Detection and Expression of Biosynthetic Genes in Actinobacteria

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George Bervanakis

DECLARATIONS

I certify that this Masters thesis entitled “Detection and Expression of Biosynthetic Genes in Actinobacteria” constitutes my own work and has been carried out by myself, unless stated otherwise. The thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief that it does not contain any material previously published or written by another person except where due reference is made in the text. The work described in this thesis was performed by myself while I was enrolled as a Masters of Science student at Flinders University of South Australia, (Biotechnology, School of Medicine), Department of Microbiology and Infectious Diseases, Faculty of Health Sciences.

Signed: _____

Date: _____

Abstract

Most microbial organic molecules are secondary metabolites which consist of diverse chemical structures and a range of biological activities. Actinobacteria form a large group of Eubacteria that are prolific producers of these metabolites. The recurrence of pathogens resistant to antibiotics and a wider use of these metabolites apart from their use as anti-infectives, has been the impetus for pharmaceutical companies to search for compounds produced by rare and existing actinobacterial cultures.

Accessing microbial biosynthetic pathway diversity has been possible through the use of sensitive and innovative molecular detection methodologies. The present study evaluated the use of molecular based screening as a rational approach to detect secondary metabolite biosynthetic genes (SMBG) in uncharacterised natural Actinobacterial populations. A polymerase chain reaction (PCR) approach was selected for ease of application and high sample processivity. Rational designed screening approaches using PCR in the discovery of SMBG, involved identifying common functions in secondary metabolite biosynthetic pathways, such as condensation reactions in polyketide synthesis, genes encoding these functions, and using conserved regions of these genes as templates for the design of primers to detect similar sequences in uncharacterised actinobacteria. Design of primers involved rigorous *in silico* analysis followed by experimentation and validation.

PCR screening was applied to 22 uncharacterised environmental isolates, eight of these displayed the presence of the ketosynthase (*KS*) gene belonging to the type I polyketide synthases and eight contained the ketosynthase (KS_α) gene belonging to the type II polyketide synthases, six of the isolates contained the presence of a presumptive dTDP-glucose synthase (*strD*) gene which is involved in the formation of deoxysugar components of aminoglycoside antibiotics and one isolate contained the presence of a presumptive isopenicillin N synthase (*pcbC*) gene involved in beta-lactam synthesis. Alignments of partially sequenced PCR products from isolates A1488 and A3023 obtained using type II PKS primers showed close similarities with KS_α genes from antibiotic producing actinobacteria. Similarly, alignments of

sequences from isolates A1113 and A0350 showed regions of similarities to *KS* genes from antibiotic producing actinobacteria.

Fermentation techniques were used for inducing expression of secondary metabolites from the uncharacterised actinobacteria isolates. By using antimicrobial guided screening it was determined that most of the isolates possessed the capacity to produce antimicrobial metabolites. Dominant antagonistic activity was detected against Gram positive bacteria and to a minor extent against fungi. Optimal fermentation liquid media were identified for certain isolates for the production of antimicrobial metabolites. Two alternative fermentation methods; solid-state and liquid-oil fermentations were evaluated to improve secondary metabolite production in the uncharacterised isolates. Solid-substrate fermentation showed that it could induce a complex metabolite pattern by TLC analysis, however this pattern varied according to the substrate being used. Liquid media supplemented with refined oils, showed a positive response indicated by higher antibacterial activities detected.

Evaluation of semi-purified organic extracts identified two isolates A1113 and A0350 producing similar antimicrobial metabolites as detected by HPLC/UV/MS, a literature database search of similar compounds containing the same molecular weight identified the compound as belonging to the actinomycin group of compounds. A complex metabolic pattern was identified for isolate A2381, database searching identified some of the compounds as having similar molecular weights to actinopyrones, trichostatins, antibiotics PI 220, WP 3688-5 and YL 01869P.

