NOVEL PHARMACEUTICALS FROM KENYAN CYANOBACTERIA

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**A REPORTED SUBMITTED TO THE WIOMSA IN RESPECT OF THE
MARG II CONTRACT**

April 2004

SUMMARY

Two projects undertaken at the Heriot-Watt University, Edinburgh, U.K as part of the WIOMSA Marg II Programme are presented here. Project 1 was on studies carried out on the Kenyan marine cyanobacterium *Lyngbya majuscula*, which was collected from Shimoni, 100 km south of Mombasa, Kenya and ferried to the U.K on 12th October 2003. Specifically, *L. majuscula* was investigated on the potential for bioprocess intensified production of secondary metabolites with therapeutic potential, the Chemistry of its secondary metabolites and that of the cultures, and on the 16S rDNA classification of its epiphytic bacteria isolates. Project 2 focused on the isolation of cell-cell signaling inducer compounds from biofilms of marine bacteria *Bacillus licheniformis* EI-34-6.

ACKNOWLEDGEMENTS

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LIST OF SYMBOLS

rDNA	Ribosomal dioxyribonucleic acid
EtOH	Ethanol
GFF	Glass fibre filter
DCM	Dichloromethane
TLC	Thin layer chromatography
Si Gel	Silica gel
^{1}H NMR	Proton nuclear magnetic resonance
MHz	Mega hertz
KOH	Potassium hydroxide
PCR	Polymerase chain reaction
AHLs	Acylhomoserine lactones
AMS	Air membrane surface
MeOH	Methanol
mM	Millimole
$MnCl_2$	Manganese chloride
μL	Microlitre
μm	Micrometre
D_2O	Deuterated water
$CDCl_3$	Deuterated chloroform

PROJECT 1

GOAL
A collection of the Kenyan marine cyanobacterium *Lyngbya majuscula* from Shimoni, 100 km south of Mombasa, Kenya was ferried to the U.K on 12th October 2003 in filtered (GFF 20µ) sea water of its natural habitat for investigations on;

1. The potential for bioprocess intensified production of secondary metabolites. The wild *L. majuscula* has an established history of being a prolific producer of a diversified range of secondary metabolites.

2. The chemistry of its secondary metabolites and that of the laboratory cultures.

However, in the process of reviewing the above objectives it became necessary to also investigate the 'microbial world' of *L. majuscula*. The *L. majuscula* surface is inhabited by myriad coloured bacteria. As a consequence therefore, the cyanobacterium was investigated on;

3. The surface bacterial composition including the strain identification and antimicrobial activity of the bacteria fermentations. Identification of the compounds produced during these fermentations was also deemed necessary.

1.1 *LYNGBYA MAJUSCULA* CULTURES
Approximately 100 mg specimen of *L. majuscula* were cultured in 250 mL conical flasks using BG11 and artificial sea water media with a constant light supply (500 lux) and filtered air (0.3 mm Whatman). The air was supplied from above. The room temperatures were 20-25 °C. No substantial growth of the cyanobacterium was observed. Some white biomass was however produced, culminating into the gradual death of the cyanobacterium within 2 weeks. In some cases, the cyanobacterium was attacked by diatoms of the *Nitschia* sp. The mass of *L. majuscula* initially used for the cultures was changed in order to find out if it is crucial for the growth process. In addition, investigations were made on whether or not dipping the cyanobacterium into 70% v/v EtOH for a few minutes influenced growth. The experiments aimed at establishing the optimum conditions for the effective culturing of *Lyngbya majuscula*. These experiments were not so successful because it requires a minimum of three months to carry them out.

1.2 SECONDARY METABOLITES ISOLATION
The *L. majuscula* species were extracted for the MeOH, hexane and dichloromethane components. The DCM fraction was be purified using a combination of TLC, Sephadex LH 20 size exclusion chromatography and Si Gel flash chromatography techniques. The purified fractions were concentrated using a rotary evaporator (25 °C) before subjection to antimicrobial screens. A rough guide of what these fractions were composed of was carried out using ^{1}H NMR spectroscopy at 200 MHz. The aim here was to find out if the compounds isolated from the cyanobacterium have a similarity with those produced by both the surface bacteria and by the media cultures. Bioassay guided isolation continued to be the strategy to achieve this objective. These experiments went hand in hand with the investigations on the secondary metabolites isolates of the surface bacteria. However, it turned out that emphasis was on the

search for antimicrobial compounds from *L. majuscula* epiphytic bacteria, which offered the best avenues of research.

