. polyisoprenivorans* VH2 (ABV68923); LcpKb1 from *G. westfalica* Kb1 (ABV68924); LcpSH22a from *N. nova* SH22a (WP025350295); LcpRPK1 from *R. rhodochrous* RPK1 (AMY60409); LcpNVL3 from *Nocardia* sp. strain NVL3 (API85527); LcpE1 from *N. farcinica* E1 (ABC59140); and LcpHR-BB from *Solimonas fluminis* HR-BB (WP104231946).

Almost all rubber-degrading Actinobacteria, including *Streptomyces*, *Nocardia*, and *Rhodococcus* species have a single *lcp* homolog¹⁵. We believe that Actinobacterial strains adapt by incorporating *lcp* genes into their chromosome through their plasmid (*G. polyisoprenivorans* VH2) which leads to the presence of more than 1 unique *lcp* homolog as seen in *Microtetraspora* sp. AC03309 and *Dactylosporangium* sp. AC04546. Previous reports indicate that different *lcp* produces a variety rubber degraded products (molecular weight, number of isoprene units, functional groups, etc)¹². This would most likely be due to the different or synergistic mechanism of *lcp*, which is yet to be explored.

Most of the clear zone producing strains (61%) were isolated from soil sample collected from Kiding Village forest area (6 separate soil samples). Kiding village settlement was founded in the 1840s and is located 1,300 m above sea level and is only accessible by a 3-hour hike. While all the other NR latex degrading strains were isolated from secondary forest soil samples. The isolation sites for all the strains have no obvious rubber wastes or rubber materials present, so there does not appear to be an apparent evolutionary pressure for the appearance of *lcp* genes. Nonetheless, these strains do degrade rubber, and it is possible

that this function may have been triggered by the presence of rubber particles dispersed in the environment. Recent studies have shown that rubber particles from sources such as tyres are widely dispersed. Sieber et al., (2020), estimated that 218 ktons rubber particles from tyres are mainly deposited on road-side soils (74%), surface water (22%) and in soils (4%). Alternatively, the presence of latex producing plants in the vicinity may have contributed to the development of *lcp* in these strains. Further studies would be required to confirm the reasons for this, but this observation highlights the importance of surveying microorganisms from diverse ecosystems (e.g. marine, or fresh water bodies) for their genomic background and functional ability for biodegradation of rubber or other pollutants.

The influence of the local environment on the development of rubber degrading genera is an intriguing question. The locations where the strains were collected are not obviously rubber dense (e.g. rubber plantations, factories or waste sites), but yet homologous *lcp* genes were found in the genome of the strains, and they are functionally capable of using latex or rubber as a nutrient source. It would be interesting to survey the collection locations for the presence of rubber pollution in the form of microparticles or other sources of latex in the vicinity. Correlative studies of this nature would help elucidate whether *lcp* genes are solely expressed based on the presence of rubber/latex in the organisms' local environment. The variation in *lcp* structure, function and between different genera and species implies that enzyme product may be evolved to break down specific compounds. This observation raises the intriguing prospect that strategic combinations of different enzymes from different *lcps* may work synergistically in the degradation of latex or rubber.

As can be seen in the rubber utilization studies, minimal differences were observed for tyre samples, similar case was observed after incubating tyre samples for up to 9 months²². Unlike other rubber products, tyres are made from vulcanized rubber and ~30% carbon black for reinforcement which makes them more resistant to degradation. Since tyre is the second largest contributor to microplastic pollution in the ocean, we need to improvise preliminary screening methods for tyre degradation by microbes²³.

4.0 Materials & Methods

- Screening for NR latex Degrading Bacteria

NR latex medium preparation: Latex agar preparation was modified from Braaz et al., 2004, 2004.; mineral salts medium (MSM) per litre [K₂HPO₄, 8.0 g; KH₂PO₄, 1.0 g; (NH₄)₂SO₄, 0.5 g; MgSO₄·7H₂O, 0.2 g; NaCl, 0.1 g; Ca(NO₃)₂, 0.1 g; 15 g agar] were added

with 1 mL 100× trace element solution and 0.02% purified NR latex concentrate. Trace elements (100×) per 100 ml [containing $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$, 5 mg; and MnSO_4 , 5 mg] were filter-sterilised ²⁴.

