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INVESTIGATIONS OF AN ANTIFUNGAL ANTIBIOTIC PRODUCED BY AN ENVIRONMENTAL ISOLATE OF *PAENIBACILLUS POLYMYXA*

by

PERRIN HUDSON BEATTY 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

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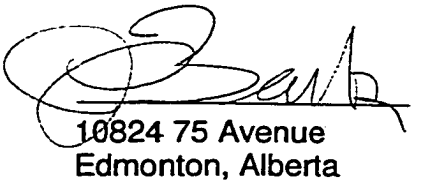

10824 75 Avenue
Edmonton, Alberta
Canada, T6E 1K2

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University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Investigations of an Antifungal Antibiotic Produced by an Environmental Isolate of *Paenibacillus polymyxa* submitted by Perrin Hudson Beatty in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology and Biotechnology.

Dr. S.E. Jensen, Supervisor

Dr. G.M. Gaucher

Dr. A.G. Good

Dr. P.D. Kharbanda

Dr. K.L. Roy

Dr. J.C. Vederas

November 29, 1999

I dedicate this thesis

to

my Mother and Father

Thank you

ABSTRACT

Canola is an economically important crop grown in Canada. One of the major diseases of canola is caused by *Leptosphaeria maculans*. A biological control of *L. maculans* was sought and found as an environmental bacteria isolated from canola stubble.

The environmental isolate was determined to be *Paenibacillus polymyxa* from biochemical and growth characteristics, fatty acid methyl ester (FAME) analysis and 16S rRNA sequence similarity. The isolate was given the stain designation PKB1. *P. polymyxa* PKB1 produces antifungal material around the onset of sporulation. The medium for optimal production of the antifungal material from *P. polymyxa* PKB1 was potato dextrose broth (PDB).

The antifungal material was extracted from *P. polymyxa* PKB1 cell/spore culture by: methanol extraction from pelleted biomass or n-butanol extraction from acidified, heated culture. The crude extracts were purified using size exclusion and reverse phase chromatography. The pure antifungal material was characterized to be a mixture of cyclic depsipeptides of molecular weights 883, 897, 948 and 961 Da, with the sequence of the 897 Da component as cyclic(thr-val-val-X-glx-ala) with an ester bond between thr and ala and a 15-guanidino-3-hydroxypentadecanoic acid moiety bound to the N-terminal amino group of thr. These compounds are similar to the cyclic depsipeptides fusaricidin A, B, C and D.

DNA fragments, corresponding to portions of peptide synthetase (PS) modules were amplified from the *P. polymyxa* PKB1 genome. Five of nine different fragments of the expected size were PS encoding fragments due to their similarity to PS enzymes. Three of these fragments were used as probes of a LambdaGEM®-11 *P. polymyxa* PKB1 genomic library to identify recombinant phage carrying PS encoding DNA. The

insert from one of the positive phage was digested with restriction enzymes, subcloned and sequenced, and determined to be similar to PS enzymes. One of the subcloned DNA fragments from the recombinant phage was incorporated into a *Bacillus* integrative vector and introduced into *P. polymyxa* PKB1 to disrupt production of the antifungal material. The transformants were able to inhibit *L. maculans* still, however integration of the recombinant *Bacillus* vector into the *P. polymyxa* PKB1 genome was successful.

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My family has always believed in my abilities and encouraged me to succeed. Thank you everyone for the summertime reunions, excursions to Maui, friendship and love. I thank Ned with all my heart for his commitment and unconditional support of me.

Although I am happy to be finished my formal education, I will always strive to learn and experience new things. I want to encourage my nieces and nephews to continue their educations and fulfill their dreams and aspirations as I did.

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LIST OF ABBREVIATIONS

A	Alanine
aa	amino acid
aaa	amino acid activating
ala	Alanine
allothr	Allothreonine
amp	ampicillin
ARC	Alberta Research Council
arg	Arginine
asn	Asparagine
asp	Aspartic acid
asx	Aspartic acid or asparagine
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BGSC	Bacillus Genetic Stock Centre
BHI	Beef heart infusion
BHI	Brain Heart Infusion
BLAST	Basic local alignment search tool
BLASTn	Nucleotide similarity search using BLAST
BLASTp	Amino acid similarity search using BLAST
bp	Base pair
BSA	Bovine serum albumin
C	Cysteine
CAM	chloramphenicol
CAT	Chloramphenicol acetyl transferease
dCTP	deoxycytosine triphosphate
D	Aspartic acid
Da	Dalton
DAB	2,4-diaminobutyric acid
DNA	deoxynucleoside nucleic acid
dNTP	deoxynucleotide triphosphates
DTT	Dithiothreitol
E	Glutamic acid
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
F	Phenylalanine
FAB	Fast atom bombardment
FAME	Fatty acid methyl ester
FDP	Forward degenerate primer
G	Glycine
GB	Glucose broth
GCG	Genetics Computer Group
GHPD	15-guanidino-3-hydroxypentadecanoic acid
gln	Glutamine
glu	Glutamic acid
glx	Glutamic acid or glutamine
h	Hour
H	Histidine
his	Histidine
HPLC	High performance liquid chromatography

I	Isoleucine
IPTG	Isopropyl-β-D-thiogalactopyranose
K	Lysine
kb	kilobase
KDa	Kilodalton
KL	Katznelson and Lochhead media
KLM1	Katznelson-Lochhead modified No. 2
λ	lambda
LB	Luria-Bertani
M	Methionine
MALDI	Matrix assisted laser desorption/ionization
MDa	MegaDalton
ms	mobile solvent system
MS	Modified Stansly's media
MS	Mass spectrometry
MS1	Modified Stansly's medium No. 1
MS2	Modified Stansly's medium No. 2
MS3	Modified Stansly's medium No. 3
MS4	Modified Stansly's medium No. 4
MS5	Modified Stansly's medium No. 5
MS/MS	Tandem mass spectrometry
mu	mass unit
MW	Molecular weight
MYM	Maltose-yeast extract-malt extract
m/z	mass per charge
N	Asparagine
NB	Nutrient Broth
NMR	Nuclear magnetic resonance
nt	Nucleotide
OD	Optical density
P	Proline
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PDB2	Potato dextrose broth modified No. 2
PDB3	Potato dextrose broth modified No. 3
PDB4	Potato dextrose broth modified No. 4
PDB5	Potato dextrose broth modified No. 5
PDB6	Potato dextrose broth modified No. 6
PEG	Polyethylene glycol
PS	Peptide synthetase
PSD	Post source decay
Q	Glutamine
R	Arginine
RDP	Reverse degenerate primer
RNA	Ribonucleic acid
rp	recombinant phage
RP-HPLC	Reverse phase high performance liquid chromatography
rpm	revolutions per minute
S	Serine

SDS	Sodium dodecyl sulfate
T	Threonine
TEMED	N,N,N', N'-Tetramethylethylenediamine
TCSS	Trypticase soy with starch media
TCSS	Trypticase soy-starch
TE	Tris EDTA buffer
TES	N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid
TES	Tris, EDTA buffer
TFA	Trifluoroacetic acid
thr	Threonine
TOF	Time of flight
Tris	Tris(hydroxymethyl)-aminomethane
V	Valine
val	Valine
v/v	volume per volume
W	Tryptophan
w/v	weight per volume
X	Unknown amino acid
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
Y	Tyrosine
YE	Yeast extract
dITP	deoxyinosine triphosphate
RE	Restriction endonuclease
ZOI	Zone of inhibition

A. Introduction

A.1 Canola

Canola is an economically important crop grown in Canada, Europe, New Zealand, China, India, eastern Africa and Australia. It is an oilseed plant of the family Crucifereae and the genus *Brassica*, and is grown for the production of oil for human consumption and meal for livestock consumption. There are four species of *Brassica* grown in different parts of the world as a source of edible vegetable oil; these are: biennial or winter forms of *B. napus* grown in Europe and China, summer annual forms of *B. napus* and *B. rapa* grown in Canada, *B. juncea* grown in India and *B. carinata* grown in eastern Africa (Rimmer *et al.* 1992). For the oil and meal derived from the plant to be graded as canola oil and meal rather than rapeseed oil and meal, the product must be low in glucosinolates (expressed as 3 mg 3-butenyl isothiocyanate/g of canola meal) and erucic acid (< 5%) (Vaisey-Genser and Eskin, 1982). The plants that produce canola grade oil are called canola plants rather than rapeseed plants. *B. napus* (summer canola), *B. campestris* (summer turnip canola) and *B. rapa* are generally low in both glucosinolates and erucic acid, whereas *B. juncea* and *B. carinata* are high in erucic acid levels. A close genetic relationship exists between these species, which has enabled plant breeders to cross hybridize useful characteristics of one species to another (Rimmer and van den Berg 1992).

Worldwide consumption of vegetable oils has been increasing every year by approximately 4% more than the world consumption of animal and marine oils; in 1996 canola represented 14% of the world's oil crops (Kindt, 1996). Canada has been a major exporter of canola grade oils since the 1980's, when Canadian rapeseed growers switched to canola grade *B. napus* and *B. campestris* cultivars. This switch to *Brassica* varieties that produce seed oil low in erucic acid was in response to the United Nations Food and Agricultural Organizations' recommendation that oil for human consumption be low in erucic acid, based on results of animal studies (Vaisey-Genser and Eskin, 1982). Rats fed a high erucic acid diet (> 6% of dietary calories) developed cardiac lipidosis which, upon further feeding with erucic acid, developed into focal necrotic lesions within the heart muscle (Vaisey-Genser and Eskin, 1982). In humans, erucic acid appears to be well digested, but may be related to a reduction in the blood platelet count. However, food scientists have also found that the relatively small amount of erucic acid present in canola oil may be beneficial because it inhibits lipoxygenase activity. Lipoxygenase

oxidizes unsaturated fatty acids (such as linolenic acid), which causes oil to have a rancid smell and flavour (Vaisey-Genser and Eskin, 1982).

Glucosinolates are secondary metabolites produced by cruciferous plants that have bioactivity against plant antagonists (Pedras and Seguin-Swartz, 1992). These compounds are not toxic to animals until they are hydrolysed and form isothiocyanates, oxazolidinethiones or cyano compounds. Hydrolysis of glucosinolates is catalysed by myrosinase, an enzyme in canola meal. Heat treated canola meal does not have any of the toxic by-products of glucosinolate hydrolysis because the enzyme is denatured. However, the growing of canola cultivars low in glucosinolates is suggested to reduce the risk of toxicity, as well as the practise of heat treating canola meal before using it as livestock feed (Josefsson and Uppstrom, 1976).

Considerable losses in canola seed quality and yield are seen every year due to fungal diseases such as blackleg, caused by the fungus *Leptosphaeria maculans*, as well as other disease-causing fungi including *Sclerotinia sclerotiorum, Rhizoctonia solani, Fusarium avenaceum* and *Alternaria brassicae,* and the fungus *Marasmius oreades* . Blackleg is considered an endemic disease of *B. napus* and *B. rapa* in most of the regions of the world where these crops are grown. It has been a serious disease of rapeseed and canola plants in Europe since 1950, and the disease virtually closed the rapeseed and canola industry in western Australia in 1972. Blackleg was found on spring canola in Saskatchewan for the first time in 1975, and has since spread to Alberta and Manitoba (Gugel and Petrie, 1992). In Canada, the economic losses due to blackleg can exceed 300 million dollars annually (Kharbanda, ARC, Vegreville, AB). Alberta has listed *L. maculans* as a provincial pest due to the economic losses (1%) that the farmers in these provinces have suffered since blackleg was introduced to these areas (Rimmer, 1992).