1.3 ISOLATION OF BRIGHTLY COLOURED *L. MAJUSCULA* EPIPHYTIC BACTERIA

ABSTRACT
The search for bioactive compounds from the Kenyan marine cyanobacterium *Lyngbya majuscula* has led to the isolation of 9 brightly coloured epiphytic bacteria associated with the cyanobacterium. Six of the nine bacteria isolated *viz.* *Pseudoalteromonas* sp., *Norcadia cornyebacteroides*, *Paracoccus macussii*, *Micrococcus luteus*, *Stappia* sp. and the endospore forming gram positive *Bacilli* sp. BacB3 were identified through either full or partial sequence of the 16S rDNA gene in addition to the more classical gram staining techniques. Initial experiments identified *Pseudoalteromonas* sp. along with an unidentified bacteria as the only bacteria likely to surround the *L. majuscula* with populations of 8.0 - 18 (x 10^5) and 7.5- 9.3 (x 10^5) cfu respectively. However, subsequent dosing of the cyanobacterium with EtOH (70 % v/v) prior to taking swarb cultures enabled the isolation of the remaining brightly coloured epiphytic bacteria. When the dominant *Pseudoalteromonas* sp. was subjected to a time series recoverability with EtOH (70 % v/v), it was established that EtOH (70 % v/v) was effective as a sterilising agent against *Pseudoalteromonas* sp. only for the first 15 minutes. Experiments carried out on the interaction between the dominant *Pseudoalteromonas* sp. and the other epiphytic bacteria isolates revealed that *Micrococcus* sp. and *Paracoccus marcusii* antagonise *Pseudoalteromonas* sp. by slowing the growth of the latter bacteria by a zone of 12 mm to 19 mm and of 6 mm to 8 mm respectively. All the other bacteria sp. did not exhibit this activity. Both *Micrococcus* sp. (yellow) and *Paracoccus marcusii* however did not show any significant antimicrobial activity against the pathogens *Staphylococcus aureus* strain MRSA9551 and *Bacillus subtilis* strain SFP on the disc diffusion assay (6 mm disc).

Key Words
Lyngbya majuscula, epiphytic bacteria, 16S rNA gene, full/partial sequence, EtOH (70 % v/v).

1.3.1 INTRODUCTION

The mat forming marine cyanobacterium *Lyngbya majuscula*, also known as *Microcoleus lyngbyaceus* (Burja *et al*, 2002) is known to be a prolific producer of a diverse range of biologically interesting secondary metabolites. The compounds isolated from *L. majuscula* worldwide in pan-tropically geographic locations include those which exhibit antimicrobial activity; anti-proliferative compounds; anti-Hiv agents and those which have shown promise as potential anti-cancer agents. Recently, homodolastatin 16 was isolated from a Kenyan collection of *L. majuscula* along with the already known antanapeptin A (Coleman-Davies *et al*, 2003). Mat forming cyanobacteria have been known to thrive and to proliferate even in extreme conditions of nitrogen deficiency because of their ability to fix atmospheric nitrogen and also because they produce defensive secondary metabolites. Recent studies have however shown that nitrogen fixation and production of requisite growth factors like vitamins, metals and chelators in *L. majuscula* is aided by the epiphytic bacteria with which they mutually co-habit (Steppe *et al*, 1996). Alternative studies have been able to demonstrate that epiphytic bacteria promote morphological development in some seaweeds (Tatewaki *et al*, 1983).

Epiphytic bacteria are culturable and have the potential to offer a sustainable supply of desired compounds for pharmacological investigations. The dream to find alternative medicine from the oceans and seas over two decades ago for the ever increasing drug resistance by microbes has now turned the tide on the search for novel bioactive compounds from epiphytic microbes particularly in marine invertebrates. Marine invertebrates such as sponges and coelenterata have been extensively studied by marine natural products chemists (Kobayashi and Ishibashi, 1993). Although a lot of studies have been carried out and are still going on in the search for novel bioactive compounds from *L. majuscula*, there is little mention on the (epiphytic) epibiotic bacteria which appear on the surface of *L. majuscula*. So far, four brightly coloured quinoline producing bacteria have been reported for *L. majuscula* (Gil-Turnes, 1988). Alkaloid 1 and 2 are quinoline compounds which have been isolated from Curacao collections of *L. majuscula* (Orjala and Gerwick, 1997), suggesting that the epiphytic bacteria associated with the cyanobacterium have a role in secondary metabolite production. Secondary metabolite production in marine organisms is known to be highly dependent on bio-geographic conditions. Following the same argument, it is therefore highly probable that epiphytic bacteria co-habiting *L. majuscula* will influence secondary metabolite production in accordance with bio-georaphic conditions.

Molecular biology approaches for eliciting microbial diversity have proven to be most useful in recent times, having an edge over culture based techniques (Webster *et al*, 2001). Cloning as well as sequencing of the 16S rDNA genes have been able to provide enough data for describing complex microbial community composition. In this current study, molecular approach was used in tandem with microscopy to describe to the genus level the composition of epiphytic bacteria appearing on the surface of the Kenyan *L. majuscula*. Complimentary experiments were carried out to investigate microbial antagonism amongst the taxon, and also on the tolerability of the more dominant *Pseudoalteromonas* sp. to 70 % ethanol. Culturability of the epiphytic bacteria taxon using various media recipes was also investigated.