NR latex concentrate: Freshly tapped crude latex was washed with 0.002 % Tween 80 by centrifugation (5 min at $19,320 \times g$); the washing step was repeated 3x. The top layer was used to prepare the latex agar. Equal amounts of 0.002 % Tween 80 were added into the concentrated purified latex, heat sterilized and stored at 4 °C as purified NR latex concentrate for further use. MSM agar with purified NR latex concentrate were then transferred into 6-well plates (4 mL per well). Cultures cultivated in liquid broth for 7 to 14 days were spotted (~ 40 µL) onto each agar well. The plates were incubated at 30 °C for up to 4 weeks. Colonies that produced translucent clearing zones, indicating the degradation of the NR latex, were recorded.

- Identification of NR latex Degrading Bacteria

Strains producing clear zones were identified based on morphological features and molecular techniques. Morphology was observed on ISP2 agar, and sporulation on soil extract agar ²⁵. Sporulation patterns were observed directly using a 50× long distance (Olympus LMPLFLN; Olympus, Tokyo, Japan). Spore chain structures and colonial morphology were recorded and photo-documented. Molecular identification was made based on the amplification of the 16S rDNA gene using primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (59-TACGGYTACCTTGTTACGACTT-39) with the following parameters: 5 min at 96 °C, 30 cycles of 45 sec at 96 °C, 2 min at 55 °C, 4 min at 72 °C and the final extension for 7 min at 72 °C. Amplified products were purified (GFX PCR DNA GE Healthcare) and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kits based on Sanger's dideoxy sequencing method.

- Genomic DNA Isolation, Nucleotide Sequencing and Sequence Analysis

Strains producing clear zones on latex agar were subsequently cultivated in 20 mL ISP2 broth (Shirling & Gottlieb, 1966) in a 250 mL flask at 28 °C, 200 rpm for three to ten days. Genomic DNA (gDNA) was extracted and purified using method from Moore et al., (2008). The DNA quantity and quality were verified by spectrophotometric means (Eppendorf Biospectrophotometer basic). The purified DNA samples were subsequently sent for genomic

sequencing (*Microtetraspora* sp. AC03309 and *Dactylosporangium* sp. AC04546) using Illumina MiSeq by service provider BioEasy Sdn. Bhd..

- Profiling of *Lcp* Genes in NR Latex Degrading Bacteria

The presence of *lcp* genes in the bacterial strains that produced clearing zones were screened and amplified using the following primers: *lcp441f* [5'-GGAG(TG)C(GC)GC(GC)GTCTACTACTC-3'] and *lcp879r* [5'-GATCGG(AG)T(TC)GAG(AGC)ACCTGC-3']²⁸, *lcp1f* [5'-ATGGAGAATCTCAGTAGACGT-3'] and *lcp1199r* [5'-ATGACCGGAATGGTGATCGG-3'], designed based on the conserved regions of published *lcp* gene sequences (*Micromonospora* sp. WMMB235 (ENA accession number MDRX01000001), *Micromonospora* sp. TSRI0369 (ENA accession number LIVU01000002), *Micromonospora* sp. NRRL B-16802 (ENA accession number NZLGEB01000064), *Micromonospora echinospora* ATCC15837 (ENA accession number NGNT01000005). The sequences were aligned using Bioedit²⁹. The gene was amplified using following parameters: 2 min at 95 °C, 30 cycles of 30 sec at 95 °C, 1 min at 55 – 65 °C, 2 min at 72 °C and the final extension for 5 min at 72 °C. Annealing temperature were optimised for each strain.

Amplified products were purified (GFX PCR DNA GE Healthcare) and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kits based on Sanger's dideoxy sequencing method.

- Utilization of Rubber Materials

To study rubber utilization on synthetic rubber; samples of rubber gloves and tyres were used as the sole carbon and energy source, fresh latex was included as control. Fresh latex was harvested from 5-year-old rubber trees at a rubber plantation site at Kulim, Kedah. The latex was brought back and left to solidify at room temperature. Solid latex pieces were then cut into 1 cm x 3 cm latex pieces. Steel free tyre granules (1 to 3 mm) were obtained from a tyre recycling factory (Gcycle Tyre Recycling) in Kedah. Latex gloves (PRO-CARE), disposable and non-powdered, were used in these studies. To remove antimicrobial substances from the latex glove and tyre granules prior to incubation with Actinobacteria, the material was treated with chloroform as follows: 1 g of sample was extracted with 100 mL chloroform for 12 h³⁰.