A.2 *Leptosphaeria maculans*: description and life cycle

Leptosphaeria maculans (Desm.) Ces. & De Not. (conidial or asexual state=*Phoma lingam* [Tode: Fr.] Desmas.) is an ascomycetous fungus that causes the disease called blackleg, canker or dry rot in crucifers, particularily in all *Brassica* species, but mainly in *B. oleracea*, *B. rapa* and *B. napobrassica* (Figure A.2.A; Commonwealth Agricultural Bureau, 1972). The fungus is teleomorphic and so can produce pycnidiospores asexually (within pycnidia) or ascospores sexually (within pseudothecia

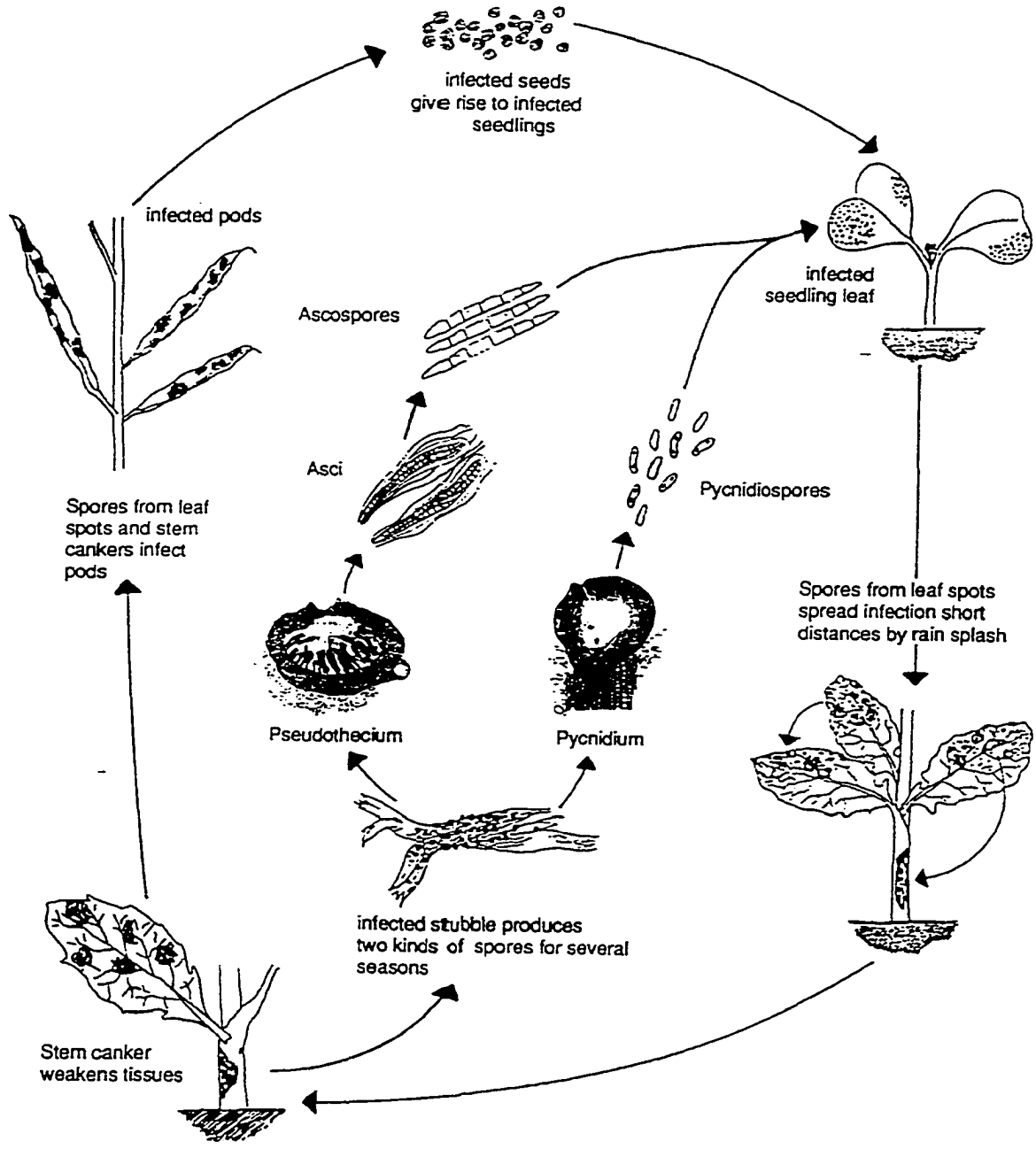


Figure A.2.A. Disease cycle of *Leptosphaeria maculans* on canola. Obtained from P. Kharbanda, Alberta Environmental Centre, Vegreville, Alberta.

or ascocarps). Ascospores will only form when two distinct strains of *L. maculans* are present on the same plant. These ascospores can be transmitted as an aerosol, travelling from 2 to 8 km in a wind, especially after a light rain or heavy dew and in temperatures between 8 and 12°C (Hall, 1992). The peak times for wind-borne spread generally coincide with the crop's most vulnerable stage of growth; the emerging seedling and young plant stage (Hall, 1992). Ascospores can survive on canola stubble left in the fields, in pseudothecia or as free ascospores. A field infested with *L. maculans* will remain contaminated until the plant stubble rots away, a process that takes approximately 4 y. Ascospores present on the canola stubble are a primary inoculum of the disease on emerging seedlings (Kharbanda, 1992); growth of infected seeds is also a primary inoculum of the disease (Hall, 1992). Pycnidiospores are not air-borne; instead they infect canola plants by being splashed onto the leaves, from soil or from a previously infected plant, during rainfall. This type of disease transmission is known as the secondary inoculum (Gugel and Petrie, 1992).

The fungus infects the canola plant through stomates or wounds in the symptomless biotrophic (early) stage, allowing its hyphae to grow intercellularly throughout the plant tissue. After this phase the fungus induces plant cell death and uses the degraded plant cell components as nutrients to produce more pycnidia (Williams, 1992). The production of pycnidiospores is also stimulated on infected plants by light. The disease spreads down the plant from the leaves to the roots. The entire plant can become affected by the disease; young plants and plants with low levels of glucosinolates and erucic acid are the most susceptible. Pycnidiospores form black lesions on all infected areas and infected stems can display girdling fungal cankers and transverse splits along the stem (Figure A.2.B). These cankers can cause premature ripening of the plant due to a lessening or restriction of nutrient and moisture flow along the stem, leading to a loss in seed quality such as shrivelled seeds and shattered seed pods. If the seed pod is infected, it will produce infected viable seeds. If planted, these can spread the disease to new geographical areas (Saskatchewan Agriculture and Food, 1996). A severe *L. maculans* infection on the stem or roots can also cause the plant to wilt or topple over.

Different isolates of *L. maculans* exhibit varying levels of virulence. Two basic groups of *L. maculans* have been identified. A virulent or aggressive group and a nonvirulent or nonaggressive group (McGee and Petrie, 1978). The nonvirulent varieties of *L. maculans* can cause disease symptoms on the plant leaves and stems that appear

A

B

Figure A.2.B. Blackleg infection on a canola leaf and stem caused by *L. maculans*. A. The leaf is exhibiting lesions, the black dots are pycnidiospores. B. The stem is exhibiting girdling and splitting. The photograph of the stem is from P. Kharbanda *et al.*, 1994.

late in the growing season and cause only mild damage. The virulent types of *L. maculans* infect the canola seedlings, then continue to infect the plants early in the growing season, causing severe damage and death of the crop plants. Comparison between the two groups demonstrates that the virulent group differs from the nonvirulent group by: causing disease in *Brassica* spp., having a slower growth rate, producing more pycnidia on agar medium and producing various sirodesmins (the causal agent of blackleg of crucifers [Boudart, 1989 and Pedras and Seguin-Swartz, 1992]). The two groups also have different electrophoretic patterns, restriction fragment length polymorphism (RFLP) patterns (Koch, 1989) and random amplified polymorphic DNA (RAPD) patterns (Mahuku *et al.*, 1997). Attempts to mate the nonaggressive and the aggressive forms to produce ascospores have not been successful (Gabrielson, 1983). Purwantara *et al.* (1999) screened *L. maculans* isolates from Australia for disease severity against *B. juncea* strains from around the world. The authors classified the *L. maculans* isolates into one of two groups with this method; *B. juncea* attackers and non-attackers. These molecular biology experiments and mating studies performed on the virulent and nonvirulent strains of *L. maculans* demostrated that they are probably two separate species of fungus that are closely associated on infected plants and stubble (Rouxel *et al.*, 1994).

The virulent species of *L. maculans* have been differentiated into three pathogenicity groups (PG2, PG3, PG4) based on their disease causing abilities in three canola cultivars: Westar, Quinta and Glacier, using the cotyledon inoculation test (Table A.2). The cotyledons of the cultivars were inoculated with fungi and after 11 d the disease symptoms on the leaves were evaluated using a scale of 0 to 9, with 0 indicating no disease symptoms and 9 indicating severe disease symptoms. PG-1 represents the nonaggressive strains, that were nonvirulent on the three cultivars tested. The PG-2, PG-3 and PG-4 groupings represent aggressive *L. maculans* strains from a wide range of geographical regions (Williams, 1992). The PG2 strains were virulent on Westar, somewhat virulent on Quinta and avirulent on Glacier. The PG3 strains were virulent on Westar and Glacier and somewhat virulent on Quinta, and the PG4 strains were virulent on all three cultivars. Other researchers, using the same cultivars as test plants, have determined different groupings of virulent isolates. This was probably due to the lack of a definite method for characterizing virulence on the plants (Rimmer, 1992).

Table A.2. The different pathogenicity groups of *L. maculans*, based on disease formation on the cotyledons of three canola cultivars.

L. maculans isolate	origin	sirodesmins produced	disease severity on canola cultivars[a] Westar	Quinta	Glacier
PG1		none	—[b]	—	—
PG2			+[c]	+/—[d]	—
PHW843	Australia	none	8.3	6.8	1.1
Leroy	Canada	yes	9.0	6.8	1.5
PHW620	France	yes	8.5	7.9	2.1
Lm2	UK	yes	9.0	5.6	1.0
PHW839	S. Africa	yes	7.0	3.2	1.0
PHW128	USA	yes	9.0	8.3	2.3
PG3			+	+/—	+
PHW888	Australia	yes	8.9	5.4	7.8
PHW478	France	yes	8.9	5.9	8.2
PHW437	Germany	yes	9.0	5.9	8.2
P146	UK	yes	8.9	4.7	8.4
PHW423	N. Zealand	yes	8.2	5.9	8.0
PHW100	USA	yes	8.9	5.9	7.5
PG4			+	+	+
PHW914	Australia	yes	9.0	8.3	7.6
PL 87-111	Australia	yes	8.8	8.8	7.8
PL 87-2	Canada	yes	8.6	9.0	8.0
PHW433	Germany	yes	9.0	8.8	8.4
VI 4	Germany	yes	9.0	9.0	8.0
41 A4	UK	yes	8.8	8.8	8.4

(adapted from Rimmer, 1992, and Williams, 1992).
[a] disease severity scaled 0 to 9, from least to most.
[b] —, no lesion formed
[c] +, lesion formed
[d] +/-, intermediate stage

A.3 Methods of blackleg control

The methods used to date to slow or stop the spread and severity of blackleg have been: use of fungicides, development of blackleg resistant canola cultivars and cultural control.

In the past decade research has been conducted into finding a biological control agent active against *L. maculans*. There is no biocontrol agent against the fungus available for use to date.

Fungicides used as seed treatments against *L. maculans*, such as seed-slurry and seed-soak treatments with 0.2% germisan or 0.2% suspensions of benomyl and thiabendazole, have been effective in reducing or eliminating seedborne inoculation of the disease on canola (Commonwealth Agricultural Bureau, 1972). Fungicide seed treatments are not, however, effective against inoculation of the growing canola plants with *L. maculans* spores that persist in infected canola stubble or blackleg infection caused by wind-borne ascospores or rain splashed pycnidiospores (Gugel and Petrie, 1992). Australian researchers reported a reduction of blackleg disease when the fungicide flutriafol was applied as a coating on phosphate fertilizer granules that were added to the field at the time of sowing (Gugel and Petrie, 1992). The same study done in Canada showed that the fungicide only provided poor and variable protection to the plants against the fungus (Gugel and Petrie, 1992). Fungicides have not been consistently successful as inhibitors of the fungal spores in the field; single application of fungicides at the time of seeding does not provide sustained protection for the plants, and considerable plant losses occur before the crop reaches a stage of growth where natural resistance to blackleg (and other fungal diseases) can develop (Kharbanda, 1992). Multiple applications of fungicide sprays early in crop growth (foliar application) has been shown to control blackleg, but this treatment is expensive, can cause environmental damage and may select for fungicide-resistant *L. maculans* (Kharbanda, 1994).

Plant breeders have designed many different cultivars of canola that vary widely in their susceptibility to *L. maculans* infection by either the virulent or nonvirulent forms (Chakraborty *et al.*, 1992). Stable blackleg resistant varieties *of B. napus* such as Jet Neuf, Major and Cresor have been developed by plant breeders for use in the European climate (Ferreira, 1995).

Certain species of *Brassica* display economically useful characteristics such as low glucosinolates and erucic acid levels, increased yield of oil, cold tolerance, drought tolerance or resistance to blackleg. Species exhibiting one or more of these desireable traits have been cross hybridized in an effort to produce an ecomomically superior plant designed for growth in specific climates. The canola species that are low in glucosinolates and erucic acid, such as *B. napus* and *B. rapa*, are more susceptible to blackleg infection than the rapeseed varieties such as *B. juncea*, which exhibits resistance to blackleg but is high in glucosinolates and erucic acid (Keri *et al.*, 1997). *B. juncea* is also not suitable for growth in cold climate areas. The genetics of the resistance phenotype displayed by certain species of canola is not fully known at the present time, making the breeding of plants that are carrying a stable genetic resistance pattern against *L. maculans* a difficult task. There has been much research recently into mapping the disease resistance genes of blackleg resistant *Brassica* species with the hope of cloning the resistance genes into canola grade species of *Brassica* to produce an economically superior plant.