1.3.2 MATERIALS AND METHODS

1.3.2.1 Surface bacteria isolation and gram stain identification

L. majuscula was treated with 70% EtOH (2 min) prior to transferring swarb cultures into marine agar plates. The cultures were grown in the incubator at 28 °C for 3 days. Gram staining of the bacteria was done using the crystal violet method and confirmed by the KOH technique. The colonies of bacteria were observed in the former method using a Leica DMLS microscope fitted with an oil objective (x 200) to morphologically reveal the gram strains of the respective bacteria. The colonal populations of the surface bacteria were determined by grinding a sufficient amount of the *L. majuscula* specimen in distilled H_2O (1 mL) followed by centrifuging the contents in an eppendorf and culturing in marine agar at 28 °C the supernatant of the serial dilutions. The assortment of coloured bacteria isolates were cultivated in columbia agar (CA), nutrient agar (NA) and marine agar (MA) in order to establish the appropriate growth media recipe for each species (Table 1).

1.3.2.2 *Pseudoalteromonas* sp. tolerance to 70 % ethanol

Bacteria pellets, so formed by vortexing and centrifuging of 1 mL of the marine broth at high speed (13000 rpm) were submerged in 1 mL EtOH (70%) with thorough mixing. Time series cultures of these bacteria in EtOH (70 %) were made in the form of a lawn at 0 min, 2 min, 15 min, 30 min, 60 min and overnight. The cultures were grown overnight in the incubator at 28 °C.

1.3.2.3 Antagonism amongst *L. majuscula* surface bacteria taxon

A supernatant of bacteria pellets dispersed in deionized water (1 mL) was made. Bacteria, representative of the various strains in filter discs (6 mm) were grown on a lawn (20 µL) of the dominant *Pseudoalteromonas* sp. and incubated at 28 °C overnight.

1.3.2.4 Genomic identification of the *L. majuscula* surface bacteria

Bacteria isolates were cultured in marine broth 2216 (50 mL, Difco) to generate sufficient biomass for DNA extraction. The cultures were incubated at 28 °C with continuous shaking until growth reached saturation (3-5 days) after which they were harvested by centrifugation. 1 mL of a bacterial single colony harvest from the shake cultures was vortexed for 3 min at 3000 rpm. The pellets were washed free of marine salts with deionized water. DNA was extracted from the aqueous stationary phase using standard phenol-chloroform procedures (San brook et al, 1989). The DNeasy Tissue Kit 04/99 from QIAGEN was used. The PCR product (Plate 3) of the 16S rDNA was achieved by reacting a mixture containing 2 × PCR buffer biomix (50 µL, BIOLINE®), 1 µL of primer 9F (5'-GAGTTTGATCCTGGCTCAG-3'), and 1 µL of primer 1492R (5'- TACGGYTACCTTGTTACGACTT -3'). The final volume of the PCR mixture was adjusted to 100 µL by adding Mill Q water. Thermal cycling was performed with a TECHNE Touchgene (U.S.A). The samples were subjected to an initial denaturing step (92 °C, 4 min; 94 °C, 30 sec) followed by annealing (40 °C, 1 min). The thermal profile used was 30 cycles consisting of 1 min of primer annealing at 55 °C, 1 min of extension at 72 °C and 1 min of denaturation at 94 °C. A final extension step consisting of 10 min at 72 °C was also included. PCR products were detected by agarose gel electrophoresis (GIBCO BRL, U.S.A) and were visualised by UV fluorescence after ethidium bromide staining. Sizes of PCR products generated by the 9F and 1492R primers were approximately 1 kb. The respective PCR product was

purified using the DNeasy Prep kit (QIAGEN) as specified by the manufacturer and sequenced bidirectionally with the primers 9F and 1492R (described above). Sequencing of the final DNA extracts was done by the MWG Genomic Technologies (U.K). The 16S rDNA sequences determined in this study have been deposited with the MWG BIOTECH (U.K).

1.3.2.5 Antimicrobial activity

The pathogenic methicillin-resistant bacteria *Staphylococcus aureus* strain MRSA9551 and *Bacillus subtilis* strain SFP were used. Marine liquid media (1 mL, Difco) from the cultures of *Micrococcue luteus* and *Stappia* sp. were transferred into 1.5 mL eppendorf tubes and centrifuged (5000 x g, 10 min, room temperature). The activities of spent media were examined after the spent media was filtered twice with a Nalgene membrane filter (pore size, 0.2 μm). Sterile paper discs (6 mm, Whatman) were saturated with the supernatants (60 μL) and left to dry in the oven (40 °C). Antimicrobial activity assays with the above pathogens were performed using diagnostic sensitive test agar (Oxoid) at 37 °C). Inhibition zones were measured after 12 hours.

1.3.3 RESULTS

Swarb cultures of the bacteria inhabiting the surface of *Lyngbya majuscula* in marine agar resulted into some distinct domineering colonies (Plate 1, upper plates) which were observed to be yellow and light yellow upon subculturing. Treatment of the *L. majuscula* with 70% EtOH (2 min) prior to culturing in marine agar inhibited the growth of the dominant bacteria species but enhanced the growth of a brightly coloured species of bacteria (Plate 1, lower plate). Examples of individual species of these coloured bacteria can be seen in plate 2.