Pre-culture of actively growing strains were cultivated in ISP2 broth, the culture (1 mL) was then transferred into 250 mL test flasks containing 50 mL MSM and 0.5% (w/v)

sole carbon source (fresh latex, samples of gloves or tyres)³⁰. The inoculated flasks were incubated at 30°C, 180 rpm for 30 days. Test flasks without culture were used as control. All sample studies were carried out in triplicate.

Sample preparation for Scanning Electron Microscope (SEM) and Attenuated Total Reflection-Fourier transform infrared (ATR-FTIR) were carried out by rinsing the inoculated rubber materials with distilled water, then immersing them in 96% ethanol for 1 hour before air-drying at ambient temperatures. For SEM viewing, samples were prepared using the hexamethyldisilazane (HDMS) method. Dried samples were mounted onto a SEM stub, coated with gold using the Quorum Q150T S (Quorum Technologies Ltd) sputter coater (15 min) and viewed using the SEM Quanta FEG 650 (Thermo Fisher Scientific).

SEM images of Actinobacteria strains were also directly viewed using Field Emission Scanning Electron Microscope (FESEM), Quattro Thermo Fisher Scientific carried out at Curtin Biovalley Sdn. Bhd. in Miri. Strains cultivated on ISP2 agar were cut (1 cm x 1 cm) and directly viewed under the FESEM.

To determine the formation of new, or disappearance of functional groups in the polymer units of the samples, post-incubation samples (fresh latex, latex glove, tyre) and non-inoculated samples were analysed using FT-IR Spectrum 400 (Perkim Elmer), equipped with ATR at more than 90% pressure ranging from 4000 cm⁻¹ to 650 cm⁻¹. The angle of incidence was set at 45 using a ZnSe crystal with 20 active internal reflections. Four scans were co-added with resolution set at 4 cm⁻¹.

5.0 Conclusions

Miniaturised random screening of the SBC's MNPL led to the successful identification of 18 NR latex degrading Actinobacteria. Two strains, *Microtetraspora* sp. AC03309 and *Dactylosporangium* sp. AC04546 were explored. Both having more than 1 *lcp* genes on their chromosome and their operon structure is similar to other reports of functional rubber degrading Actinobacteria. Both strains were able to colonise and degrade rubber-based materials within 30 days of incubation. The discovery of novel *lcp* genes and confirmation of the strain's biodegradation activity in this study further indicate several important directions to explore to better understand the potential of using Actinobacteria in biodegradation processes. Continuing to collect, screen, catalogue, characterize genomically and confirm the activity of Actinobacteria from diverse environments will help us identify the fundamental environmental or biological factors that result in the ability to degrade rubber or

latex. In turn this framework will help guide the development of bio-processes that can be used to degrade rubber products on an industrial scale.

Author Contributions:

A.A.B: Data Curation, Writing-Original Draft, Investigation, Formal Analysis, Visualization, Funding Acquisition, **J.N.:** Resources, Writing-Review and Editing, **T.C.Y.:** Project Administration, Funding Acquisition, **K.S.:** Supervision Project, Conceptualization, Writing-Review and Editing.

Funding: This work was supported by the Government of Sarawak under USM grant number 304.PBIOLOGI.6501009.J136.

Acknowledgements: We would like to acknowledge the Sarawak Government for providing research fund to conduct this study. Also acknowledging the Sarawak Biodiversity Centre staff especially those working in the Microbiology Group and the Molecular Group. Thank you to Dr. Mohd Farith bin Kota (SBC) for the FESEM image. We also like to express our appreciation to the Ecobiomaterial Lab members of USM for their support.

Conflicts of Interest: The authors declare no conflict of interest.

References:

1. Manurung, R., Abdullah, Z. C., Ahmad, F. B. & Kuek., C. Biodiversity-Biotechnology: Gateway to Discoveries, Sustainable Utilization and Wealth Creation. in *International Symposium held in Kuching Sarawak, Malaysia 19th – 21st November 2008*. (2008).
2. Azadi, D., Shojaei, H., Mobasherizadeh, S. & Naser, A. D. Screening , Isolation and Molecular Identification of Biodegrading Mycobacteria from Iranian Ecosystems and Analysis of their Biodegradation Activity. *AMB Express* (2017) doi:10.1186/s13568-017-0472-4.
3. Hassan, Q. P., Bhat, A. M. & Shah, A. M. Bioprospecting Actinobacteria for Bioactive Secondary Metabolites from Untapped Ecoregions of the Northwestern Himalayas. in