Researchers from the University of Alberta in 1994 reported that development of *B. napus* L. cultivars that could produce superior oil, grow in the cold western Canadian climate and be resistant to blackleg was well underway. A double haploid breeding program was used to allow a quicker development process than the conventional approach to plant breeding (Stringam *et al.*, 1994). One of the results of this breeding program was the *B. napus* cultivar Quantum, that exhibited higher levels of resistance to blackleg than any other cultivar registered in Canada (Mayerhofer, 1997).

Other researchers have conducted studies to determine the genetic source of resistance to blackleg in *B. juncea*; they found that resistance was likely due to two epistatic genes. One of the genes conferred inherited resistance in a dominant manner and the other gene in a recessive manner. These genes appeared to act together to confer resistance to blackleg at the emerging seedling and young plant stages. This research was aimed toward finding the genetic source of resistance so that it could be transferred to *B. napus* to make a blackleg resistant, canola oil grade cultivar. However, it was suggested by the authors that this could be a difficult transgenic cultivar to construct if both genes were required in *B. napus* in order to produce a stable resistance trait (Keri *et al.*, 1997).

In another study, the genetic source of blackleg resistance in two Australian canola cultivars; Shiralee and Maluka, was investigated by University of Alberta

researchers. They found that genetic resistance to blackleg in Shiralee at the cotyledon stage appeared to be from a single major locus. This was in agreement with results previously obtained from genetic analysis of resistance in double haploid *B. napus* lines, which suggested that the blackleg resistance shown by these plants at the cotyledon and adult plant stages was conferred by a single major locus (Mayerhofer *et al.*, 1997).

Ferreira *et al.* (1995) mapped the genetic source of resistance by *B. napus* to *L. maculans* to a single major locus using double haploid plant lines. The level of resistance was determined by the plant phenotypic response to *L. maculans* in greenhouse cotyledon inoculation experiments. These authors also found four other genomic regions that were involved in a significant response to *L. maculans* inoculation. One of the genes showed similarity to known plant chitinase genes. Analysis of the genetic resistance response of the plants in field conditions showed that the resistance response came from two genomic regions that were different from the regions mapped in the greenhouse experiments (Ferreira *et al.*, 1995).

The canola cultivars presently commercially available for use in Canada all show varying degrees of susceptibility to blackleg. Figure A.3. illustrates the mean disease severity and the mean yield of canola of the *B. napus* and *B. rapa* cultivars that are registered for use in western Canada (Kharbanda, ARC, Vegreville, AB).

Cultural control (conscientious farming techniques) is very important in controlling the spread of blackleg. *L. maculans* can remain on a field for as long as there is organic matter such as canola stubble present for its survival. It can take four years for canola stubble to rot away, suggesting that infested fields should be left fallow for four years, or that nonsusceptible crops such as cereals should be grown on infested land for that period of time. In regions of Alberta that have a high rate of blackleg infection, planting a field with canola when it had canola on it the year before, can result in 49 to 100% of the stems being infected with blackleg, depending on the type of canola cultivar planted (Alberta Agriculture, 1992). Research suggests that if the stubble is buried while the field is fallow, it degrades more quickly and does not allow spreading of the ascospores by wind (Gugel and Petrie, 1992). Volunteer canola and wild mustard plants can also harbour the fungus and so must be cleared from around canola fields. Use of canola seeds that have been certified to be free of *L. maculans* is also an important control measure against the disease (Gugel and Petrie, 1992).

There have been two reports in the literature of biocontrol agents against *L. maculans*.

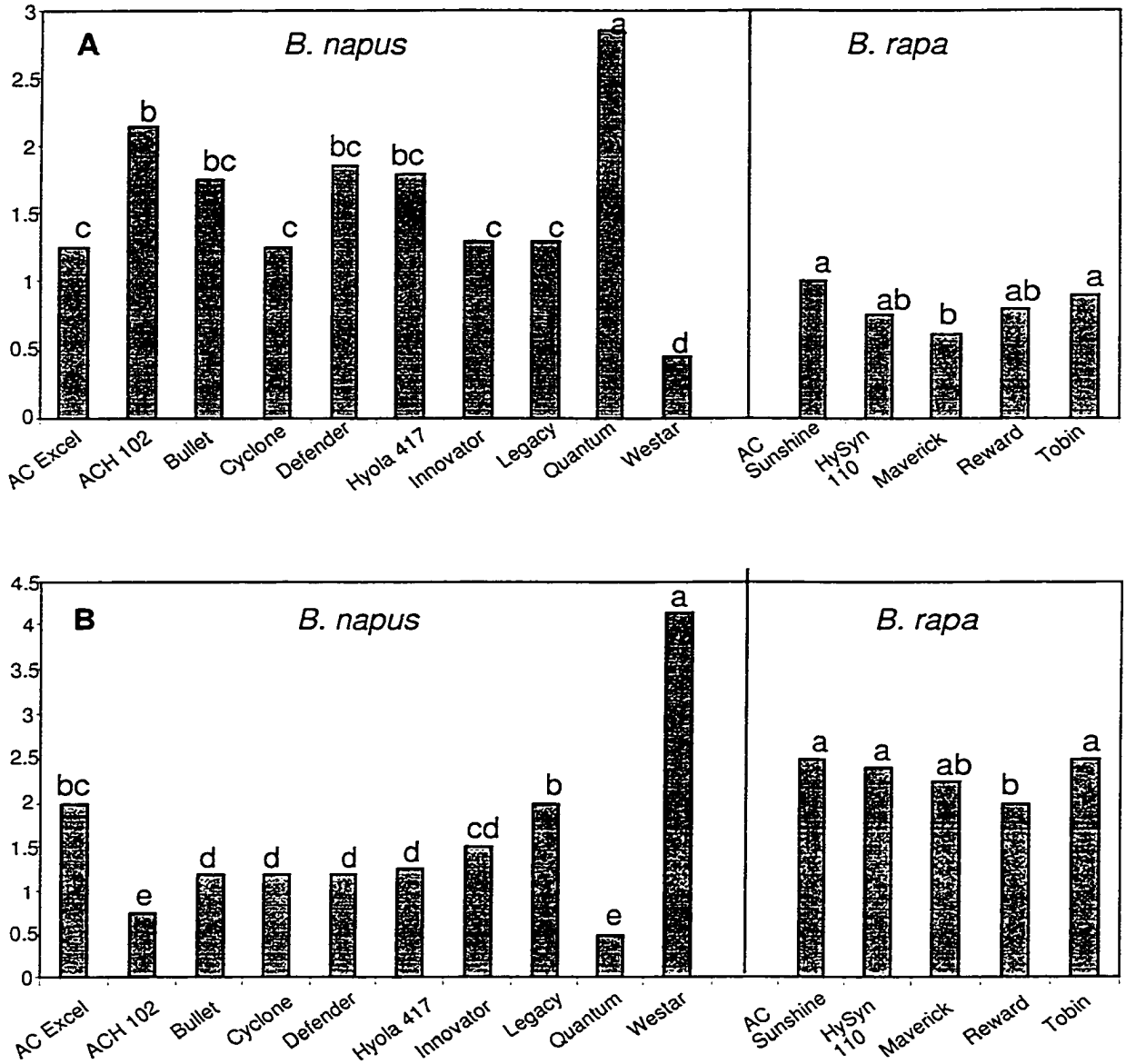


Figure A.3. Characteristics of canola cultivars registered for use in Canada as of 1995, grown on blackleg infested land near Warburg. A. Mean yield of seed harvested from a small plot and extrapolated to 10^3 X kg/ha for each cultivar. B. Mean disease severity of blackleg on the canola cultivars, measured on a 0 to 5 scale: 0, no disease; 1, visible pycnidiospores on leaf; 2, small lesion with pycnidiospores; 3, half of stem is girdled; 4, whole stem girdled and plant alive; 5, dead plant. The *B. napus* and *B. rapa* cultivars were analysed separately. Cultivars with the same letter designation above the bar are not significantly different according to Duncan's multiple range test with a 95% confidence (p=0.05).

Kharbanda and Dahiya (1990) found a strain of *Penicillium verrucosum* that was able to inhibit *L. maculans*, *S. sclerotiorum* and *R. solani*. The researchers determined that the source of the inhibition was a previously characterized secondary metabolite produced by many different *Penicillia* called citrinin. Use of this organism as a biocontrol agent is not feasible however; citrinin is a toxin that has been reported to cause kidney damage in farm animals.

Chakraborty *et al.* (1994) screened organisms found in the phyllosphere (leaves) of *B. napus* for antagonism towards *L. maculans*. One organism in particular, *Erwinia herbicola*, was found to be able to inhibit the fungus in both *in vitro* and *in vivo* tests. This bacterium secreted a thermolabile antifungal compound into the culture broth that was thought to be the main source of inhibition against *L. maculans*.

The use of a strain of *Paenibacillus polymyxa* (*Paenibacillus polymyxa*, formerly named *Bacillus polymyxa*, reclassified by Ash *et al.*, 1993) isolated from canola stubble, as a biocontrol agent against *L. maculans* has been studied by Kharbanda *et al.*, (1996) in greenhouse pot trials. One of the goals of this thesis was to determine the source of the inhibition of this strain of *P. polymyxa* against *L. maculans*.

Fungicides, blackleg resistant cultivars and conscientious farming have all met with variable success in some geographical areas, however for greater success of the control of blackleg, these methods should be used in conjunction with one another. If fungicide-treated seeds of a canola cultivar that is resistant or highly tolerant to blackleg are planted on a blackleg infested field, a loss of crop yield due to infection will result. If proper farming techniques are used, blackleg infection can be minimized. The most vulnerable times in canola growth are the emerging seedling and young plant stages (cotyledon to 8th leaf stage; Hall, 1992), particularily for canola cultivars designed to be low in glucosinolates and erucic acid. It is at this susceptible time that another method for control of *L. maculans* is required.

A.4 Biocontrol agents of Agricultural pests

Research to discover biocontrol agents against agricultural pests has increased recently due to public pressure on the agricultural industry to reduce the use of chemicals. Awareness and concern about the effects of pesticides and herbicides on humans, animals and the environment is growing. As much as food safety is a concern,

food production is also important, which has led to an increased desire to find natural biocontrol agents to inhibit the action of yield-reducing pests.

Organisms or mixtures of organisms that exhibit some form of inhibitory action against agricultural pests as control agents of disease in field and storage conditions are being used in North America and Europe. Some of these bacteria are listed in Table A.4. These biocontrol organisms, whether they be bacteria or fungi, tend to be species that occur naturally in soil. They may be free living or symbiotic species. In some cases, mode of inhibitory action of the biocontrol agent is known, as with the use of the crystalline toxin produced by *Bacillus thuringiensis* against species of Lepidoptera (Feitelson *et al.*, 1992). In other cases the mode of action is not known; for instance the activity of *Bacillus subtilis* Quantum-4000™ against fungal diseases of peanut plants, and the whole organism is used as the biocontrol agent (Reddy *et al*, 1993). Another example of a biological control agent with an unknown mode of action is *B. subtilis* GB03 Kodiak®. Cotton seeds are treated with this bacterium before planting to suppress seedling diseases and chronic long term diseases of cotton. *B. subtilis* GB03 is known to produce peptide antibiotics called iturins that are active against fungi, however the protective effects are considered to be due to a combination of antibiosis, niche competition and induced systemic resistance (Brannen and Kenney, 1997).

Use of the protective bacterium or fungus as a biocontrol agent, instead of the inhibitory agent(s) produced by the organism, can be advantageous. It is more economic to grow large quantities of an organism and prepare it for commercial distribution than to grow the organism, purify the inhibitory agent, then prepare it for commercial distribution. The inhibitory agent(s) produced by a bacterium or fungus that has the ability to control an agricultural pest may be unknown. Inhibition of the agricultural pest may be due to a synergistic relationship between many bacteria or between the bacteria and/or fungi and plant, or there may be two or more types of inhibitory agent produced by the biocontrol organism that all act to enhance the biocontrol of the pest.