Experiments conducted to determine the potential for the dominant bacteria *Pseudoalteromonas* sp. to tolerate EtOH (70 %) revealed that *Pseudoalteromonas* sp. are fairly sensitive to the introduction of EtOH at the first instance. However EtOH appeared to promote the growth of this species of bacteria from 15 min onwards. 70 % ethanol is widely acknowledged to have strong sterilising activity against common bacteria worldwide.

Early experiments on inter-bacterial antagonism indicated that in the absence of exposing the cyanobacteria to EtOH (70 %), populations of the bacteria in marine agar plates were dominated by two distinct gram −ve bacteria. In trying to mimick the natural microbial environment of the bacteria on the surface of *L. majuscula*, growing of the various strains of bacteria in filter discs (6 mm) over a lawn of the dominant *Pseudoalteromonas* sp. revealed that *Micrococcus* sp. (yellow) and *Stappia* sp (pink) antagonise *Pseudoalteromonas* sp. by slowing the growth of the latter bacteria by a zone of 12 mm to 19 mm and of 6 mm to 8 mm respectively. All the other bacteria sp. did not exhibit this activity.

The dominant bacteria isolate TDLD1 whose gram stain was gram −ve rods by microscopy, was identified by 16S rDNA studies to belong to the sulfur-oxidizing symbiont relatives of γ-*Proteobacteria*. *Pseudoalteromonas* sp are commonly associated with sea water and are known to synthesise biologically active metabolites displaying anti-bacterial, bacteriolytic, algicidal and some toxins (Holmstrom and Kjelleberg, 1999). We suggest that *Pseudoalteromonas* sp. is likely to form a greater

part of the external mucilage of *L. majuscula*. However, it may be the hard to find minor epiphytic bacteria taxon that are likely to define the microbial world of *L. majuscula*. The red TDLR gram +ve rods bacteria isolate was identified by full 16S rDNA sequence to match the description of *Norcadia corynebacterioides*. *Norcadia corynebacterioides* belongs to the genus *Norcadiopsis*. Prior to the 16S rDNA identification techniques, the genus *Norcadiopsis* was defined on the basis of chemotaxonomic markers (Rainey *et al*, 1996) with phospholipids type III and the phospholipids phosphatidylcholine and phosphatidylmethylethanolamine as salient chemotaxonomic features (Kroppenstedt 1992; Lechevalier *et al*, 1977). *Norcadia corynebacterioides* has previously been reported as unpublished reference (Venkateswaran, 2002) vide strain SAFR-015 with accession number AY167850.1 gi:27497670 in the MWG 16S rDNA data-bank. Partial sequence of the 16S rDNA gene of the brightly coloured orange bacteria which microscopy identified as consisting of gram –ve cocci of short rods matched that of *Paracoccus marcusii*. The species *P. marcusii* belongs to the genus *Paracoccus* that is in the α-3 subclass of the *Proteobacteria*. The genus *Paracoccus* is acknowledged to possess substantial metabolic versatility by growing both aerobically and anaerobically on a wide range of compounds (Harker *et al*., 1998). The yellow Gram- +ve strain TDLY was identified to belong to the genus *Micrococcus* by partial sequence of the 16S rDNA gene. *Micrococcus luteus* was found to be the closest relative of the *Micrococcus* strain. The genus *Stappia* to which the pink coloured TDLP strain was classified belongs to the rather ecologically important carboxydotrophic bacteria. Carboxydotrophic bacteria are within the α-*Proteobacteria* and the *Firmicutes*, which utilise carbon monoxide as the ultimate carbon and energy source whilst expressing relatively low affinity carbon monoxide. It has been suggested that the global carbon monoxide budget in the oceans is likely to be mediated by the presence of this genus of bacteria as well as the recent discovery of the actinomycetes in the deeper hot ocean vents (Feling *et al*., 2003). Soil microbes especially actinomycetes, fungi and bacteria are already known to remove up to 15 % of the annual carbon monoxide flux to the atmosphere. The white/milky endospore forming gram +ve bacteria as established by the gram stain methods was confirmed to belong to the genus Bacillus sp. BacB3. through partial sequence of the 16S rDNA gene. Similar Bacillus sp. BacB3 have been reported from the rhizospheres of transgenic potatoes expressing the lytic peptide cecropin B (Sessitsch *et al*., 2003). We were unable to characterise the 16S rDNA of TDLD2, TDLD3 and TDLDY.

