CHARACTERIZATION OF ANTARCTIC BACTERIA AND THEIR ANTIMICROBIAL ACTIVITIES

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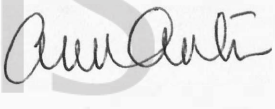

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ABSTRACT

CHARACTERIZATION OF ANTARCTIC BACTERIA AND THEIR ANTIMICROBIAL ACTIVITIES

A total of 2582 bacterial strains were isolated from 16 soil and water samples from the King George Island and Schirmacher Range, Antarctica. Twenty three Antarctic bacterial strains inhibited the growth of one or more Gram-negative and Gram-positive food pathogens such as *Escherichia coli* 0157:H7, *Salmonella* spp., *Klebsiella pneumonia*, *Enterobacter cloacae* and *Vibrio* spp. and a Gram-positive food pathogen *Bacillus cereus* K15. Seven out of the 23 strains, BG5, CG21, HKAM1, MTC3, MA2, WEA1 and WEK1 were identified based on their 16S rDNA sequences and biochemical analyses. They were *Pseudomonas* sp. MTC3, *Pseudomonas* sp. CG21, *Pseudomonas* sp. MA2, *P. corrugata* WEK1, *P. migulae* WEA1, *Janthinobacterium lividum* HKAM1 and *Pedobacter cryoconits* BG5. Although most of them were affiliated to the same genus or closely related species, their biochemical, phenotypic characteristics and antibiotics resistance profiles varied. Inhibitors produced by strains MTC3, CG21 and BG5 were sensitive to protease suggesting that they have proteinaceous structures while strains WEA1, WEK1, HKAM1 and MA2 were not sensitive to catalase, lipase, α-amylase, and protease indicating four of these inhibitors have complex structures. Three out of seven Antarctic bacterial strains WEA1, WEK1 and MA2 were found to encode polyketide synthase gene, indicating the antimicrobial agent was probably produced by polyketide synthase. Antimicrobial resistance profiles of 45 Antarctic bacterial isolates were obtained. Most of the bacteria were resistance to at least of three or more types of antimicrobial agents tested while one of the bacterial isolate was susceptible to all the antimicrobial agents. These data revealed that the existence of many antimicrobial resistant strains among the Antarctic bacterial population. The plasmid sequence of pHK1 of *Pseudomonas* sp. CG21 revealed that there was no gene encoding the antimicrobial production and antimicrobial resistance on the plasmid. Basically the pHK1 plasmid carried genes encoding for plasmid replication, stability and maintenance, mobilization and genes for unknown function.

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ABSTRAK

Sebanyak 2582 bakteria telah diasingkan dari 16 sampel tanah dan air yang diperolehi dari Antartika. Dua puluh tiga bacteria dari Antartika yang dapat merencatkan tumbesaran bakteria seperti Escherichia coli 0157:H7, Salmonella spp., Klebsiella pneumonia and Vibrio spp. dan patogen Gram-positif Bacillus cereus K15. Tujuh daripada dua puluh tiga bakteria tersebut telah dikenalpasti melalui jujukan DNA 16S rDNA dan analisis biokimia yang terdiri daripada Pseudomonas sp. MTC3, Pseudomonas sp. CG21, Pseudomonas sp. MA2, P. corrugata WEK1, P. migulae WEA1, Janthinobacterium lividum HKAM1 dan Pedobacter cryoconits BG5. Walaupun spesis ini tergolong dalam kumpulan genus yang sama, namun terdapat perbezaan dari segi biokimia. Bahan antibiotik yang dihasilkan oleh bakteria MTC3, CG21 dan BG5 adalah sensitif kepada protease manakala bahan antibiotik yang dihasilkan oleh bakteria WEA1, WEK1, HKAM1, MA2 tidak sensitif kepada katalase, lipase, α-amilase dan protease yang menunjukan bahan ini mempunyai struktur yang kompleks. Tiga bakteria WEA1, WEK1 dan MA2 didapati membawa gen polyketide synthase, menunjukan bahan antibiotik dihasilkan oleh polyketide synthase. Profil kebolehan pertahanan antibiotik daripada 45 bakteria dari Antartika menunjukan kebanyakan bakteria dari Antartika mempunyai kerintangan peka kepada sekurang-kurangnya tiga atau lebih jenis antibiotik yang diuji. Walau bagaimanapun, dua bakteria tidak peka kepada semua antibiotik yang diuji. Data ini menunjukan bakteria dari Antartika mempunyai kerintangan kepada antibiotik. Jujukan DNA plasmid pHK1 yang dibawa oleh Pseudomonas sp. CG21, menunjukan tiada gen berkaitan dengan penghasilan antibiotik dan pertahanan antibiotik yang dibawa oleh plasmid. Plasmid ini membawa gen yang berkaitan dengan replikasi plasmid, kestabilan serta penyelenggaraan plasmid, kemobilan dan gen yang membawa protein yang tidak dikenali.