Many of the biocontrol organisms used or being studied are plant growth promoting asymbiotic rhizosphere (root tissue colonization) or spermosphere (seed colonization) bacteria (PGPR; Petersen *et al.*, 1996). It has been shown that the rhizosphere soils support a much larger number of microorganisms than nonrizosphere soils (Mavingui *et al.*, 1992). Not all antibiotic producing bacteria are PGPR, but most, and probably all PGPR show antagonistic qualities to a variety of plant disease causing organisms. PGPR bacteria are free living soil bacteria that can exhibit some or all of

Table A.4. Organisms used, or being studied, as biocontrol agents for fungal diseases and/or insects.

biocontrol organism	active against	inhibitory agent(s)	reference
Bacillus cereus UW85	fungal diseases of alfalfa, tobacco, cucumber, peanuts (*Phytophtora medicaginis*)	zwittermicin A	He *et al.* 1994.
B. laterosporus strains 921 and 615	mosquito larvae (*Aedes aegypti, Anopheles stephensi, Culex pipiens*)	spores and crystalline inclusions	Orlova *et al.*, 1998.
B. sphaericus	some species of mosquitoes and blackflies	crystalline toxin	Orlova *et al.*, 1998
B. subtilis APPL-1	bean rust (*Uromyces phaseoli* [Reben.] Wint.)	heat stable culture filtrate component	Baker *et al.* 1983
B. subtilis (Quantum-4000™)	fungal diseases of peanuts	unknown	Reddy *et al.*, 1993.
B. subtilis GB03 (Kodiak™, Gustafson Inc, Plano TX)	fungal diseases of cotton plants (*Fusarium* spp., *Rhizoctonia* spp.)	PGPR bacterium; increases root mass, diseases suppressed by unknown means	Brannen and Kenney, 1996.
B. subtiltis (B-3)	bean rust (*Uromyces phaseoli* [Raben.]) brown rot of stone fruit (*Monilinia fructicola* [Wint.])	iturins	Gueldner *et.al.*, 1988.
B. thuringiensis subsp. israelensis	some species of mosquitoes and blackflies	crystalline toxin	Orlova *et al.*, 1998
B. thuringiensis var. *kurstaki* spores, wettable powder (Dipel™)	some species of Lepidoptera (caterpillars), especially Painted Lady caterpillar (*Vanessa cardui* L.)	crystalline toxin	Miranpuri *et al.*, 1993
dead *Pseudomonas fluorescens* genetically engineered with *B. thuringiensis* crystal toxin genes (MVP™, M-Trak™; Mycogen Corp., San Diego, CA)	some species of Lepidoptera (caterpillars), Diptera (mosquitoes, blackflies) and Coleoptera (beetles)	crystalline toxin encapsulated in dead *P. fluorescens* cells	Feitelson *et al.*, 1992.

Table A.4. continued

biocontrol organism	active against	inhibitory agent(s)	reference
Enterobacter aerogenes B8	apple crown and root rot (*Phytophtora cactorum*)	antibiotic-like substance	Chernin *et al.*, 1995
E. cloacae with chitinolytic enzymes from *Trichoderma harzianum*	seed rotting fungus (*Pythium ultimum*), cucumber wilt (*Fusarium solani*) (*Botrytis cinerea, Uncinula necator*)	agglutinins, hydroxamate, siderophores, chitinases, ammonia, antifungal metabolites	Lorito *et al.*, 1993
Erwinia carotovora, E. rhapontici, P. putida, P. fluorescens	damping-off of safflower, white beans and chickpea (*Pythium* sp. group G.)	competition for nutrients, pyoluteorin, oomycin	Liang *et al.*, 1996
Plant growth promoting rhizosphere bacteria *Azotobacter, Bacillus, Clostridium, Serratia, Pseudomonas spp.*	soil-borne fungal diseases; take-all of wheat (*Gaeumannomyces graminis* var. *tritici*)	synergistic, enhanced plant growth, competition for nutrients, chitinolytic enzymes, antibiotics	Reddy *et al.*, 1993
P. fluorescens	cotton seedling diseases (*Rhyzoctonia solani, Theilaviopsis basicola, Alternaria* spp., *Verticillium dahliae*)	pyrrolnitrin 2,4-diacetylphloro-glucinol	Howell and Stipanovic, 1978. Bonsall *et al.*, 1997
Streptomyces lydicus WYEC108	broad spectrum biocontrol agent of fungi (*Pythium* spp., *Aphanomyces* spp.)	extracellular chitinases	Mahadevan and Crawford, 1997
S. platensis	herbicidal activity, inhibitory to phtopathogenic fungi	resormycin	Igarashi., *et al.* 1997

these traits: fix atmospheric nitrogen, solubilize soil phosphates, produce plant growth promoting compounds (such as gibberellin-like compounds, or the phytohormone indoleacetic acid [Shishido *et al.*, 1996]), excrete chitinolytic compounds, siderophores and antibiotics and colonize the roots or the mycorrhizosphere (area of fungal colonization of plant roots) of certain plants. These organisms can compete for nutrients with the fungi that comprise the mycorrhizosphere. (Burr and Caesar, 1985). All these traits can aid in the biocontrol of agricultural pests.

There are factors that can influence the effectiveness of PGPR bacteria (by influencing population size and metabolic activity) used in commercial applications such as; method of crop inoculation, light intensity, the moisture content, pH and aeration of different soils, climatic conditions, irrigation, choice of fertilizers and the planting dates of the crop (Burr and Caesar, 1985). Another factor that has been investigated is effect of fungicides on the PGPR bacteria. Seven fungicides that are used on potato seedpieces were tested for inhibition of the PGPR bacteria that are applied to potato crops as a commercial application in California. One of the seven chemicals tested in *in vitro* studies inhibited growth and population size of the PGPR bacteria, and the other six had no effect on these bacteria (Burr and Caesar, 1985). The effect of the fungicides: propiconazole, prochloraz, iprodione and herbicides: clopyralid, sethoxydim and ethametsulfuron-methyl, registered for use on canola crops in western Canada, was tested on *P. polymyxa*. None of these chemicals inhibited the growth of the bacterium (Kharbanda *et al.*, 1996)

A.5 *Paenibacillus polymyxa* as a PGPR bacterium and a biocontrol agent

P. polymyxa is a PGPR bacterium that has been isolated from the rhizosphere or mycorrhizosphere of white clover, perennial ryegrass, crested wheatgrass (Holl *et al.*, 1988), lodgepole pine (Holl and Chanway, 1992), Douglas fir (Shishido *et al.*, 1996), wheat (Heulin *et al.*, 1994), *Phaseolus vulgaris* (Petersen *et al.*, 1996), the rhizosphere of garlic (Kajimura and Kaneda, 1996; 1997) and canola stubble (Kharbanda, 1994).

Kado *et al.* (1987) patented the process of using *P. polymyxa* isolate 9A to inhibit verticillium wilt of potatoes. The authors reported that the young potato plants were protected from disease formation by applying the bacterium to potato seed pieces before they were planted.

Holl and Chanway (1992) reported using *P. polymyxa* isolate L6-16R as a successful growth promoter of three out of four lodgepole pine species. Pine seedlings showed a mean increase in shoot and root dry weight of up to 35%, 8 wk after inoculation with *P. polymxa*, when compared to uninoculated controls. The researchers concluded that the level of increase in pine seedling growth was a function of the size of the *P. polymyxa* rhizosphere population.

Smid *et al.*, (1993) isolated bacteria from soils that had been used to grow Narcissus and tulip bulbs. They found four strains of *P. polymyxa* that could inhibit the growth of *Penicillium hirsutum*, a fungus that causes storage rot of tulip and hyacinth bulbs. The bacteria excreted low molecular weight compounds into the growth medium that were thermostable and could inhibit the fungus over a broad range of pH values.

Mavingui and Heulin (1994) tested several strains of *P. polymyxa* that had been isolated from nonrhizosphere soils (NR) and from the rhizosphere soils (RS) and rhizoplane soils (RP) of spring wheat for chitinase activity and antifungal activity against *Gaeumannomyces graminis* var. *tritici* (*Ggt*). These researchers analysed the strains for chitinase activity by assaying for chitobiase activity (that develops concomitantly with chitinase activity) by the O'Brien-Colwell (1987) method and for antifungal activity by growing the strains on PDA seeded with a plug of *Ggt* and measuring the inhibition of the fungus after 6 days. The authors found that chitinase activity and antifungal activity was not necessarily correlated. Some strains of *P. polymyxa* that exhibited strong chitinase activity were weakly inhibitory to *Ggt* and vice versa. The researchers determined that 72.5% of the NR *P. polymyxa* strains were positive for both chitinase and antifungal activity (though not necessarily the same strains for both), 81.5% of the RS *P. polymyxa* strains were positive for chitinase activity and 92% of them were positive for antifungal activity and 100% of the RP *P. polymyxa* strains were positive for both activities. The authors suggest from this work that selective pressures from the ecological niches that the rhizoplane and rhizosphere provide appear to create specifically adapted strains of *P. polymyxa* that are not found in the nonrhizosphere soils.

Shishido *et al.* (1996) used known plant growth promoting *P. polymyxa* strains L6, Pw-2 and S20 to investigate the interactions between the bacteria and the fungal strains colonizing the roots of pine and spruce seedlings. Certain strains of fungi colonize the root tips of the seedlings, forming a mycorrhizosphere that acts to stimulate seedling growth. Some bacterial species can also be found in the mycorrhizosphere.

The researchers wanted to determine whether the bacteria in the mycorrhizosphere were enhancing plant growth indirectly, by stimulating the mycorrhizosphere populations to grow, or directly, by stimulating the plant to grow. They found that *P. polymyxa* did not increase the level of fungal colonization of the seedling roots or stimulate fungal growth. However, all three *P. polymyxa* strains increased the biomass of both the pine and spruce seedlings, with or without the presence of the fungus and so stimulated plant growth directly.

Jisha and Alagawadi (1996) studied the interaction of two known phosphate-solubilizing bacteria, *Pseudomonas striata* and *P. polymyxa*, with a cellulolytic fungus, *Trichoderma harzianum*, and added organic matter (cotton stalks) on the growth of sorghum. Grain yield was the highest (by 28-30%) from plants that were treated with either one of the phosphate-solubilizing bacteria, together with the cellulolytic fungus plus the organic matter when compared to plants that were treated with either the phosphate-solubilizing bacteria (either one or both) or the cellulolytic fungus and the organic matter. The researchers concluded that the growth of the phosphate-solubilizing bacteria was stimulated by the presence of the actively growing cellulolytic fungus. They hypothesized that this effect was due to the release of simple sugars from the cotton stalks into the rhizosphere, that were degraded by fungus, where they were used as a carbon and energy source by the phosphate-solubilizing bacteria. The increase in bacterial growth led to a concomitant increase in solubilization of inorganic phosphate from the soil; this was used by the plant as a ready source of phosphate.

Petersen *et al.* (1996) investigated the relationship between the symbiotic rhizosphere bacterium *Rhizobium etli* and the asymbiotic bacterium *P. polymyxa* on bean plants (*P. vulgaris*). A synergism between these two bacterial species had previously been reported to enhance plant growth more than either bacterial species acting separately (Chanway *et al.*, 1991). The researchers found that when *P. polymyxa* was used as a co-inoculant with *R. etli*, the symbiotic bacterial population increased when compared to the population of *R. etli* without *P. polymyxa*. This increase in the *R. etli* population allowed an increase in fixed nitrogen that the plant could utilize. When *R. etli* was grown in minimal medium with *P. polymyxa*, there was no concomitant increase in its cell density. The *R. etli* population was also not stimulated to increase in population by the addition of plant exudate to the medium. The researchers concluded that *P. polymyxa* indirectly stimulates an increase in the *R. etli* population via the plant host.

Moharram *et al.* (1997) studied whether the addition of *P. polymyxa* to the soil as a free living nitrogen fixing bacteria could replace the more expensive nitrogen containing fertilizers for optimum growth of wheat by farmers who operate small-scale farms in Egypt. They found that the dry weight of wheat inoculated with *P. polymyxa* was higher than for the uninoculated controls, but was approximately the same for wheat grown with nitrogen containing fertilizers. The use of *P. polymyxa* as a source of fixed nitrogen for wheat growth as opposed to the use of nitrogen containing fertilizers, was therefore preferable due to cost effectiveness.

Nielson and Sorensen (1997) isolated thirteen strains of *B. pumilus* and two strains of *P. polymyxa* from barley rhizosphere that were both antagonsitic to plant pathogenic fungi such as *Aphanamyces cochleoides*, *Pythium ultimum* and *Rhizoctonia solani.* These bacteria all produced a variety of cell-wall-degrading enzymes such as cellulase, mannanase and xylanase, that were not found to be produced by non-antagonistic bacteria isolated from the same source. The authors found that the two *P. polymyxa* strains appeared to be antagonistic to *A. cochleoides* in a medium-independent manner. The authors suggested that this was due to constitutive production of the degrading enzymes by the *P. polymyxa* strains.