1.3.4 DISCUSSION

The chemistry of the circumtropic marine cyanobacterium *Lyngbya majuscula* is replete with a whole diverse range of linear lipopetides as well as cyclic peptides and macrolides. However, there is little mention on whether or not a majority of these compounds truly originate from the cyanobacterium or are a product of symbiotic relationships between the cyanobacterium and the epiphytic bacteria that are associated with it. Gerwick and co-workers (Gerwick *et al*, 2001) have studied for example the biosynthesis of barbamide, the antimolluscicidal lipopeptide isolated from a Curacao collection of *L. majuscula*. This however is an isolated case. With an exception of the thesis publication by Gil-Turnes (Gil-Turnes , ibid) which mentions the isolation of four brightly coloured epiphytic bacteria inhabiting *L. majuscula*, literature on the symbiotic relationship of *Lyngbya majuscula* is quite minimal. Orjala *et al* (ibid) was able to establish that indeed the four brightly coloured bacteria isolated from the Curacao *L. majuscula* may have a symbiotic relationship with the

cyanobacterium by isolating quinoline compounds from the bacteria which had earlier been isolated from cyanobacterium. It is most puzzling that EtOH(70%) was a discriminating factor in the isolation of the brightly coloured epiphytic marine bacteria from *L. majuscula* in this study because EtOH(70%) is widely acknowledged to be an effective sterilising agent. It is most likely that in the first instance that the *Pseudoalteromonas* are curtailed from proliferating in the first 15 minutes, they are undergoing some lysis. Representatives of *Pseudoalteromonas* are commonly found in oceanic waters (Hentschel *et al*, 2001) and therefore it may not have been surprising to have it associated with *L. majuscula*. This dominating group of γ-*Proteobacteria* has been known to synthesise compounds possessing antimicrobial properties (Holmstrom and Kjelleberg, ibid) and is therefore likely to play a protective role as it appears on the mucilage of the cyanobacterium. It has also been shown that aquatic microorganisms are sometimes unable to elicit antimicrobial properties in conventional laboratory shake cultures as the conditions may not exactly mimick the natural marine environment (Yan *et al*, 2003). The lack of antimicrobial activity by the spent media of *Micrococcus luteus* and *Stappia* sp., which exhibited antagonism against the dominant *Pseudoalteromonas* may therefore be as a result of the lack of coherence of the laboratory conditions and those in the field. The genus *Micrococcus* has close resemblance to *Arthrobacter*, which are organisms that have primarily been isolated from the soil. *Arthrobacter* are hardly known to produce any bioactive compounds (Wieser *et al.*, 2002). The complimentary role between the production of antibiotics and secondary metabolites by epiphytic symbiotic bacteria demands that *Micrococcus* cannot mediate for secondary metabolite production. In this study, *M. luteus* was found to antagonise the dominant *Pseudoalteromonas* sp. We postulate here that this antagonism of *Pseudoalteromonas* sp by *M. luteus* is largely due to antibiosis and not due to secondary metabolite production. Although the tiny bacteria TDLD2 and the extremely tiny TDLD3 albeit shiny bacteria were observable on the marine agar plates, they were only associated with the dominant bacteria TDLD1. It is highly probable that these two tiny bacteria require special growth media if their DNA were to be isolated fully. This could be supported by the fact that the PCR product of both TDLD2 and TDLD3 was always weak.

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Plate1. Normal bacteria growth (top two plates) and bacteria growth on treatment of *L. majuscula* with 70% EtOH (2min,bottom).
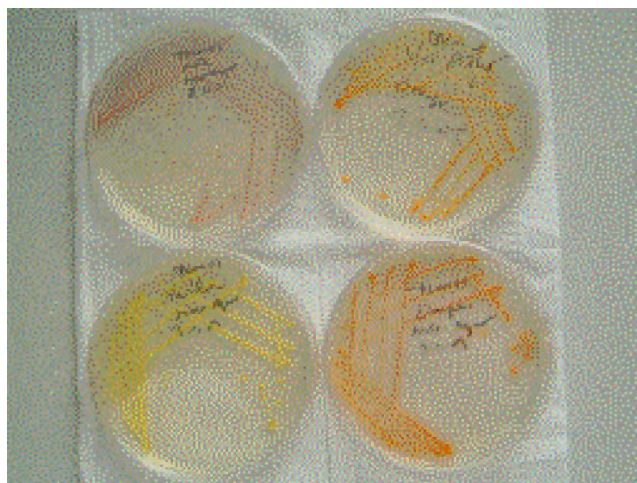

Plate 2A. White (milky) bacteria

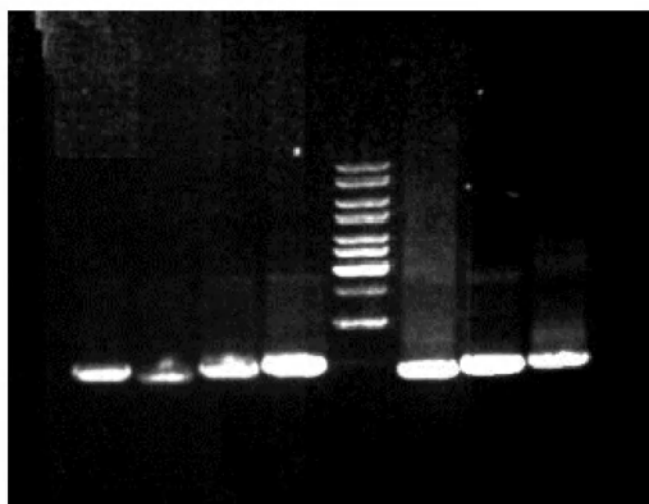Plate 2B. Red, Orange and yellow bacteria

Plate 3. PCR product of the epiphytic bacteria isolates.
Norcadia cornyebacteriodes< Paracoccus < Micrococcus
< Bacillus sp. BacB3< Marker< Stappia < Pseudoalteromonas
< unidentified (Left to right)

Table: Colouring and gram stain of the bacteria isolated from the surface of
L. majuscula as well as the efficacy of the growth media.

