

**ISOLATION AND CHARACTERIZATION
OF ANTARCTIC ACTINOBACTERIA,
BACTERIA AND FUNGI WITH
ANTIMICROBIAL
ACTIVITIES**

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ABSTRACT

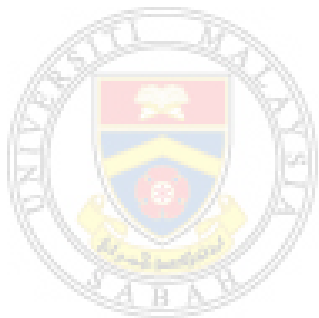
Antimicrobial resistance (AMR) is a serious health problem worldwide. The escalating prevalence of AMR is driving the need for new antimicrobial drugs. Exploration of extreme environments such as Antarctica may give us hope to discover promising drug candidates. This study aimed to discover Antarctic spore-forming soil microorganisms that produce novel antimicrobial compounds. The microbial isolation was carried out using a combination of low- and high-nutrient isolation agar media. A total of 90 strains were isolated from two soil samples, which were further clustered into 19 groups at a similarity of 60% using random amplified polymorphic DNA fingerprinting (RAPD) fingerprinting technique. Antimicrobial activities of the isolates were tested against 13 Gram-positive and -negative bacteria, and strain Im33 was chosen for further characterization due to its capability to inhibit all bacteria. It was described on the basis of morphological, physiological and molecular phylogenetic analyses, as well as carbon utilization and antimicrobial capabilities, along with strains E22 and INACH3013. They were identified as *Talaromyces*, *Penicillium* and *Streptomyces* spp. respectively based on 16S rDNA, 18S rDNA or internal transcribed spacer (ITS) sequences. The strains were capable of producing enzymes to degrade a wide variety of carbon sources, suggesting that they might play a role in the nutrient cycling of Antarctic terrestrial ecosystem in order to obtain nutrients for survival and growth. Both strains Im33 and E22 showed high levels of resistance to cycloheximide, which could probably be a defence mechanism that confers them a competitive advantage over cycloheximide-sensitive species in a nutrient scarce environment. Strain INACH3013 might have a narrow spectrum β -lactamase or a different mechanism of resistance, given that it was susceptible to ampicillin but not to other β -lactam antibiotics. The inhibitory effect of strains Im33, E22 and INACH3013 against multiple Gram-positive and Gram-negative bacteria indicated that they produce broad-spectrum antimicrobial compounds. The antimicrobial compounds synthesized by strain Im33 were pH-stable, thermostable, non-polar and non-toxic, which might be good candidates for drug development. The estimated genome size of strain INACH3013 is 9,357,559 bp with 70.5% G+C content. A total of 8,551 coding sequences (CDSs) in 432 subsystems were annotated by the Rapid Annotation using Subsystems Technology (RAST) server. The secondary metabolite biosynthetic potential of strain INACH3013 was assessed using the antibiotics and Secondary Metabolites Analysis SHell (antiSMASH). Thirty-two biosynthetic gene clusters (BGCs) were predicted to be involved in the production of 1 bacteriocin-terpene hybrid, 2 bacteriocins, 8 NRPSs, 2 T1PKSs, 1 T2PKS, 1 T3PKS, 1 ectoine, 1 lantipeptide, 1 lassopeptide, 2 melanins, 2 siderophores, 2 terpenes, 1 T1PKS-NRPS hybrid, 1 bacteriocin-T1PKS hybrid, 1 T1PKS-butyrolactone hybrid, 1 T2PKS-T1PKS hybrid, 1 lantipeptide-terpene hybrid and 3 secondary metabolite-related proteins that did not fit into any category. Fifteen clusters that displayed low similarities (<40%) to known BGCs in other strains are most probably species specific and might encode metabolites with previously unreported novel chemical structures and biological activities. Eight clusters that showed no relatedness to any known BGCs might synthesize potentially unknown natural products, or their compounds could only be partially predicted from the genes' organization. These preliminary findings support that Antarctic spore-forming microorganisms are a great source of novel bioactive metabolites with biotechnological and pharmaceutical potentials.