Dijksterhuis *et al.* (1999) investigated the interaction of *P. polymyxa* with the fungus *Fusarium oxysporum*, that causes dry rot of tulips. This was a continuation of the study by Smid *et al*, (1993) to find a biocontrol agent active against the fungus *Penicillium hirsutum.* The authors had noticed during the previous study that motile *P. polymyxa* cells were attracted to the conidia of the fungus while the fungal spores swelled during germination. Dijksterhuis *et al.* wanted to determine if this attraction was involved in the antagonism of the fungus by *P. polymyxa.* They found that the presence of living bacteria with the fungus was necessary for continued antagonism toward fungal growth. Addition of antibiotics active against *P. polymyxa* to cultures of the bacterium and the fungus relieved the suppression of fungal growth. The authors proposed a possible mode of antifungal activity by *P. polymyxa* could be the continuous production of antifungal compounds by bacteria localized on the fungal hypha; this would provide the means of delivering antibiotic directly to the site of action; the fungal hyphae. As well, *P. polymyxa* could suppress fungal growth by competing for nutrients, since bacteria were found at high cell densities around the fungal hyphae. The authors also found that the addition of magnesium ions to the paired cultures increased fungal growth, which they attributed to inhibition of the antifungal compounds by the magnesium ions. An

alternative theory is that inhibition of the fungus could be due to a lack of magnesium ions that are needed for its growth. This would suggest that *P. polymyxa* produces a compound that sequesters and transports magnesium into the bacterial cell, thus the addition of excess magnesium to the culture relieves the competition for magnesium between the fungus and *P. polymyxa.* Haavik and Froyshov (1975) suggested that the antibiotic bacitracin, produced *by Bacillus licheniformis*, can be produced in continuous culture in a nutrient dependent manner, and that its function may be to carry manganese ions through the bacterial cell membrane for use by the cell.

A.6 Secondary metabolites

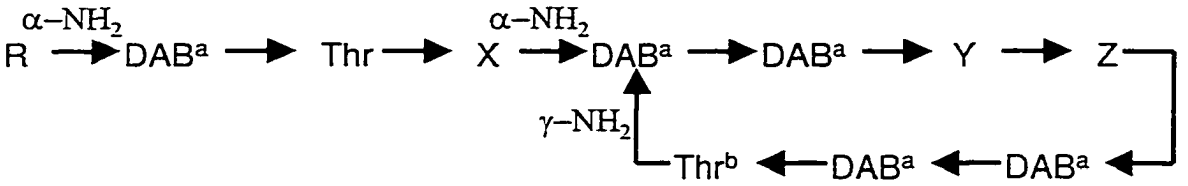
Secondary metabolites are compounds produced by an organism that are involved in or responsible for interactions between the producer organism and its environment. They are believed to have no role in the internal workings of the producer organism; if the genes responsible for the production of the secondary metabolite were eliminated, the organism would still thrive (Davies, 1992). There is much debate on why organisms produce these energy expensive compounds. Some suggested activities of secondary metabolites are: antibiotics against other organisms for self-protection or competition for nutrients, metal transporting compounds, plant-, nematode- or insect-microbe symbiosis agents, pheromones, differentiation effectors between cells of the same species and within the producer cell, excretion of unwanted products, reserve pools for potential new pathways or products of 'selfish' DNA (Davies, 1992). Secondary metabolites are produced by many bacteria and fungi and by some plants (eg. phytoalexins from *Brassica* sp. [Pedras *et al.*, 1992]) and animals. However, research into secondary metabolism has mainly been on microbes, so there may be many more secondary metabolites produced by the more complex organisms that have not yet been found (Kleinkauf and von Dohren, 1995). The function that secondary metabolites perform for the producing organism may not be well understood, but their ability to act as antibiotics against specific organisms (bacteria, fungi, nematodes and insects) or biological components (such as viruses and tumours), immunosuppressants or biosurfactants has been used by the medical, agricultural and industrial fields for decades (Bodansky and Perlman, 1969; Kleinkauf and von Dohren, 1988; Davies, 1992).

Many genera of bacteria produce antibiotics, but most of the familiar antibiotics are produced by *Streptomyces* and *Bacillus* soil bacterial species. There are different classes of antibiotics such as: aminoglycosides (kanamycin), β-lactams (penicillins), polyketides (tetracyclines; a subclass of polyketide are the macrolides; erythromycin) and peptides (cyclosporin; Hash, 1975; Kleinkauf and von Dohren, 1988; Kirby, 1992). Many of the antibiotics used medically and in the agriculture industry are peptide antibiotics; bialophos, cyclosporin, polymyxins, gramicidin S and tyrocidine are a few examples (Sonenshein, 1993).

Peptide antibiotics can range from molecular weights of 270 (bacilysin) to 4500 (licheniformin); they differ from proteins in that D-amino acids, basic amino acids such as ornithine and diaminobutyric acid, β-amino acids, dehydroamino acids and other modified amino acids (approximately 300 different amino acids have been found to date) are common in the peptides. As well, bonds between peptide components other than peptide bonds can be formed such as the ester bonds in enniatin (Zocher *et al.*, 1982; Katz and Demain, 1977) and the amide bond in bacitracin (Ishihara *et al.*, 1989). Conversely, arginine, histidine and methionine, which are common amino acids in proteins, are uncommon in peptides (Bodansky and Perlman, 1969). The peptide antibiotics can be linear (gramicidin), branched (polymyxins) or cyclic peptides (gramicidin S) and they can contain amino acid modifications such as N-methylation, glycosylation and acylation (Bodansky and Perlman, 1969, Katz and Demain, 1977). Peptide antibiotics also tend to be produced as families of peptides that may differ by one or two amino acids or by a modification of the existing peptide. An example of this is the family of peptides called polymyxins, produced by *P. polymyxa*, *Aerobacillus polyaerogenes* and *Bacillus circulans* (Figure A.6; Umezawa *et al.*, 1978; Komura and Kurahashi, 1985). Fungal and bacterial peptide antibiotics are made differently than proteins; they are produced non-ribosomally by multienzyme complexes called peptide synthetases (Kleinkauf and von Dohren, 1990; von Dohren, 1993; Turgay and Marahiel, 1994; Stein and Vater, 1996).

A. 7 Peptide synthetase multienzyme complexes

Peptide synthetases (PS) are enzymes that construct peptides. They are made up of structured domains that carry highly conserved amino acid sequences, that in turn are grouped into modules that are responsible for the addition of a certain amino acid



antibiotic name	R	X	Y	Z
polymyxin B_1	6-methyloctanoic acid	DAB[a]	D-phe	leu
polymyxin B_2	6-methylheptanoic acid	DAB[a]	D-phe	leu
polymyxin C (P)	6-methyloctanoic acid	D-ser	phe	thr
polymyxin D_1	6-methyloctanoic acid	D-ser	D-leu	thr
polymyxin D_2	6-methylheptanoic acid	DAB[a]	D-leu	thr
polymyxin E_1 (colistin A)	6-methyloctanoic acid	DAB[a]	D-leu	leu
polymyxin E_2 (colistin B)	6-methylheptanoic acid	DAB[a]	D-leu	leu
polymyxin M (A)	6-methyloctanoic acid	DAB[a]	leu	thr
polymyxin S	6-methyloctanoic acid	D-ser	D-phe	thr
polymyxin T[b]	6-methyloctanoic acid	DAB[a]	D-phe	leu
circulin A	6-methyloctanoic acid	DAB[a]	D-leu	ile
circulin B	6-methylheptanoic acid	DAB[a]	D-leu	ile

[a] DAB, 2,4-diaminobutyric acid.
[b] polymyxin T has a leu substituted for the thre at this position.

Figure A.6. The polymyxin group of antibiotics. All amino acids are in the L configuration unless indicated otherwise. Adapted from Umezawa *et al.*, 1978 and Shoji *et al.*, 1977.

into the peptide product. These amino acid activating (aaa) modules and the domains that are within them are found in peptide synthetase enzymes produced by bacteria and fungi. There is a co-linearity between PS enzymes and the peptides that they synthesize; for every amino acid in the peptide antibiotic there is a corresponding aaa module in the PS enzyme that is responsible for activation and insertion of that amino acid into the growing peptide chain. As well, the aaa modules in the PS enzymes are in the same order as the amino acid sequence of the peptide antibiotic. These enzymes can be comprised of one multi-functional enzyme, as in the cyclosporin synthetase of the fungus *Tolypocladium niveum* (CYSYN; 11 modules, 1.7 MDa; Weber *et al.*, 1994), or of several multifunctional enzymes arranged in a multienzyme complex, such as the enzymes TY1 (1 module, 122 KDa), TY2 (3 modules, 230 KDa) and TY3 (6 modules, 460 KDa) for tyrocidine production by *Brevibacillus brevis* (Table A.7.A; Gocht and Marahiel, 1994; Stein and Vater, 1996).

Each enzyme is multifunctional because each aaa module in the PS enzyme is segregated into domains that perform distinct functions (Figures A.7.A. and A.7.B.). Every aaa module (1000 to 1400 amino acid in length) has an amino acid adenylation domain, a 4'-phosphopantetheine (pan) carrier motif and an elongation domain. Some aaa modules have additional domains for epimerization or methylation of the amino acid that it is responsible for activating and placing into the growing peptide chain (Stein and Vater, 1996).

The amino acid adenylation domain (approximately 500 amino acid in length) contains nine highly conserved amino acid sequences (Table A.7.B), that are involved with ATP binding and amino acid adenylation, and two variable amino acid regions that are involved with recognition of the amino acid that the aaa module activates and transfers to the peptide product. These conserved sequences are also found in other enzymes such as: acyl-CoA synthetases, amino acid adding enzymes (D-ala activating enzyme), siderophore synthetases and luciferase enzymes in insects. The enzymes that carry these conserved motifs are considered to be a part of a superfamily of acyl-adenylate-forming enzymes (von Dohren, 1993).

The pan carrier motif (core 6/J), also called the thiolation site, is a highly conserved amino acid sequence: LGGHSLK. The PS enzyme cofactor, pan (a moiety of coenzyme A), is covalently bound by a phosphodiester bond to the serine in this conserved motif (Stachelhaus *et al.*, 1998; Quadri *et al.*, 1998). There is one covalently bound pan cofactor in each module (Vater *et al.*, 1997). The activated amino acyl

Table A.7.A. Selection of peptides and the peptide synthetase enzymes responsible for their construction[a].

peptide		peptide synthetase multienzymes (in order)					
peptide name	list of aa in structure (in order)	enzyme name(s)	no. modules	size (KDa)	producing organism(s)	properties	reference
tripeptide precursor of penicillin /cephalo-sporin	L-α-aminoadipate-cys-D-val	ACVS	3	420[b]	*Penicillium chrysogenum*, *Cephalosporium acremonium*, *Streptomyces clavuligerus*	antibiotics	Aharonowitz *et al.*, 1993
enniatins A,B,C (depsi-peptides)	(D-Hyiv[c]-Meile[d])$_3$(A) (D-Hyiv[c]-Meval[d])$_3$(B) (D-Hyiv[c]-Meleu[d])$_3$(C)	Esyn	2	347	*Fusarium oxysporum*, *F. scripti*	antifungal, cationophor, immuno-modulator	Zocher *et al.*, 1982, Stein and Vater, 1996
lichenysins (lipopeptide)	β-hydroxy fatty acid-glx-leu-D-leu-val-asx-D-leu-ile/leu/val[e]	LicA LicB LicC	3 3 1	~210 ~210 ~70	*Bacillus licheniformis*	antimicrobial, surfactant	Konz *et al.*, 1999
surfactins (lipopeptide)	β-hydroxy fatty acid-glu-leu-D-leu-val-asp-D-leu-ile/leu/val[e]	SRFS2 SRFS3 SRFS4	3 3 1	402 401 114	*B. subtilis*	surfactant, antimicrobial	Besson, 1994, Stein and Vater, 1996
syringomycins	ser-D-ser-D-dab[f]-dab[f]-arg-phe-dhb[g]-(3-OH) asp[h]-(4-Cl) thr[i]-fatty acid (C_{10}, C_{12} or C_{14})	SyrE SyrB	8 1	1038 105	*Pseudomonas syringae* pv. *syringae*	phytotoxic, antimicrobial, antifungal	Guenzi *et al.*, 1998
tyrocidine	D-phe-pro-phe-D-phe-asn-glu-tyr-val-orn-leu	TY1 TY2 TY3	1 3 6	123 230 460	*Brevibacillus brevis*	antibiotics	Pfiefer *et al.*, 1995, Stein and Vater, 1996