	Description	Gram Stain	Media Preference	Approximate No. Colonies
TDLR *Norcadia cornyebacter-iodes*	Red	Gram +ve rods		Nil
TDLO *Paracoccus macussii*	Orange	Endospore forming Gram -ve bacilli	NA≤MA	Nil
TDLY *Micrococcus*	Yellow	Gram +ve cocci	NA<MA	1×10^4
TDLW *Bacillus sp. BacB3*	White/Milky	Endospore forming Gram +ve	MA<NA<CA	Nil
TDLP *Stappia*	Pink	Gram -ve rods	CA<NA<MA	Nil
TDLD1 *Pseudoaltero monas*	Cream	Gram -ve rods	MA (ideal)	$8.0 - 18 \ (\times 10^5)$
TDLD2 *unidentified*	Cream and tiny	Gram -ve rods	Nd*	$7.5- 9.3 \ (\times 10^5)$
TDLD3 *unidentified*	White/Misty, extremely tiny	Gram +ve cocci	Nd*	Nil
TDLDY *unidentified*	Deep yellow	Gram -ve	MA ideal	Nil

Pseudoalteromonas sp. tolerance to 70 % EtOH
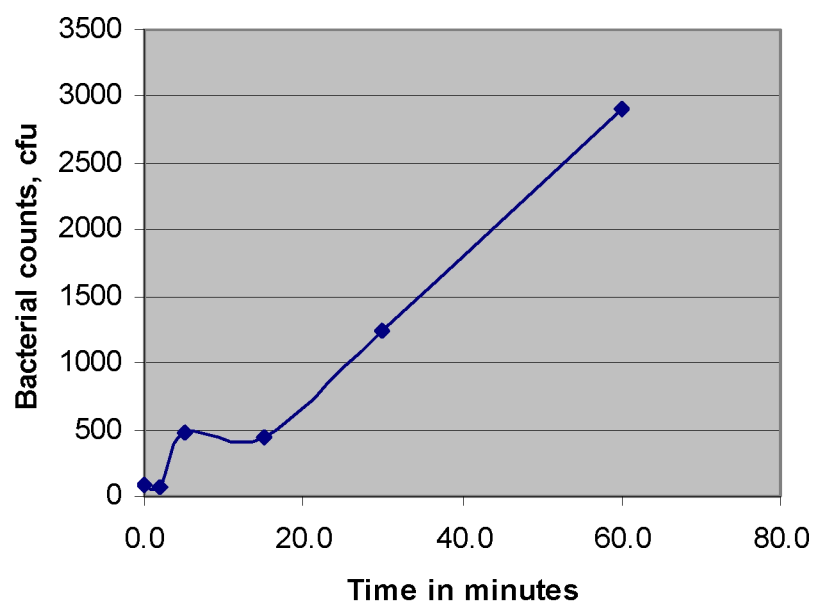
PROJECT 2:

2.0 WHAT REALLY ARE THE COMPOUNDS RESPONSIBLE FOR CELL-CELL SIGNALLING IN BACTERIA BIOFILMS?

2.1 INTRODUCTION

Microorganisms exist predominantly in sessile communities rather than as free–living planktonic cells. In these sessile communities, bacteria organise themselves in micro-colonies that could otherwise be quite complex. Biofilms are therefore the macromolecular matrix of differentiated surface localised communities into which individual bacteria cells are embedded. Biofilms have a profound role in the pathogenesis of many infectious diseases as well as in biofouling of the marine biota in the aquatic environment. Previously, it was thought that microbial life was anything but individual organisms single-mindedly pursuing growth by cell division (Dunny, 2002). However, it has become increasingly clear that the formation of biofilms, virulence, genetic exchange as well as antibiotic production result from well coordinated multicellular processes controlled by cellular communication (cell-cell signalling). Amongst the gram negative bacteria such as *Pseudomonas*, *Agrobacterium* and *Vibrio, acylhomoserine* lactones (AHLs) have been shown to be responsible for the cell-density dependent behaviour (quorum sensing). Quorum sensing is not restricted to the gram negative bacteria, it is a phenomenon which has also been observed in the build up of cell densities of gram positive bacteria such as *Bacilli, Streptococci, Enterococci*, etc. However, unlike in the former type of bacteria where AHLs are involved, the signal molecules responsible for quorum sensing in gram positive bacteria are peptides of about 5-20 amino acids in length (Fuqua, 1994; Dunny, 1997). However, the real nature of the inducer compounds responsible for cell-cell signaling in bacteria biofilms is far from being understood.