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LIST OF ABBREVIATIONS

rRNA	-	Ribosomal ribonucleic acid
rDNA	-	Ribosomal deoxyribonucleic acid
WHO	-	World Health Organization
STEC	-	Shiga toxin-producing *Escherichia coli*
EHEC	-	Enterohemorrhagic *Escherichia coli*
ETEC	-	Enterotoxigenic *Escherichia coli*
EPEC	-	Enteropathogenic *Escherichia coli*
EIEC	-	Eenteroinvasive *Escherichia coli*
HUS	-	Hemolytic–uremic syndrome
THF	-	Tetrahydrofolate
DHF	-	Dihydrofolate
PABA	-	Paraminobenzoic acid
mRNA	-	Messenger RNA
tRNA	-	Transfer RNA
ATP	-	Adenosine triphosphate
LAB	-	Lactic acid bacteria
Gly	-	Glycine
Tyr	-	Tyrosine
Asn	-	Asparagine
Val	-	Valine
Xaa	-	Histidine
Cys	-	Cysteine
FAO	-	Food and Agriculture Organization
Ala	-	Alanine
ABA	-	Aminobutyric acid
Ala-S-Ala	-	Lanthionine
ABA-S-Ala	-	*p*-methyllanthionine
TSA	-	Tryptic Soy Agar
LBA	-	Luria-Bertani agar
ABM	-	Antarctic Bacterial Medium
NB	-	Nutrient broth
NA	-	Nutrient agar

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KOH	-	Potassium hydroxide
dNTP	-	2′-dexoyribonucleoside-5′-triphospates
EDTA	-	Ethylenediaminetetraacetic acid
PCR	-	Polymerase chain reaction
TAE	-	Tris-acetate-EDTA
SDS	-	Sodium lauryl sulfate
NaCl	-	Sodium chloride
$MgCl_2$	-	Magnesium chloride
BLAST	-	Basic local alignment search tool
MEGA	-	Molecular Evolutionary Genetics Analysis
MCS	-	Multiple cloning sites
CDS	-	Coding sequence
ORF	-	Open reading frame
RBS	-	Ribosome binding site
CDD	-	Conserved Domain Database

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LIST OF SYMBOLS & UNITS

β	-	Beta
°C	-	Degree Celsius
<	-	Less than
>	-	More than
γ	-	Gamma
α	-	Alpha
%	-	Percent
cfu	-	Colony forming units
g	-	Gram
g	-	Graviti
S	-	Subunit
ml	-	Milliliter
mM	-	Millimolar
M	-	Molar
mg	-	Milligram
w/v	-	Weight per volume
s	-	Second
cm	-	Centimeter
X	-	Times
µl	-	Microliter
min	-	Minute
-	-	Minus
rpm	-	Rotation per minute
pmol	-	Pico mol
U	-	Unit
µM	-	Micromolar
V	-	Volt
Kb	-	Kilo base
g	-	Gravity

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CHAPTER 1

INTRODUCTION

1.1 Background

In the late 1960 and early 1970s, notable achievements in developing antibiotics to combat pathogenic diseases had lead to the misconception that the war between infectious diseases and human was over (Spellberg *et al.*, 2004). However, currently, medical centers are experiencing a rise in antimicrobial resistance of pathogenic bacteria and the susceptibility of pathogens against antibiotics is steadily decreasing. The emergence of multiple drug resistance in clinical *Escherichia coli* O157:H7, *Vibrio cholerae* and *Salmonella enterica* from the tropics such as Malaysia is one of the manifestations of this phenomenon (Radu *et al.*, 2001; Radu *et al.*, 2002; Tunung *et al.*, 2007). Many isolates were found to be resistant not only to the β-lactam family of antibiotics but also to the new aminoglycosides such as tobramycin and gentamicin and this poses a serious therapeutic problem for the clinicians and public health agencies (Hogan and Kolter, 2002; Haryani *et al.*, 2007). The exhibition of resistance indicates to us that there is a need to look for new antibiotics to keep pace with emergence of resistance caused by microbiological agents and genetic mutation of pathogenic bacteria.