Table A.7.A. continued

peptide name	peptide: list of aa in structure (in order)	peptide synthetase multienzymes: enzyme name(s)	no. modules	size (KDa)	producing organism(s)	properties	reference
gramicidin S	D-phe-pro-val-orn-leu-	GS1	1	127	*B. brevis*	antibiotic	Conti *et al.*, 1997
	D-phe-pro-val-orn-leu-	GS2	2	510			
fengycin (lipopeptide)	glx-D-orn-ile-D-allo-thr-glx-D-tyr-pro-glx-D-ala (D-val)-tyr/ile[e] plus fatty acid component	PpsA	2	~256	*B. subtilis*	antifungal	Lin *et al.*, 1998, Tosato., 1997
		PpsB	2	~256			
		PpsC	2	~256			
		PpsD	3	~360			
		PpsE (FenB?)	1	144			
cyclosporin	Mebmt[jd]-abu[k]-sar-Meleu[d]-val-Meleu[d]-ala D-ala-Meleu[d]-MeLeu-Meval[d]-	CYSYN	11	1689	*Tolypocladium niveum*	immuno-suppressive, antifungal	Weber *et al.*, 1994
bacitracin	[ile-cys][L]-leu-glu-ile-(α-lys-D-orn-ile-D-phe-his-D-asp-asnε[m])	BA1	5	335	*B. licheniformis*	antibiotic	Ishihara *et al.*, 1989
		BA2	2	240			
		BA3	5	380			

[a] amino acids are in the L conformation unless otherwise stated.
[b] Average MW of ACVS enzymes from different producer organisms.
[c] D-Hyiv, 2-hydroxyisovaleric acid in the D conformation
[d] Me, methylated amide bond at these amino acids
[e] the amino acid in this location can vary
[f] Dab, 2,4-diaminobutyric acid
[g] Dhb, 2,3-dehydroaminobutyric acid
[h] (3-OH) Asp, 3-hydroxyaspartic acid
[i] (4-Cl) Thr, 4-chlorothreonine
[j] Bmt, (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine
[k] α-aminobutyric acid
[L] [ile-thr] condenses to form thiazoline
[m] ε. the epsilon amino group of lys forms an amide bond to asn, forming a ring structure

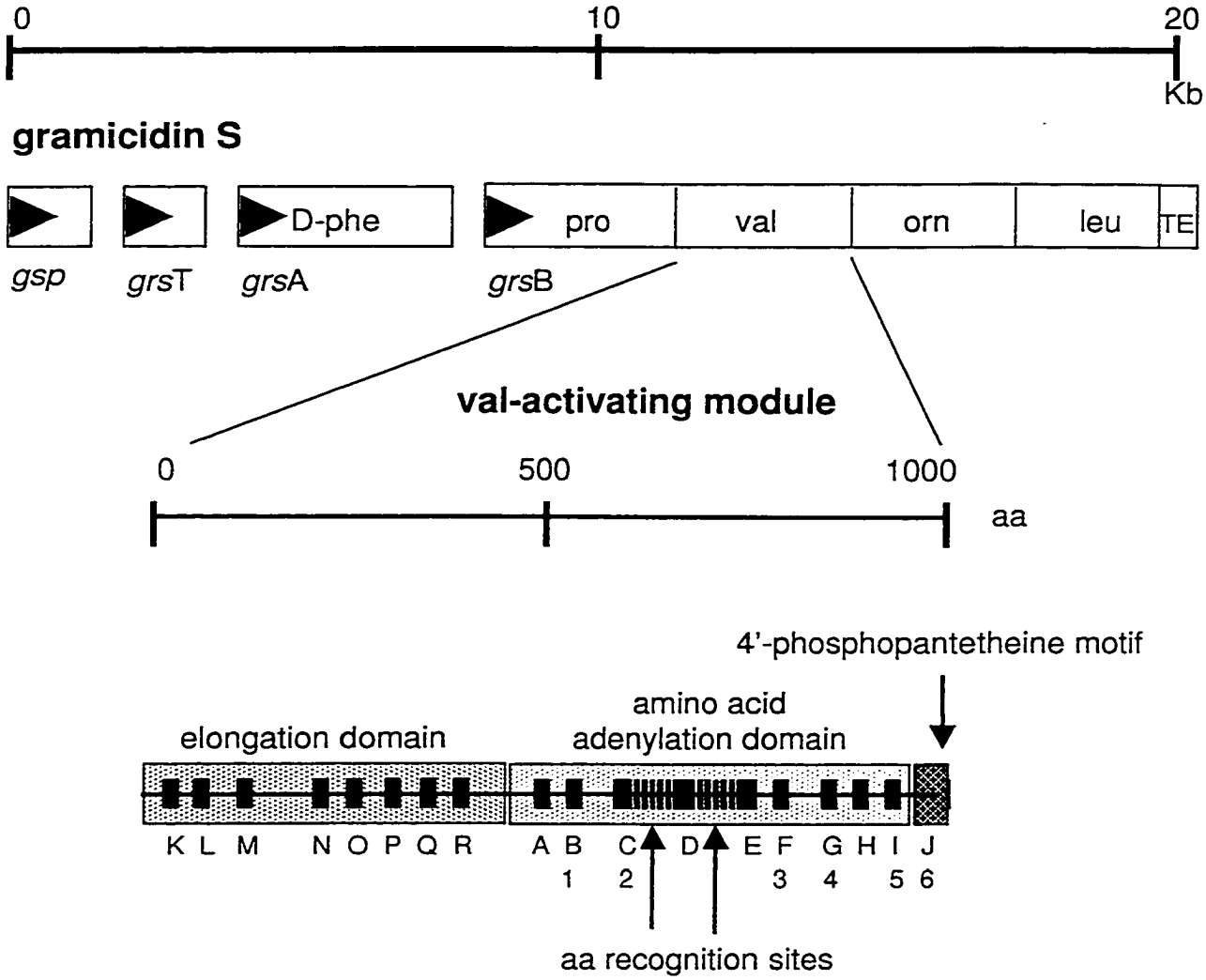


Figure A.7.A. Genes in the gramicidin S operon: *gsp* PPTase encoding gene; *grs*T thioesterase encoding gene; *grs*A encodes GS1, that activates and epimerizes phe; *grs*B encodes GS2, that activates pro-val-orn-leu and accepts D-phe from GS1; TE encodes a thioesterase domain. The domains found in the val-activating module, are shown. The conserved sequence sites A-R (1-6 alternate labels) and the variable aa recognition sites are also indicated. Adapted from Stachelhaus and Marahiel, 1995; Stein and Vater, 1996; Stachelhaus *et al.*, 1998; Vater *et al.*, 1998.

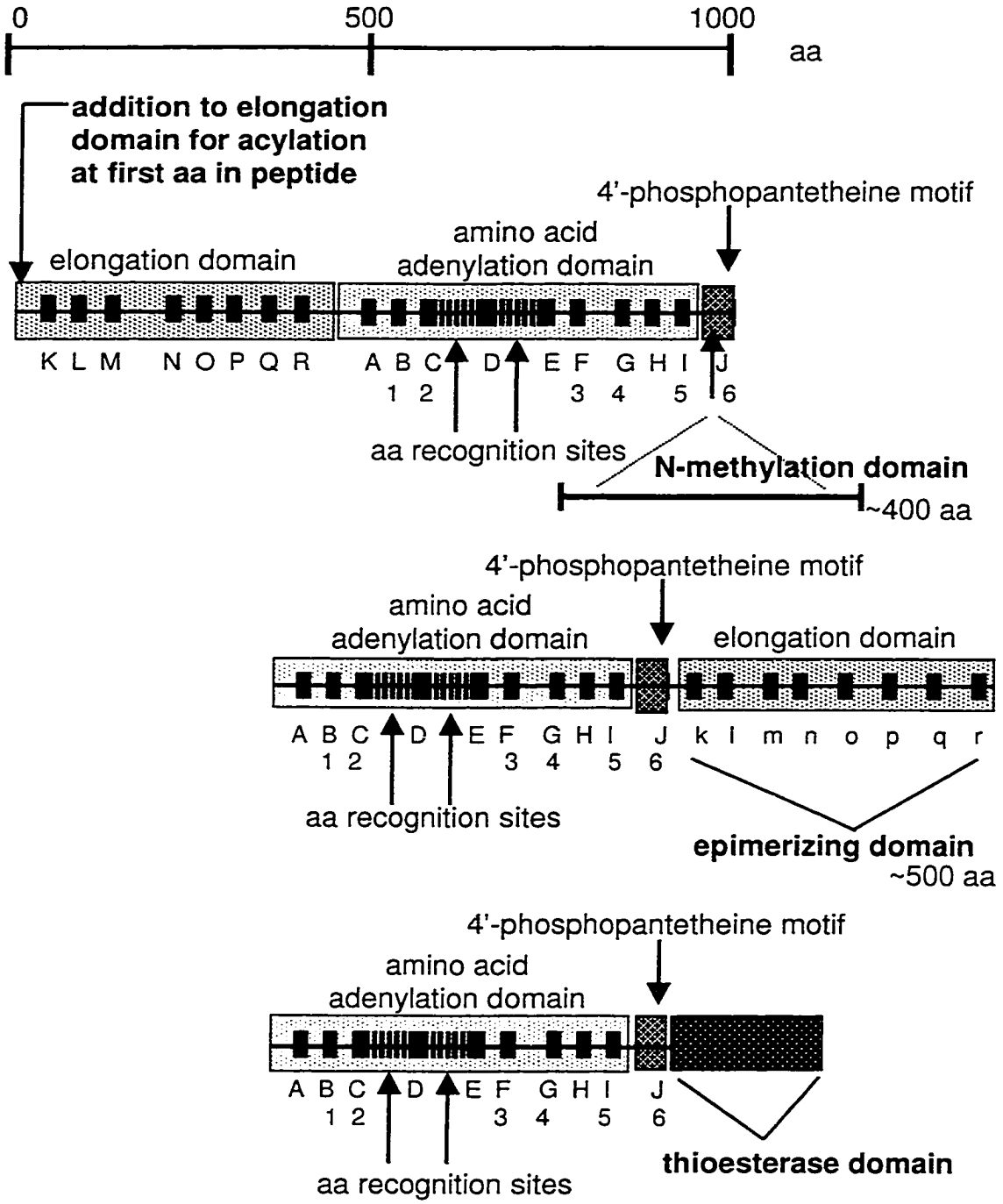


Figure A.7.B. Modified domains: the type II domain with the N-terminal methylation domain, additional sequence at the N-terminal elongation domain for acylation, the thioesterase domain and the epimerization domain. The epimerization domain is similar to the elongation domain with some differences in the conserved sequences K-R; this is depicted by naming these sequences k-r.

adenylate is thioesterified by its carboxyl group to the sulfhydryl group at the end of the pan arm, which is a term called 'loading'. This pan carrier motif is not found in the other adenylate-forming superfamily enzymes; those enzymes appear to transfer the activated component to an external thiol-carrying cofactor (Stein and Vater, 1996).

The elongation domain (between 300 and 400 amino acid in length), also called the condensation domain, contains eight conserved amino acid sequences (K-R; Table A.7.B), that are involved in aminoacyl or peptidyl transfer from consecutive pan arms with the consequential formation of a peptide bond. The bond formation is thought be catalyzed by the second histidine in the conserved sequence that is called M; HHIIXDGXSXXIL, that closely resembles the active site motif HHXXXDG of acyl transferases, where the second histidine is the catalytic base for bond formation between two substrates (Stachelhaus *et al.*, 1998). It is also theorized that the elongation domains have an acyl or peptidyl donor site and an acyl or peptidyl acceptor site that acts in a similar manner to the donor and acceptor sites found in ribosomal protein synthesis (Stachelhaus *et al.*, 1998).

Bacterial PS enzymes (and ACV synthetases of fungi) carry a thioesterase domain next to the pan carrier motif of the aaa module that activates the last amino acid to be incorporated into the peptide chain. Fungal PS enzymes do not appear to have this domain (Stachelhaus *et al.*, 1998). The thioesterase domain has a conserved GXSXG sequence that is also found in lipase and esterase enzymes (Stein and Vater, 1996).

Some amino acids in peptides produced by PS enzymes are modified by epimerization while at the thiolation step of peptide production. An aaa module that is responsible for epimerizing the amino acid from the L to the D conformation (such as GS1 of gramicidin S: L-phe to D-phe) has an epimerizing domain (between 300 and 400 amino acid in length) that contains eight conserved amino acid sequences, k-r, that are similar to the eight sequences, K-R, of elongation domains.