The conventional shaky flask culture media approach has been found wanting in the cultivation of marine bacteria which can elicit for antimicrobial activity just in much the same manner as it happens in the natural marine environment. Infact, most aquatic marine bacteria isolates stop producing antimicrobial compounds when cultivated in shaky flasks as opposed to the air membrane technique (AMS) (Yan, 2003). Cultivation of marine bacteria isolates using the AMS technique is therefore one approach by which the concept of cell-cell signalling could be understood. An understanding of cell-cell signalling and the manner in which biofilms could be disrupted would have enormous implications in both environmental and medical microbiological studies. Recent studies have established that certain marine bacteria such as *Bacilli* are capable of disrupting biofilms of the *Micrococcus* sp. in response to competition (Burgess, unpublished).

The aim of this project was therefore to establish the nature of the compounds responsible for biofilm formation and to understand the cell-cell signalling phenomenon. Cultures of the marine *Bacillus licheniformis* strain EI-34-6 were used. The following strategy was adopted;

1. Extraction of the biofilm signalling molecules from *B. licheniformis* strain EI-34-6 biofilms followed by discrimination between lipophilic and hydrophilic components of the biofilms attained by partitioning with water.

2. Separation of the fairly polar compounds according to their molecular sizes using sephadex-LH 20 prior to further purification with either flash Si Gel chromatography or with sepak cartridges.

2.2 METHODS

2.2.1 Culturing *Bacillus licheniformis* strain EI-34-6 in AMS
Nutrient broth (13 g) was dissolved in MeOH (200 mL), shaken thoroughly and centrifuged. The supernatant after centrifuging the contents was rotar evaporated at 35 °C. The residue was thereafter dissolved in and made to 500 mL with distilled H_2O. 1% glycerol (10mL of 1:1 glycerol/H_2O) was added followed by 100mM $MnCl_2$ (1 mL) prior to autoclaving. *Bacillus licheniformis* EI-34-6 bacteria (100 µL) was cultured in the form of a swarb on the surface of a sterile semipermeable membrane disc (47mm; pore size, 0.2µm; Whatman) that was in contact with air. The EI-34-6 had been previously grown in a liquid suspension in marine broth at 28 °C for 4 days. The membrane discs were resting on tops filled with the nutrient broth media formulation. The inoculated membrane as it rested on the tops was placed in a deep petri dish (diameter, 55 mm; height, 18 mm; Bibby-Sterilin) to maintain sterility and incubated at 28 °C for 7 days. The biofilms were harvested after 7 days (by evaporation of the spent media) and the antimicrobial activity of the liquid beneath the membrane investigated. The production of bacitracin, which is a red pigment typical of inducer compounds in *B. licheniformis* EI 34-6 biofilms, was also determined.

2.2.2 Isolating the signalling compounds from *Bacillus licheniformis* biofilms.
Bacillus licheniformis EI 34-6 was cultured using AMS bioreactor at 28 °C using methanolic nutrient (see details above). The spent media of the AMS cultured *Bacillus licheniformis* EI 34-6 was evaporated down and the solid dissolved in 1:1 DCM/MeOH prior to solvent partitioning with an equal volume of water. The water soluble components were concentrated down using a rotary evaporator at 35 °C. A 700 mg slurry of the concentrate in water was eluted through a sephadex LH-20 and four fractions pooled up in accordance with their TLC-chromatography similarities. Fraction 1 was made up of test tubes A1, A2,A3 and A4; Fraction 2 consisted of A5,A6,A7,A8,A9 whereas fraction 3 comprised test tubes A10,B1,B2,.....B7,B8. Fraction 4 consisted of the eluant tails. Each test tube in the four fractions contained 10 mL of the eluted material. The pooled up fractions were similarly concentrated down using a rotary evaporator. The organic dichloromethane phase was similarly investigated for the signalling components. The 30 mg of residue obtained from the dichloromethane phase was not adequate for eluting through a column.
^{1}H NMR spectra (200 MHz, D_2O) were recorded for each of fractions 1 through 4 to find out if the biofilm signalling molecules were still retained in any of the four fractions. A further ^{1}H NMR spectrum (200 MHz, $CDCl_3$) was recorded for the dichloromethane phase.

2.3 RESULTS
Fractions 2 and 3 had similar spectra to the original spectrum for whose compound bioactivity was observed. Fractions 2 and 3 showed a weak pigment elicitation effect in shake flask cultures but not antimicrobial activity after cultivation for one week. The activity of induction was low (weak red).

2.4 CONCLUSION

It is most probable that a column approach to isolating the signalling molecules may be flawed because of the very insignificant amounts of these compounds in the biofilms. Nevertheless, sequential elimination of extraneous material from the biofilms through column chromatography coupled with careful selection of appropriate solvent systems by TLC may in deed assist in the isolation process. The low magnitude of induction observed in fractions 2 and 3 mediates for chemically defined media as a suitable alternative to eliminating errors from media components.

NOTE:

This project is still ongoing in Dr. Burgess research laboratory and the results indicated here were only preliminary observations. Unfortunately, I had to leave the project prematurely in order to take up a Ph.D studentship at the Macquarie University, Australia.