- New and Future Developments in Microbial Biotechnology and Bioengineering: Microbial Secondary Metabolites Biochemistry and Applications* 77–85 (Elsevier, 2019). doi:10.1016/B978-0-444-63504-4.00006-2.
4. Clark, T. Enhancing the Biodegradation of Waste Rubber Discarded Rubber Materials. *Int. Latex Conf. 2015* (2015).
 5. Shivilata, L. & Satyanarayana, T. Thermophilic and Alkaliphilic Actinobacteria: Biology and Potential Applications. *Front. Microbiol.* **6**, 1–29 (2015).
 6. Cornish, K. Alternative Natural Rubber Crops: Why Should We Care? in *Technology & Innovation* 244–255 (2017).
 7. Blaettler, K. G. The Manufacturing Process of Rubber. <https://sciencing.com/manufacturing-process-rubber-5206099.html> (2018).
 8. Spence, D. & Van Niel, C. B. Bacterial Decomposition of the Rubber in *Hevea* Latex. *Ind. Eng. Chem.* **28**, 847–850 (1936).
 9. De Vries, O. Decomposition of Rubber Hydrocarbons by Fungi. *Zentbl Bakteriол Parasitenkd Infekt.* **74**, (1928).
 10. Shaposhnikov, V., Rabotnova, I., Iarmola, G., Kuznetsova, V. & Mozokhina-Porshniakova, N. N. Destructive Effect of Bacterial Development on Natural Rubber. *Mikrobiologiya* **21**, 147–54. (1952).
 11. Birke, J. *et al.* Production of Functionalized Oligo-isoprenoids by Enzymatic Cleavage of Rubber. *Microb. Biotechnol.* **10**, 1426–1433 (2017).
 12. Rose, K. & Steinbüchel, A. Biodegradation of Natural Rubber and Related Compounds: Recent Insights into a Hardly Understood Catabolic Capability of Microorganisms. *Appl. Environ. Microbiol.* **71**, 2803–2812 (2005).
 13. Kasai, D. Poly(cis-1,4-isoprene)-Cleavage Enzymes from Natural Rubber-Utilizing Bacteria. *Biosci. Biotechnol. Biochem.* **84**, 1089–1097 (2020).

- 456 14. Basik, A. A., Juboi, H., Shamsul, S. S. G., Sanglier, J. J. & Yeo, T. C. Actinomycetes
457 Isolated from Wetland and Hill Paddy during the Warm and Cool Seasons in Sarawak,
458 East Malaysia. *J. Microbiol. Biotechnol. Food Sci.* **9**, 774–781 (2020).
- 459 15. Gibu, N. *et al.* Characterization of the Genes Responsible for Rubber Degradation in
460 Actinoplanes sp. strain OR16. *Appl. Microbiol. Biotechnol.* **104**, 7367–7376 (2020).
- 461 16. Nanthini, J., Ong, S. Y. & Sudesh, K. Identification of Three Homologous Latex-
462 clearing protein (lcp) Genes from the Genome of Streptomyces sp. strain CFMR 7.
463 *Gene* **628**, 146–155 (2017).
- 464 17. Birke, J. & Jendrosseck, D. Rubber Oxygenase and Latex Clearing Protein Cleave
465 Rubber to Different Products and Use Different Cleavage Mechanisms. *Appl. Environ.*
466 *Microbiol.* **80**, 5012–5020 (2014).
- 467 18. Birke, J., Röther, W. & Jendrosseck, D. Latex clearing protein (Lcp) of Streptomyces
468 sp. strain K30 is a b-type Cytochrome and Differs from Rubber oxygenase A (RoxA)
469 in its Biophysical Properties. *Appl. Environ. Microbiol.* **81**, 3793–3799 (2015).
- 470 19. Braaz, R., Fischer, P. & Jendrosseck, D. Novel Type of Heme-dependent Oxygenase
471 Catalyzes Oxidative Cleavage of Rubber (poly-cis-1,4-isoprene). *Appl. Environ.*
472 *Microbiol.* **70**, 7388–7395 (2004).
- 473 20. Jendrosseck, D., Tomasi, G. & Kroppenstedt, R. M. Bacterial Degradation of Natural
474 Rubber: A Privilege of Actinomycetes? *FEMS Microbiol. Lett.* **150**, 179–188 (1997).
- 475 21. Sieber, R., Kawecki, D. & Nowack, B. Dynamic Probabilistic Material Flow Analysis
476 of Rubber Release from Tires into the Environment. *Environ. Pollut.* **258**, 113573
477 (2020).
- 478 22. Nanthini, J. & Sudesh, K. Biodegradation of Natural Rubber and Natural Rubber
479 Products by Streptomyces sp. Strain CFMR 7. *J. Polym. Environ.* **25**, 606–616 (2017).
- 480 23. Kole, P. J., Löhr, A. J., Van Belleghem, F. G. A. J., Ragas, A. M. J. & Nl, F. V. Wear