Drug discovery screening can serve to benefit from PCR detection of biochemical genotypes in initial screens, providing a rapid approach in identifying secondary metabolite producing capabilities of microorganisms prior to the commencement of costly and time consuming fermentation studies. Additionally the identification of biochemical genotypes allows a directed approach in using fermentation media designed to induce biosynthetic pathways of specific classes of compounds.

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List of Abbreviations

AA	amino acid
ACP	acyl carrier protein
ACV	δ-(L-a-aminoadipyl)-L-cysteinyl-D-valine
ARS	Agriculture Research Service
AT	acyl transferase
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
AU	absorbance units
BA	bioassay agar
BDH	British Drug House
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSN	broth supernatant
C	carbon
CLF	chain length factor
cm	centimeters
CoA	coenzyme A
CU	codon usage
Da	Dalton
DDBJ	DNA Database of Japan
DEBS	deoxyerythronolide B synthase
DEX	dextrin
DNA	deoxyribonucleic acid
6DOHS	6-deoxysugars
DSM	German Culture Collection
EDTA	ethylenediamine tetra-acetic acid
EMBL	European Molecular Biology Laboratory
EMS	electrospray mass spectrometry
ESI	electrospray ionisation source
EtAc	ethyl acetate
EtOH	ethanol
g	gram
gDNA	genomic deoxyribonucleic acid
h	hour
IPNS	isopenicillin N synthase
kb	kilobase
KR	ketoreductase
KSα	ketosynthase alpha
LC-MS	liquid chromatography-mass spectrometry
LSA	linseed, safflower, and almond crushed seeds
ME	mycelial extracts
MeOH	methanol
min	minutes
mg	milligram
mAU	milli-absorbance units
ml	millilitres

µl	microlitres
µM	micromolar
MS	mass spectrometry
MSA	multiple sequence alignment
mM	milli molar
m/z	mass-to-charge ratio
nm	nanometer
NA	nucleic acid
NRRL	Northern Regional Research Laboratories Culture Collection
^{o}C	degrees Celsius
OLV	olive Oil
ORF	open reading frame
pcbAB	δ-(L-a-aminoadipyl)-L-cysteinyl-D-valine synthase
pcbC	isopenicillin N synthase
PCR	polymerase chain reaction
PDA	Photo Diode Array
PK	Polyketide
PKS	Polyketide Synthatase
R_f	relative mobility
rpm	revolutions per minute
RP-HPLC	reverse-phase high performance liquid chromatography
rRNA	ribosomal ribonucleic acid
σ	sigma
SAF	safflower oil
SBM	soya bean meal
SDS	sodiumdodecylsulphate
SI	sucrose inorganic salts medium
SM	secondary metabolites
SMBG	secondary metabolite biosynthetic genes
SmF	submerged fermentation
SOY	soya oil
SSF	solid-substrate fermentation
spp.	species
SUN	sunflower oil
TLC	thin-layer chromatography
TBE	tris-borate buffer
T_m	melting point temperature
TSB	Tryptic Soy Broth
UV-Vis	Ultra-Violet Visible
VO	vegetable Oil
v/v	volume per volume
WAL	walnut oil
w/v	weight per volume
YME	Yeast-Malt Extract Agar

Aims of the Project

1) Identify biochemical genotypes in a range of actinobacteria cultures from different environments, which are related to the productivity of secondary metabolites.

2) Induce the expression of secondary metabolites from actinobacteria, with an emphasis on genera that are poor producers.

Experimental Objectives

1) To develop and implement PCR screening methodologies for the detection of biosynthetic genes in actinobacteria.

2) To formulate a fermentation media that exhibits broad range applicability for the production of secondary metabolites.

Null Hypothesis

1) The presence of biosynthetic genes in an actinobacterial strain indicates the presence of a biosynthetic pathway and that the organism may have the capability to produce a specific secondary metabolite under defined fermentation conditions.

2) The ability of an actinobacterial strain to produce secondary metabolites is not influenced by the isolation source.