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The emerging of food-borne microbial pathogens has been a serious threat to human's health resulting in food poisoning (Haryani *et al.*, 2007). Furthermore, the threat of bioterrorism with multiple drug resistant pathogens such as Anthrax and Cholera highlight the need for continued intensification of antimicrobial research (Gilligan, 2002; Thomson *et al.*, 2004). Another factor is the downtrend of the antibiotic research and development even though there is an increase in pathogen resistance and the fact that no new classes of antibiotics have been developed since 1963. Although there were 2 new antibiotics with narrow spectrum drugs (linezolid and daptomycin), that have been approved, there is no new structural classes of antibiotic that have been launched into the market since 1963, when the quinolone, nalidixic acid was approved (Carpenter and Chambers, 2004; Hancock, 2007). However, a new class of antimicrobial agent, oxazolidinones was introduced in 1999

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after 40 years of effort. Thus, there is a need to discover new classes of antibiotics with new mechanisms of action, directed against new antibacterial targets. These antibiotics should be free of cross-resistance to previously existing antibiotics, to slow down resistance development against antibiotics (Labischinski, 2001). Without innovative public policy and additional financial support, fewer and fewer antibiotics will be available to treat the increasing number of drug-resistant and dangerous microbes that threaten the global community.

Antibiotic resistance requires a renewed effort to screen for antimicrobial agents effective against pathogenic bacteria resistant to existing antibiotics. Antarctica offers an interesting environment to seek for new classes of antibiotics because of its extremes of climate, habitat and biogeography. This region offers a vast potential for the development of novel applications for those natural products produced by Antarctic bacteria such as application of bioactive compounds and cold-adapted enzymes in the food, cosmetic and pharmaceutical industries (Franzmann, 1996). The microbial biomass can be immense in Southern Ocean blooms and freshwater cyanobacterial mats, species richness is generally more restricted than it is in temperate regions. This microbial biomass provides a broad variety of taxa with a diverse gene pool (Wynn-Williams, 1996). However, the antagonistic properties of cold-loving organisms have not yet been explored as extensively as those of the mesophiles. These antimicrobial agents produced in cold environments need to function at low temperatures for the organisms to gain a competitive advantage during their growth cycle in these extreme environment. Such cold-active antimicrobial compounds may be amenable to exploitation in industrial applications including chilled-food preservation or as new antibiotics against increase resistance of pathogens (O'Brien *et al.* 2004).

The emergence of multiple antibiotic resistant strains of pathogens is common due to constant exposure to various antibiotics. However, it is not known whether there is any multiple resistant strain of bacteria that is not constantly exposed to antibiotics such as the bacteria from Antarctica. Antarctic ecosystem is one of the pristine environments on Earth and offers researchers a unique opportunity to study microbial diversity and evolution (Franzmann, 1996; Vincent, 2000). Ecological studies have reported that antibiotic resistance is becoming a global phenomenon.

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The ability to resist to antibiotics is usually due to the repetitive exposure of the bacteria to antibiotics (Baquero and Blazquez, 1997) but there are cases in antibiotics resistance have been found in bacteria populations without apparent antibiotic selection pressures as shown in Antarctic ecosystems (Kobori *et al.*, 1984; De Souza *et al.*, 2006). However, antibiotics resistance is probably intrinsic and endemic to a particular bacterial species, or selected due to antibiotics occurring naturally in the environment (Bonnedahl *et al.*, 2008). A review of available literature indicates that not many studies have been conducted on antibiotics resistance of the Antarctic bacteria.

1.2 Objectives

The objectives of the study were:

i. To screen for strains with antimicrobial activity against food pathogens.
ii. To identify and characterize Antarctic bacteria with antimicrobial activities.
iii. To screen for antibiotics resistance profiles of Antarctic bacteria.
iv. To sequence and characterize the plasmid genome of a bacteria exhibiting antimicrobial activity.