ABSTRAK

Pemencilan dan Pencirian Actinobacteria, Bakteria dan Fungi Antartika dengan Aktiviti Antimikrob

Rintangan antimikrob (AMR) adalah satu masalah kesihatan yang serius di seluruh dunia. Peningkatan kelaziman AMR mendorong keperluan untuk ubat antimikrob baru. Penerokaan persekitaran yang melampau seperti Antartika mungkin memberi kita harapan untuk menemui calon-calon yang menjanjikan. Kajian ini bertujuan untuk menemui mikroorganisma tanah pembentuk spora Antartika yang menghasilkan sebatian antimikrob baru. Pemencilan mikroorganisma telah dijalankan dengan menggunakan gabungan media pengasingan nutrien rendah dan nutrien tinggi. Sebanyak 90 pencilan telah dipencilkan daripada dua sampel tanah, dan seterusnya dikelompokkan kepada 19 kumpulan berdasarkan 60% persamaan dengan menggunakan teknik amplifikasi rawak DNA polimorfik (RAPD). Aktiviti antimikrob pencilan telah diuji terhadap 13 bakteria Gram-positif dan Gram-negatif, dan strain Im33 dipilih untuk pencirian selanjutnya disebabkan kemampuannya untuk menghalang semua bakteria. Ia digambarkan berdasarkan analisis morfologi, fisiologi dan filogenetik molekul, serta penggunaan karbon dan keupayaan antimikrob, bersama dengan strain-strain E22 dan INACH3013. Mereka dikenali sebagai *Talaromyces*, *Penicillium* dan *Streptomyces* spp. masing-masing berdasarkan jujukan 16S rDNA, 18S rDNA atau kawasan penjarak dalaman (ITS). Strain-strain mampu menghasilkan enzim untuk memecahkan pelbagai sumber karbon, menunjukkan bahawa mereka mungkin memainkan peranan dalam pengitaran nutrien ekosistem terestrial Antartika untuk mendapatkan nutrien untuk kelangsungan hidup dan pertumbuhan. Kedua-dua strain Im33 dan E22 menunjukkan tahap rintangan yang tinggi terhadap sikloheximide, yang mungkin merupakan satu mekanisme pertahanan yang memberikan mereka kelebihan daya saing terhadap spesies sensitif sikloheximide dalam persekitaran kurang nutrien. Strain INACH3013 mungkin mempunyai spektrum sempit β -laktamase atau mekanisme rintangan yang berbeza, memandangkan ia sensitif kepada ampicillin tetapi tidak kepada antibiotik β -laktam yang lain. Kesan halangan kuat strain-strain Im33, E22 dan INACH3013 terhadap pelbagai bakteria Gram-positif dan Gram-negatif menunjukkan bahawa mereka menghasilkan sebatian antimikrob yang berspektrum luas. Sebatian antimikrob yang disintesis oleh strain Im33 adalah pH stabil, termostabil, tidak berkutub dan tidak toksik, yang mungkin merupakan calon-calon yang baik untuk pembangunan ubat. Anggaran saiz genom strain INACH3013 adalah 9,357,559 bp dengan 70.5% kandungan G+C. Sebanyak 8,551 jujukan kod (CDSs) dalam 432 subsistem telah dianotasi oleh pelayan Rapid Annotation using Subsystems Technology (RAST). Potensi biosintetik metabolit sekunder strain INACH3013 telah dinilai dengan menggunakan antibiotics and Secondary Metabolites Analysis SHell (antiSMASH). Tiga puluh dua kelompok gen biosintetik (BGC) dijangka terlibat dalam penghasilan 1 hibrid bakteriosin-terpen, 2 bakteriosin, 8 NRPS, 2 T1PKS, 1 T2PKS, 1 T3PKS, 1 ectoine, 1 lantipeptide, 1 lassopeptide, 2 melanin, 2 siderofor, 2 terpen, 1 hibrid T1PKS-NRPS, 1 hibrid bakteriosin-T1PKS, 1 hibrid T1PKS-butirilakton, 1 hibrid T2PKS-T1PKS, 1 hibrid lantipeptide-terpen dan 3 protein berkaitan dengan metabolit sekunder yang tidak dapat dimasukkan dalam sebarang kategori. Lima belas kelompok yang memperlihatkan persamaan rendah (<40%) kepada BGC yang diketahui dalam