REFERENCES

1. Dunny G M (2002) Microbiology: Group effort in toxin synthesis. *Nature 415, 33-34*
2. Fuqua W C, Winans S C, Greenberg E P (1994). *J. Bacteriol., 176, 269-275*
3. Dunny G M, Leonard B A B (1997) *Annu. Rev. Microbiol. 51, 527-564*
4. Yan L, Boyd K G, Adams D R, Burgess J G (2003). Biofilm-specific cross-species induction of antimicrobial compounds in *Bacilli. J. Appl. Environ. Microbiol, 69, 7, 3719-3727.*

RECOMMENDATIONS

Project 1
Further studies on the three unidentified bacteria probably by using16S rDNA and fluorescence in-situ hybridization (FISH) techniques could prove quite helpful. In any case the study of *Stappia* sp. for the sourcing of bioactive compounds and antibiotics is worthy attempting. In both these studies and also on the utilisation of *L. majuscula* epiphytic bacteria to better understand the chemistry of cell-cell signalling, the air-membrane surface technique is the right way to go. If the secondary metabolites produced by the cyanobacterium could in fact be similar to those which the epiphytic bacteria are capable of producing, then this will be a breakthrough in studying *L. majuscula* from the symbiotic point of view, an aspect that has all along been overlooked in the study of this cyanobacterium.

Project 2
I believe that a lot will come out from this research, which is currently on going at the Heriot-Watt University in Scotland. It would therefore be prejudicial for me to speculate on the nature of recommendations to make regarding this project otherwise it would be my pleasure for any interested party to get in touch with Dr. Burgess and see what possibilities of collaboration may exist.

EXPENDITURE OTHER THAN UPKEEP

1. **Collection Expenses**
    Per Diem expenses to

		KSh
1 Researcher (T.M. Dzeha) for 3 days @ Ksh. 2500	---------	7,500/=
1 Technician (S. Tunje) for 3 days @ Ksh. 1500	---------	4,500/=
1 Coxwain (J. Ayoyi) for 3 days @ Ksh. 1500	---------	4,500/=
1 Driver (P. Chiteri) for 3 days @ Ksh. 1500	---------	4,500/=

    Sub-total   22,000/=

2. **Heriot-Watt University Identity Card** @ GBP 50 ----------7,100/=
3. **Travel cancellation KLM** @ USD 100------------------------ 7,900/=
4. **Mombasa – Nairobi – London**   42,000/=
5. **Travel London-Edinburgh** @GBP 88   12,320/=

Total US$ 1170   **or**   **KSh. 91,320/=**

NB.
1. Bench fees (USD 300)— These fees were waived for me by Dr. J. Grant Burgess after realizing that the grant was not going to take me through the whole period of my research stay in Edinburgh.
2. After my research stay on December 23, I still had to get back to Heriot-Watt University to get the 16S rDNA results, accessible only through a secret code.

ABOUT SCOTLAND AND HERIOT-WATT UNIVERSITY

Scotland, England, Wales and Northern Ireland together form what is called the United Kingdom or Great Britain. Scotland, which lies to the north of England, has a very rich history and a number of very beautiful castles. The most important of these castles is undoubtedly the Edinburgh castle, which is a big landmark of the city of Edinburgh. The queen of England has a home in Edinburgh in what is known as the Holyrood palace, which occasionally serves as her official residence. Scotland has a beautiful mountaneous terrain in the North-West and has quite a number of islands in the North and West Coasts, features which attract thousands of tourists yearly from all over the world. It is also not complete to talk about Scotland if one did not mention scientific discoveries that have been associated with the Scottish. Examples of these include the discovery of helium gas by Sir William Ramsay in 1895, that of the telephone by Graham Bell (1876), the discoveries of penicillin and television by Sir Alexander Fleming (1926) and by John Logi Baird (1923) respectively and even more recently the discovery of animal cloning by Keith Kampbell and Ian Wilmut in1995. Scotland to me is therefore a very nice place and a home of science for me to be and certainly I enjoyed my stay there.

Heriot-Watt University was incorporated by Royal Charter in 1966. Its origins date back to the Edinburgh School of Arts and Mechanics Institute (1821), which in 1854 became the Watt Institution and School of Arts, named after James Watt (1736 - 1819). Heriot-Watt College was established in 1885 when the Institute amalgamated with the endowments of George Heriot's Hospital (C 17th). The University has a modern outlook to research and is not conservative. The University has a lot of facilities required for research and boasts strong collaborations with the industry such as pharmaceutical companies. The facilities at the Life Sciences department together with those that it shares with the college of Engineering and Physical Sciences cater for modern cutting edge research. My research experience at the Heriot-Watt University therefore suggests that a strong collaboration with this University could give a facelift to our research in marine sciences in the region and there are all the possibilities. Dr. J. Grant Burgess who offered me the fellowship has very close ties with the Scottish Association for Marine Sciences situated at the Dunstaffnage in the West coast of Scotland and I believe that he will be more than willing to work with serious scientists from the Western Indian Ocean (WIO) region.