How the elongation, or condensation domains function to produce a peptide moiety from the amino acyl loaded pan arms of each module is currently under investigation by many researchers. There has been some discrepancy in the literature as to whether the elongation domain of a particular aaa module is at the carboxy-terminal (Vater *et al.*, 1997) or the amino-terminal (Stachelhaus *et al.*, 1998; Guenzi *et al.*, 1998) of that module. The uncertainty of the positioning of the elongation domains is fueled because modules that epimerize the loaded amino acyl residue before it is passed to the next module, carry an epimerizing domain at the carboxy-terminus of the

Table A.7.B. The conserved amino acid sequences found in the domains of PS enzymes and the possible activity of each sequence[a]

letter /no.	domain	conserved aa sequence[b]	activity
A	adenylation	LTYXELNXXAN	unknown
B/1	adenylation	ILAVLKAGGAYPVIDF	lysine may form H-bond to carboxyl group of activating aa
C/2	adenylation	TSGTTGKPKG	phosphate binding loop, signature sequence of adenylate forming superfamily of enzymes
D	adenylation	YGPTE	active site in adenylate formation
E	adenylation	GELCIRGXGV	unknown
F/3	adenylation	RMYRTGDLA	ATP binding
G/4	adenylation	GRXDXQVKIRGXRIEL	ATP binding
H	adenylation	LPXYMVP	ATP binding
I/5	adenylation	NGKXD	ATP binding
J/6	thiolation site	LGGHSLK	pan carrier at serine, active site recognized by PPTases
K	elongation	YPVSSAQKRMY	unknown
L	elongation	LXXRHEALRTXF	unknown
M	elongation	HHIIXDGXSXXIL	amino acyl and/or peptidyl transfer, similar to active site of acyl transferases, second H acts as a catalytic base
N	elongation	TLYXVLXXXYXVL	unknown
O	elongation	DIIVGTPXAGRXXP	unknown
P	elongation	VGMFVNTLXLR	unknown
Q	elongation	VKXXLXAFXX-QDYPF	unknown
R	elongation	SRHPLLFXXXF	unknown
k	epimerization	GEXLTPIQXWFF	unknown
l	epimerization	LXXHHDALRMXY	unknown
m	epimerization	HHLVVDGVSWXIL	amino acyl and/or peptidyl transfer, similar to active site of acyl transferases, second H acts as a catalytic base
n	epimerization	EXNDILLTAXGLAL	unknown
o	epimerization	LEGHGREII	unknown
p	epimerization	SRTVGWFTSMTP	unknown
q	epimerization	VPXKGVGYGILXY	unknown
r	epimerization	PXXXFNYLGQF	unknown
TE	thioesterase	G(HY)SXG	thioesterase active site

[a] adapted from Stein and Vater, 1996.
[b] single letter amino acid designations. X indicates that the amino acid in that position is not conserved.

module. As mentioned previously, elongation domains have eight conserved sequences, K to R, with the sequence M showing similarity to acyl transferase active sites. Epimerization domains also have eight conserved sequences called k to r, that are similar to the elongation domain sequences. In particular, epimerization domains have a conserved sequence, HHLVVDGVSWXIL, (m) that is similar to the conserved sequence M of elongation domains HHIIXDGXSXXIL, that in turn closely resembles the active site motif of acyl transferases, HHXXXDG (Stachelhaus *et al.*, 1998). However a recent report by Belshaw *et al.* (1999) described the elongation domain of a particular amino acid module as at the amino-terminal of the module based on kinetic studies of the formation of peptide bonds between two amino acyl residues loaded onto pan arms within two PS modules. If the gramicidin S synthetase enzymes GS1 and GS2 are used in a model to describe the amino-terminal view of elongation domain placement within an aaa module (Figure A.7.C), then the epimerizing domain at the C-terminal of the phe-activating module (GS1, initiator module) is not considered an elongation domain because, with this view, no initiator module ever contains an elongation domain. The first elongation domain is a part of the acceptor module that activates proline. Each elongation domain appears to have a donor and acceptor site that allows the amino acyl residue that is loaded onto the pan arm to 'dock' at the correct site, depending on whether the pan arm amino acyl residue load is being passed along to the next module or if the loaded pan arm is taking the amino acyl residue (or peptidyl group) from the upstream pan arm. Further studies into the roles of the conserved sequences found in these elongation and elongation-type domains would elucidate how these domains actually work in PS multienzyme complexes.

Some fungal PS enzymes produce peptides with amino acids that have been methylated at their amino group (N-methylation, such as the aaa modules 2,3,4,5,8 and 10 of CYSYN that produces cyclosporin). The aaa modules that methylate their amino acyl adenylate have a methylation domain (approximately 400 amino acid in length) between the amino acid adenylation domain and the pan carrier motif of the aaa module. The addition of a methyl group to the amino acid occurs at the thiolation stage. Modules that carry this extra domain are called Type II modules (Weber *et al.*, 1994; Dittman *et al.*, 1994).

Some peptides are produced by complexes of PS enzymes (bacterial, except ACV synthetases of bacterial origin) that carry one or more aaa modules and others are made by one PS enzyme (fungal) that carries all the aaa modules needed for production

Amino-terminal view of positioning of the elongation domain within the aaa module

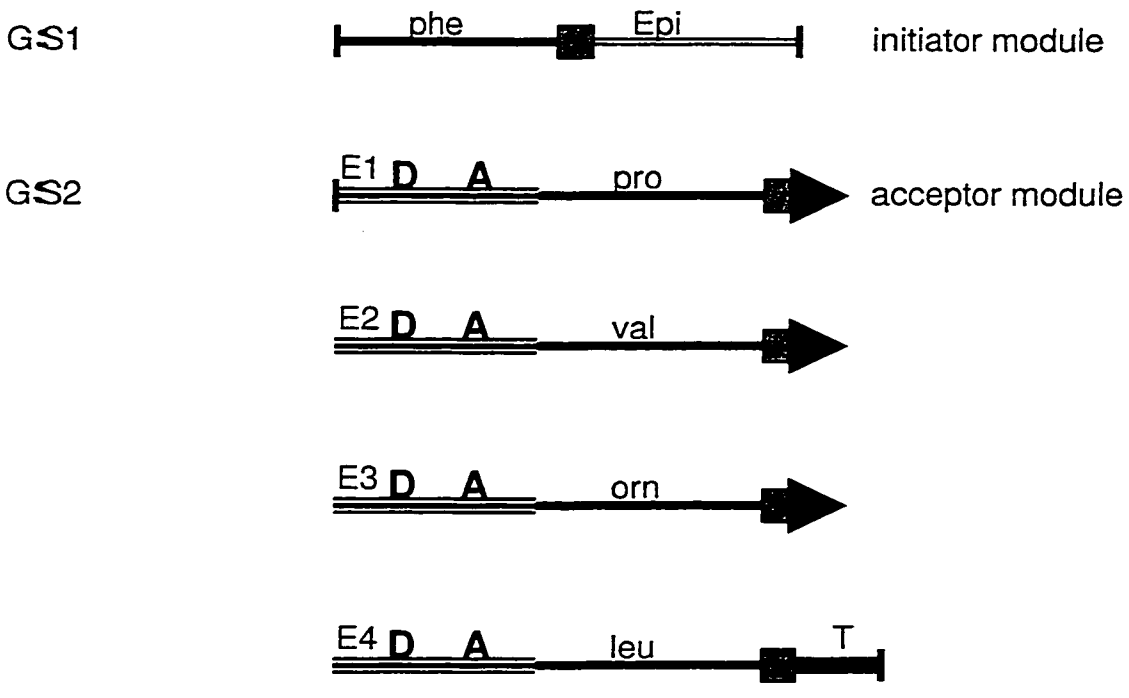


Figure A.7.C. The amino-terminal (Stachelhaus *et al.*, 1998; Belshaw *et al.*, 1999) view of the placement of the elongation domain in a particular amino acid activating (aaa) module. Gramicidin S synthetases 1 and 2 are used as a model PS enzyme system. Epi: epimerizing domain; E1-4: elongation domains; D and A refer to the donor and acceptor sites in the elongation domains; T: thioesterase domain; grey box: pan carrier motif; black line: the amino acid adenylation domain that activates the amino acid listed above it.

of the peptide (Mootz and Marahiel, 1997). The aaa module that activates the amino-terminal amino acid of the peptide is called the 'initiator module'; the rest of the aaa modules are called 'acceptor modules' (Conti *et al.*, 1997).

Each aaa module is specific for a certain amino acid, however some aaa modules can make substitutions, for instance; L-valine activating domains can also activate D or L-isoleucine (gramicidin S), L-phenylalanine can be replaced with L-tyrosine (gramicidin S and tyrocidine) and L-ornithine can be replaced with L-lysine, L-arginine or D-ornithine (gramicidin S; Katz and Demain, 1977). This substrate laxity is why peptide antibiotics are generally produced as families of related compounds.

In previous years, a multienzymatic thiotemplate model of peptide biosynthesis was proposed to explain the mode of action of PS enzymes. This model suggested that each amino acid in the peptide was activated by an aminoacyl adenylation step, then the activated amino acid was thioesterified to the thiol group of a cysteine that would have been in the conserved amino acid sequence of the thioester motif (Kleinkauf and von Dohren, 1990). The thioesterified amino acid was then tranferred to the growing peptide chain by a centrally located pan arm in a stepwise elongation reaction (Kleinkauf *et al.*, 1971). Recent studies on gramicidin S synthesis have benefitted from advances in mass spectroscopy techniques that have allowed more gentle studies of the thioester motif of PS enzymes. Previous research on the nature and amino acid sequence of the thioester motif was done by Edman degradation; the first step in the procedure would have removed the pan cofactor by a β-elimination (Vater et al., 1997).

The thiotemplate model has been amended to a 'multiple carrier model' of synthesis that suggests that a pan cofactor is attached to each domain at the conserved motif LGG(H/D)S(L/I) (core 6/J; Figure A.7.D; Stein *et al.* 1996) rather than a central pan arm responsible for elongation of the peptide. The amino acid is activated by the amino acid adenylation domain at the expense of ATP, which undergoes hydrolysis at the α,β-linkage to form AMP and PP_i. The resulting amino acyl adenylate is esterified to the thiol end of the pan arm by its carboxyl group. Each pan arm in each aaa module in the PS enzyme is loaded with the carboxy-thioesterified amino acyl adenylate that is specific for that aaa module. The peptide chain is synthesized in an N-terminal to C-terminal direction. The first aaa module in the initiator PS enzyme activates what will be the N-terminal (unless the peptide is cyclized) amino acid of the peptide product, and the last aaa module of a PS enzyme activates the amino acid that will be the C-terminal of the peptide chain.

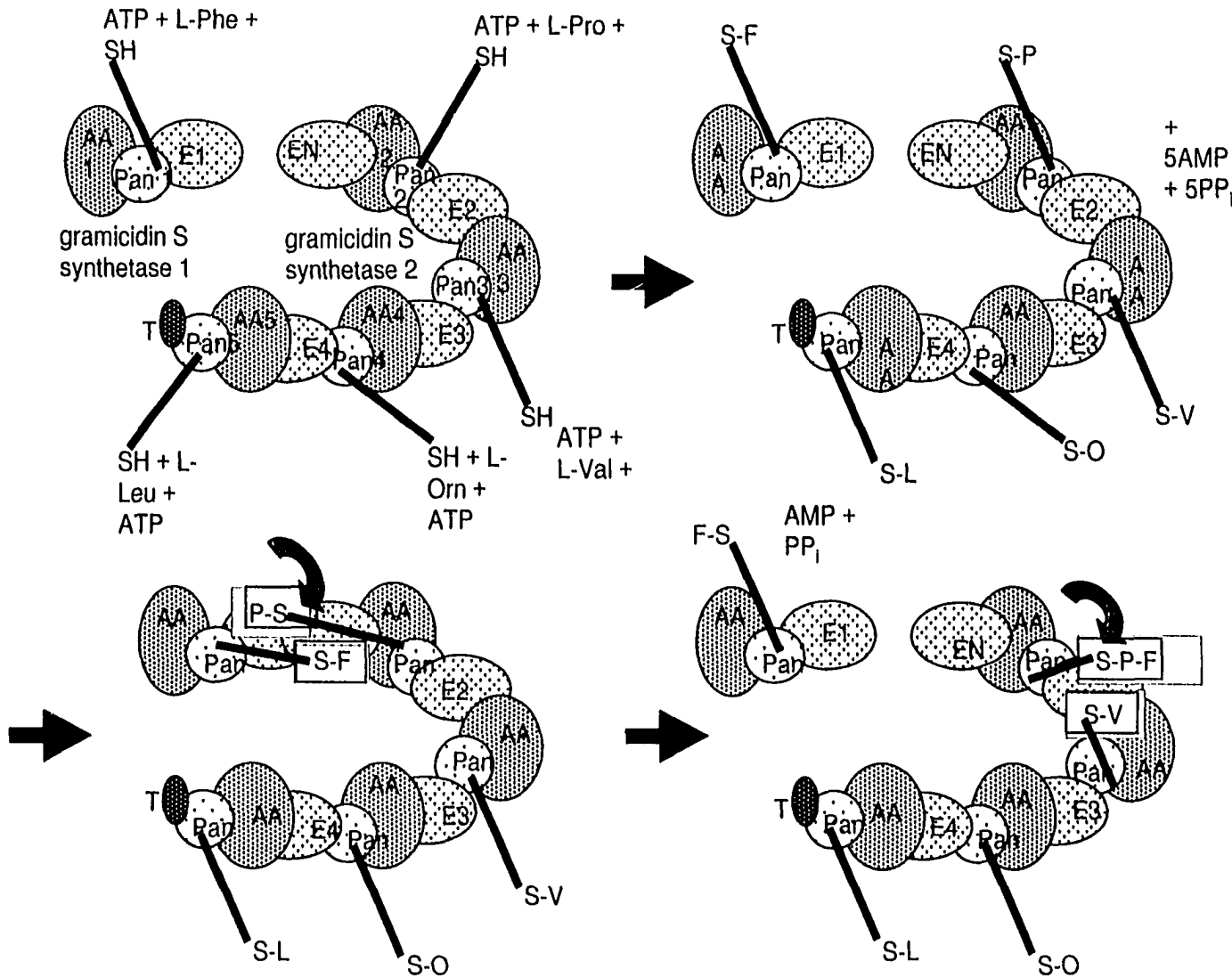


Figure A.7.D. The multiple carrier model of nonribosomal peptide biosynthesis by peptide synthetase enzymes. Gramicidin S synthetases 1 and 2 are used to demonstrate the first two steps in the biosynthesis of gramicidin S. AA is the amino acid activating region, Pan is the conserved motif that binds with the cofactor 4'-phosphopantetheine, the black line is the 4'-phosphopantetheine cofactor, EN is the N-terminal elongation domain of the acceptor module in GS2, E_n are the elongation domains in the other modules and T is the thioesterase domain. Adapted from Stein *et al.*, 1996.