strain-strain lain kemungkinan besarnya adalah spesies spesifik dan mungkin mengekod metabolit yang mempunyai struktur kimia dan aktiviti biologi baru yang belum pernah dilaporkan. Lapan kelompok yang tidak menunjukkan kesalinghubungan kepada mana-mana BGC yang diketahui mungkin mensintesis produk semula jadi yang berpotensi tidak diketahui, atau sebatianannya hanya dapat diramal sebahagiannya daripada organisasi gen. Penemuan awal ini menyokong bahawa mikroorganisma pembentuk spora Antartika merupakan satu sumber metabolit bioaktif baru yang hebat dengan potensi bioteknologi dan farmaseutikal.



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LIST OF SYMBOLS

| | | |
|--------------|---|--------------------------|
| % | - | Percent |
| °C | - | Degree celcius |
| µg/ml | - | Microgram per millilitre |
| Da | - | Dalton |
| g | - | Gram |
| h | - | Hour |
| mg | - | Milligram |
| mg/l | - | Milligram per litre |
| min | - | Minute |
| mm | - | Millimetre |
| mM | - | Millimolar |
| ng | - | Nanogram |
| nm | - | Nanometre |
| O.D. | - | Optical density |
| pmol | - | Picomole |
| rpm | - | Revolutions per minute |
| s | - | Second |
| v/v | - | Volume per volume |
| w/v | - | Weight per volume |
| X | - | Times |
| β | - | Beta |
| µg | - | Microgram |
| µl | - | Microlitre |

LIST OF ABBREVIATIONS

| | |
|---|--|
| ABA | - Actinomycetes Agar |
| ACP | - Acyl Carrier Protein |
| AIA | - Actinomycetes Isolation Agar |
| AMPs | - Antimicrobial Peptides |
| AMR | - Antimicrobial Resistance |
| antiSMASH | - Antibiotics and Secondary Metabolites Analysis Shell |
| ARO | - Aromatases |
| ASPA | - Antarctic Specially Protected Area |
| BenA | - β -tubulin |
| BGC | - Biosynthetic Gene Cluster |
| BLASTn | - Nucleotide Basic Local Alignment Search Tool |
| CaCl₂.2H₂O | - Calcium Chloride Dihydrate |
| CaCO₃ | - Calcium Carbonate |
| CaM | - Calmodulin |
| CDSs | - Coding Sequences |
| CLF | - Chain Length Factor |
| CoA | - Coenzyme A |
| Csp | - Cold Shock Protein |
| CuSO₄.5H₂O | - Copper (II) Sulphate Pentahydrate |
| cUTI | - Complicated Urinary Tract Infections |
| CYA | - Czapek Yeast Extract Agar |
| CYB | - Czapek Yeast Extract Broth |
| CYC | - Cyclases |
| DHPS | - Dihydropteroate Synthase |
| DNA | - Deoxyribonucleic Acid |
| dNTPs | - 2'-deoxyribonucleoside-5'-triphosphates |
| EPS | - Extracellular Polymeric Substance |
| ESBLs | - Extended-spectrum β -lactamases |
| EtBr | - Ethidium Bromide |
| FeSO₄.7H₂O | - Iron(II) Sulphate Heptahydrate |
| FUR | - Ferric Uptake Regulation Protein |