- and Tear of Tyres: A Stealthy Source of Microplastics in the Environment. *Int. J. Environ. Res. Public Health* **14**, (2017).
24. Heisey, R. M. & Papadatos, S. Isolation of Microorganisms able to Metabolize Purified Natural Rubber. *Appl. Environ. Microbiol.* **61**, 3092–3097 (1995).
25. Hamaki, T. *et al.* Isolation of Novel Bacteria and Actinomycetes using Soil-Extract Agar Medium. *J. Biosci. Bioeng.* **99**, 485–492 (2005).
26. Shirling, E. B. & Gottlieb, D. Methods for Characterization of Streptomyces species. *Int. J. Syst. Bacteriol.* **16**, 340 (1966).
27. Moore, E., Arnscheidt, A., Krüger, A., Strömpl, C. & Mau, M. Section 1 Update: Simplified Protocols for the Preparation of Genomic DNA from Bacterial Cultures. *Mol. Microb. Ecol. Man.* 1905–1919 (2008) doi:10.1007/978-1-4020-2177-0_101.
28. Chia, K. *et al.* Identification of New Rubber-Degrading Bacterial Strains from Aged Latex. *Polym. Degrad. Stab.* **109**, 354–361 (2014).
29. Hall, T. A. BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acid Symp. Ser.* **41**, 95–98 (1999).
30. Berekaa, M. M., Linos, A., Reichelt, R., Keller, U. & Steinbüchel, A. Effect of Pretreatment of Rubber Material on its Biodegradability by Various Rubber Degrading Bacteria. *FEMS Microbiol. Lett.* **184**, 199–206 (2000).

Figures

Figure 1

Size of clearing zone (mm) on NR latex agar in 6-well plate in relation to the day of clear zone formation for 18 rubber degrading Actinobacteria detected in this study.

Figure 2

Rubber degrading strains 16S rDNA evolutionary relationships of taxa together with top 1 blast homology in NCBI database. The evolutionary history was inferred using the Neighbor-Joining method conducted in MEGA X.

Figure 3

The organization and transcription of the *lcp* gene clusters in strain (a) *Microtetraspora* sp. AC03309 (b) *Dactylosporangium* sp. AC04546. Open arrows indicate the genes. Location (bp) are indicated above/below the genes.

Figure 4

Cis-1,4-polyisoprene biodegradation pathway by oxygen attack at the double bond (modified from Linos et al., 2000)

Figure 5

Phylogenetic tree of characterised lcps and lcps from *Microtetraspora* sp. AC03309 and *Dactylosporangium* AC04546. Enzymes: LcpK30 from *Streptomyces* sp. strain K30 (AAR25849); Lcp1VH2 from *G. polyisoprenivorans* VH2 (ABV68923); LcpKb1 from *G. westfalica* Kb1 (ABV68924); LcpSH22a from *N. nova* SH22a (WP025350295); LcpRPK1 from *R. rhodochrous* RPK1 (AMY60409); LcpNVL3 from *Nocardia* sp. strain NVL3 (API85527); LcpE1 from *N. farcinica* E1 (ABC59140); and LcpHR-BB from *Solimonas fluminis* HR-BB (WP104231946).

Figure 6

Strain morphology (i) ISP2 agar plate (ii) SEA agar (iii) FESEM/ SEM (iv) NR latex agar at Day 15 using 100 μ L of 1×10^6 cells/ mL (a) *Microtetraspora* sp. AC03309 and (b) *Dactylosporangium* sp. AC04546

Figure 7

SEM images for (i) fresh latex (ii) latex glove (iii) tyre granules – (a) controls (b) *Dactylosporangium* sp. AC04546 and (c) *Microtetraspora* sp. AC03309 incubated for 30 days, 180 rpm, 30 °C

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFigures.pdf](#)