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CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Antarctica

2.1.1 Introduction

Antarctica (Figure 2.1) is characterized by an extremely cold environment, gigantic icebergs and permanent ice shelves with the temperature rarely rises above freezing point. Microorganisms may have existed in the continental crust and their descendants may live in sub-glacial rock crevices, lakes, and sediments before the permanent ice cap formed in million years ago. The extreme conditions of Antarctica (low temperature, low humidity and high radiation) offer great opportunity for studies relating to modeling living organisms in other planets, model systems for adaptation and cell growth at low temperature, intercontinental contacts and effect on the organisms between changes of glacial and post-glacial periods (Price, 2000; Satyanarayana *et al.*, 2005).

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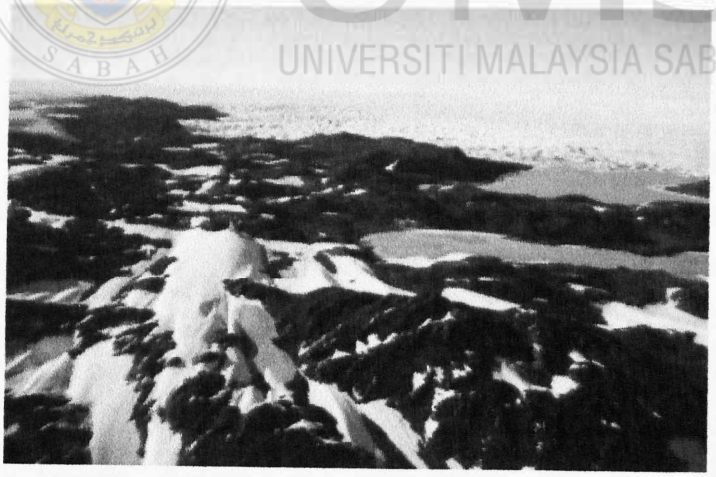

Figure 2.1 : Schimarcer Oasis.
Source : Bruenjes, 2003.

Recent evidence has suggested that microbial communities can survive on wind-deposited sediment particles within liquid water inclusions in permanently ice-covered Antarctic lakes (Price, 2000). In coastal areas, seal and penguin rookeries may contribute significant quantities of organic material to soils; which are high in nutrients. However, soils from inshore waters that have undergone a rapid freeze-

thaw cycle can be lethal to the survival of microorganisms (Wynn-Williams, 1996). Although these unique and extreme environments limit the diversity of organism, microorganisms are dominant in Antarctica. However, they survive and grow in the Antarctic soils under condition of low temperature, high osmotic stress and the strain of the freeze-thaw phase during the transition of winter to summer which is critical for the onset of microbial activity. Microorganisms need to be efficient at rapidly switching their metabolism on and off according to prevailing conditions (Russell, 2006).

Antarctica is a novel environment and its unique biodiversity has attracted companies, bioprospectors, scientists, academicians and scholars to visit Antarctica every year to conduct research in order to find useful bioactive compounds. The lack of knowledge regarding to Antarctic biota initiates the effort to isolate novel organisms. Besides, the biochemistry of Antarctic organisms involves in adaptation in extreme environments offers an opportunity to discover novel bioactive compounds (Lohan & Johnston, 2005). Moreover, studies about psychrophilic bacteria are lesser compared to thermophiles and it is not clear whether life on earth originated from a hot or cold environment.

Antarctica can be classified into three major soil zones based on climate and moisture availability, the dry valleys and bare ground on the Trans Antarctic Mountains, the oases of coastal greater Antarctica, and the maritime Antarctic Peninsula (Claridge and Campbell, 1984). The maritime Antarctica is defined as the southern polar region where the mean air temperature in a month is above freezing point in the summer and the mean monthly temperature only occasionally falls below -10°C. However, the sub-Antarctic and maritime Antarctic regions have milder climate, higher water content and greater biodiversity compared to southernmost and continental Antarctic regions where both regions have much lower temperature and arid conditions (Holdgate, 1964). The maritime Antarctica includes South Sandwich Islands, South Orkney Islands, South Shetland Islands (King George Island, Deception Island, etc.), western coastal fringe of the Antarctic Peninsula south to Marguerite Bay, Bouvetoya and Peter I Oy (Spaull, 1973).

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