| | | |
|---|---|--|
| GAA | - | Glycerol-Asparagine Agar |
| HGT | - | Horizontal Gene Transfer |
| Hsp | - | Heat Shock Protein |
| HTS | - | High-throughput Screening |
| iChip | - | Isolation Chip |
| IM2 | - | Gause Modified Agar |
| ISSA | - | Inorganic-Salts-Starch Agar |
| ITS | - | Internal Transcribed Spacer |
| K₂HPO₄ | - | Dipotassium Phosphate |
| KCH₃CO₂ | - | Potassium Acetate |
| KCl | - | Potassium Chloride |
| KGI | - | King George Island |
| KH₂PO₄ | - | Potassium Dihydrogen Phosphate |
| KNO₃ | - | Potassium Nitrate |
| KR | - | Ketoreductases |
| LB | - | Luria Bertani |
| LCA | - | Lignocellulose Agar |
| LCB | - | Lignocellulose Broth |
| LeuRS | - | Leucyl-tRNA Synthetase |
| LPS | - | Lipopolysaccharide |
| MEA | - | Malt-Extract Agar |
| MEB | - | Malt-Extract Broth |
| MgCl₂ | - | Magnesium Chloride |
| MgSO₄ | - | Magnesium Sulphate |
| MgSO₄.7H₂O | - | Magnesium Sulphate Heptahydrate |
| MgSO₄.H₂O | - | Magnesium Sulphate Monohydrate |
| MHA | - | Muellar-Hinton Agar |
| MHB | - | Muellar-Hinton Broth |
| ML | - | Maximum Likelihood |
| MIB | - | 2-Methylisoborneol |
| MnCl₂.4H₂O | - | Manganese (II) Chloride Tetrahydrate |
| MRSA | - | Methicillin-Resistant <i>S. aureus</i> |
| MS | - | Mass Spectrometry |

| | | |
|---|---|---|
| NA | - | Nutrient Agar |
| NaCl | - | Sodium Chloride |
| NADPH | - | Reduced Nicotinamide Adenine Dinucleotide Phosphate |
| NaNO₃ | - | Sodium Nitrate |
| NaOAc | - | Sodium Acetate |
| NDM-1 | - | New Delhi Metallo-β-lactamase 1 |
| NGS | - | Next Generation Sequencing |
| (NH₄)₂SO₄ | - | Ammonium Sulphate |
| NMR | - | Nuclear Magnetic Resonance |
| NRPs | - | Non-Ribosomal Peptides |
| NRPSs | - | Non-Ribosomal Peptide Synthetases |
| OA | - | Oatmeal Agar |
| OD | - | Optical Density |
| PABA | - | <i>p</i> -Aminobenzoic Acid |
| PAHs | - | Polycyclic Aromatic Hydrocarbons |
| PBP2a | - | Penicillin-Binding Protein 2a |
| PBPs | - | Penicillin-Binding Proteins |
| PCR | - | Polymerase Chain Reaction |
| PDA | - | Potato Dextrose Agar |
| PDB | - | Potato Dextrose Broth |
| PKs | - | Polyketides |
| PKSs | - | Polyketide Synthases |
| PYA | - | Peptone-Yeast Extract Iron Agar |
| R2A | - | Reasoner's 2A Agar |
| RAPD | - | Random Amplification of Polymorphic DNA |
| RAST | - | Rapid Annotation using Subsystems Technology |
| ROS | - | Reactive Oxygen Species |
| RPB2 | - | RNA Polymerase II Subunit |
| SA | - | <i>Streptomyces</i> Agar |
| SDA | - | Sabouraud Dextrose Agar |
| SDB | - | Sabouraud Dextrose Broth |
| SDS | - | Sodium Dodecyl Sulphate |
| SEM | - | Scanning Electron Microscope |

| | | |
|---|---|--|
| SOD | - | Superoxide Dismutase |
| sub-MIC | - | Sub-Minimum Inhibitory Concentration |
| T1PKS | - | Type I Polyketide Synthase |
| T2PKS | - | Type II Polyketide Synthase |
| T3PKS | - | Type III Polyketide Synthase |
| TA | - | Tyrosine Agar |
| <i>thpD</i> | - | Ectoine Hydrolase Gene |
| UPGMA | - | Unweighted Pair Group Method Using Arithmetic Mean |
| VP | - | Variable Pressure |
| VISA | - | Vancomycin Intermediate <i>S. aureus</i> |
| WHO | - | World Health Organization |
| YMA | - | Yeast-Extract Malt-Extract Agar |
| YMB | - | Yeast-Extract Malt-Extract Broth |
| ZnSO₄·7H₂O | - | Zinc Sulphate Heptahydrate |
| ZUR | - | Zinc Uptake Regulation Protein |



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