It is hypothesized that the first loaded pan arm in the initiator aaa module translocates the thioesterified amino acyl group to the donor site of the elongation domain, where it is met by the second loaded pan arm that is in the acceptor site of the same elongation domain. A peptide bond between the two amino acyl groups is formed by a nucleophilic attack from the second amino acyl group on the thioesterified carboxyl C atom of the first amino acyl group. The reaction takes place at the elongation domain. It is catalyzed by the second histidine in the conserved sequence motif M, that acts as a base and deprotonates the amino group of the second amino acyl adenylate, thus making the amino group reactive (Stachelhaus *et al.*, 1998). All types of bonds (amide and ester bonds in addition to peptide bonds) found between peptide components are thought to be formed by this condensation reaction. The specialized terminal condensation or cleavage reactions appear to be performed by either the elongation or the thioesterase domains, for example; the peptide bonds between the two pentapeptides that form gramicidin S, the three ester bonds between the three dipeptides that form enniatin, the amide bond formed with the ε-amine of lys and the carboxyl C of asn that makes a branched cyclic bacitracin and the release of the linear ACV peptide from the last pan arm. The mechanism of formation is not understood at this time (Stachelhaus *et al.*, 1998).

Once a pan arm is relieved of its amino acyl group, the domain activates another specific amino acid and the pan arm is loaded again with an amino acyl group for another round of synthesis.

Phosphopantetheinyl transferases (PPTases), are enzymes associated with PS enzymes and have been found to be essential for peptide synthesis (Schneider *et al.,* 1998). The gene encoding these enzymes is found within the PS operon (Quadri *et al.*, 1998). PPTases make up a superfamily of enzymes that are involved with acyl, peptidyl and aryl carrier enzymes to covalently attach the pan cofactor by a phosphodiester linkage to serine (Quadri *et al.*, 1998; Weinreb *et al.*, 1998). The PPTases will only recognize the pan carrier motif, LGGHSL, when it is presented within a certain tertiary structure in the enzyme substrate. The tertiary structure appears to place the serine residue of the motif in a bend at the end of an α-helix. Once the PPTase binds the cofactor to each of the carrier motifs that are in the PS enzyme, the synthetase is converted from an apo-enzyme (inactive form) to a holoenzyme (active form; Quadri *et al.*, 1998).

The genes *gsp* and *sfp* were found within the operons for gramicidin S (*B. subtilis*) and surfactin (*B. brevis*) production, respectively. Both encode PPtases (Gsp and Sfp) that can be interchanged with each other and with a PPtase from *E. coli*, EntD, that activates the siderophore synthetase that produces enterobactin. As well, Sfp could phosphopantetheinylate a peptide carrier protein from *Saccharomyces cerevisiae* and an acyl carrier protein from *E. coli*, although there was an 80-fold decrease in catalytic efficiency of Sfp when it was used in reactions involving systems from other species (Stachelhaus and Marahiel, 1995, Quadri *et al.*, 1998). Two conserved amino acid sequences separated by 40 to 45 non-conserved sequences were found among the enzymes in the PPTase superfamily: GXD-X_{40-45}–F/WXXKES/AXXK (Quadri *et al.*, 1998).

Some PS operons contain genes that encode enzymes that are similar to thioesterases. These separate thioesterase-like enzymes are found in bacterial (and ACV producing fungus) PS operons that already contain genes encoding PS enzymes carrying a thioesterase domain, as well as in PS operons that do not contain genes encoding PS enzymes with an attached thioesterase domain. Separate thioesterase genes have been found in the gramicidin S operon (*grs*T; Kratzschmar *et al.*, 1989), the tyrocidine operon (*tyc*F; Mootz and Marahiel, 1997), the surfactin operon (*srf*A-TE; Cosmina *et al.*, 1993) and the bialophos gene cluster *of S. hygroscopicus* (ORF1 and 2; Raibaud *et al.*, 1991). The gene products of *grs*T, *tyc*F, *srf*A-TE, ORF1 and ORF2 show approximately 30% homology to rat and mallard duck fatty acid thioesterase II enzymes, and they all share the conserved amino acid signature sequence of thioesterases GXSXG, that is also present in the thioesterase domains attached to bacterial PS enzymes (Kratzschmar *et al.*, 1989; Raibaud *et al.*, 1991; Stein and Vater, 1996). The specific function of these separate thioesterase enzymes that are found within bacterial PS operons is unknown; it is theorized that the finished peptide chain is cleaved from the last pan arm and/or cyclized by the thioesterase domain that is attached to the last aaa module of the PS enzyme (Mootz and Marahiel, 1997; Schneider *et al.*, 1998). For fungal peptide synthetases that do not appear to have thioesterase domains, the separate thioesterase enzymes are thought to be responsible for release of the finished peptide chain from the PS enzyme (Raibaud *et al.*, 1991).

Two genes, *tyc*D and *tyc*E, encoding enzymes that are homologous to the ABC superfamily of transporters, have been found within the tyrocidine synthesis operon (Mootz and Marahiel, 1997). An ABC transporter encoding gene, *exi*T, was found just upstream of genes that encode synthetase enzymes responsible for exochelin

(siderophore) production in *Mycobacterium smegmatis* (Zhu *et al.*, 1998). Two tandem ABC transporter encoding genes were also recently found within a PS enzyme locus in *Proteus mirabilis* (Mootz and Marahiel, 1997). An open reading frame (ORF3) was found in the bialaphos gene cluster of *S. hygroscopicus* that was similar to citrate uptake protein- and arabinose transport protein-encoding genes of *E. coli*, as well as to genes encoding membrane transport proteins in general. The authors suggested that ORF3 may encode a protein involved in an antiporter-like transport system where carbon supplies for the production of bialaphos are imported and bialaphos is exported (Raibaud *et al.*, 1991). The function of these gene products in peptide synthesis is not known, however it has been speculated that they could be responsible for excretion of the synthesized peptide from the producer cell (Zhu *et al.*, 1998). It was suggested that these ABC transporter gene products that are in the tyrocidine operon are involved in conferring resistance against tyrocidine to the producer cell by secretion of the peptide (Mootz and Marahiel, 1997). Research on the effect of polymyxins added to early exponential phase growing *P. polymyxa* (that could produce polymyxins) indicated that the culture was sensitive to the presence of polymyxins. The culture became resistant to added polymyxin during late exponential growth. This increase in resistance coincided with the cultures own increase in production of polymyxins and with the increase of a membrane-bound protein complex that was part of the synthetase complex responsible for production of the polymyxins. This membrane-bound portion of the synthetase complex appeared to activate DAB; a constituent of polymyxins. When polymyxins were added to the late exponential phase culture, the cells responded by increasing the activity of the membrane-bound DAB activating portion of the synthetase complex. These authors suggested that polymyxin plays a role in sporulating *P. polymyxa* culture by interacting with the producer bacterium's cell membrane, and that this membrane interaction results in development of resistance to polymyxin by the producer bacterium (Balakrishnan *et al.*, 1980). There are no other reports to date on putative ABC transporter protein encoding genes within nonribosomally produced peptide synthetase operons. There are, however, reports of ABC transporter encoding genes within gene clusters that encode the ribosomally produced bacteriocins, such as microcin J25 of *E. coli*, that confer resistance against the antibiotic to the producer cells. The gene product of *mcj*D, a gene found within the microcin J25 synthesis gene cluster, was found to be similar to many membrane translocator proteins of the ABC transporter protein family. Disruption of this gene, and disruption of TolC; an exporter protein of *E. coli*, resulted in

the *E. coli* producer cells becoming sensitive to microcin J25 (Delgado *et al.*, 1999). The gene cluster for ribosomal production of subtilin, a lantibiotic bacteriocin of *B. subtilis* ATCC 6633, also contains genes that appear to encode a trans-membrane export system, *spa*I, *spa*F and *spa*G. The gene product of SpaF shows similarity to the ABC transporter family of proteins, SpaI is a lipoprotein and SpaG is a membrane protein. Disruption of these genes leads to *B. subtilis* mutants that are sensitive to subtilin (Klein and Entian, 1994).

Studies have recently been done on targeted alteration of the substrate specificity of PS enzymes (reprogramming), specifically on altering surfactin producing PS enzymes from *B. subtilis* (Schneider *et al.*, 1998). The researchers 'swapped' minimal modules in the PS enzymes by molecular alteration of the PS enzyme encoding genes. A minimal module comprises the domains that are essential for recognition, activation and thiolation of a specific amino acid; these are the amino acyl adenylation domain and the pan carrier motif. Minimal modules of the five aaa modules in gramicidin S PS enzymes from *B. brevis*; phe, pro, val, orn and leu, and of the cys and val aaa modules in ACV synthetase from *Penicillium chrysogenum*, were successfully inserted into the second leu aaa module of surfactin that is within the surfactin PS enzyme, SrfA-A, by a double crossover event. The altered PS enzymes produced peptides with the expected amino acid changes; however, the new peptides were produced by the recombinant PS enzymes at a much reduced rate compared to surfactin production. This was seen even when a minimal module for leu-activation was crossed-over into SrfA-A to replace the existing leu. As well, the researchers noted that unexpected cyclization of the peptide product occurred when leu_2 was substituted by orn that resulted in the lack of one or two of the original amino acid in the finished peptide product (Schneider *et al.*, 1998). These results suggest that the double crossover event disrupts the optimum enzyme capacity, and that the placement of specific aaa modules in the PS enzymes is important in the production of a complete peptide (Schneider *et al.*, 1998).

A. 8 Research design

The objectives of this research were fourfold. The first objective was to characterize the environmental bacterial isolate that was able to inhibit the growth of *L. maculans*, a pathogen of canola, as well as other disease causing fungi of canola: *A. brassicae, F. avenaceum, Pythium* sp., *R. solani* AG2-1, and *S. sclerotiorum*. The

second objective was to establish cultivation conditions that optimized production of the antifungal antibiotic by the producer organism. The third objective was to purify and characterize the antifungal antibiotic responsible for inhibition of *L. maculans*. The fourth objective was to investigate the peptide synthetase gene(s) responsible for the production of the antifungal antibiotic. A detailed list of the goals for each chapter is given in the introduction to that chapter.

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I.1 Introduction: Characterization of a Bacterial Environmental Isolate as *Paenibacillus polymyxa*

I.1.1 The genus *Paenibacillus* and characteristics of *Paenibacillus polymyxa*

Paenibacillus polymyxa was a member of the genus *Bacillus* until Ash *et al.* (1993) determined that it should belong to a new genus *Paenibacillus* and described it as the type species. Previously, the genus *Bacillus* consisted of bacteria that were grouped together based on three characteristics: rod-shape, aerobic or faculatively anaerobic metabolism and endospore-formation. This meant that the genus *Bacillus* included a wide variety of phenotypes such as strict aerobes, faculative anaerobes, acidophiles, alcalophiles, chemolithotrophs, halophiles, psychrophiles, and thermophiles, and that the DNA G+C content varied from 33 to 64% (Ash *et al.*, 1993). With the increased molecular biology techniques and equipment available to microbiologists came the idea that bacteria could be grouped on the basis of something more elemental than phenotype, such as direct molecular characteristics (Jurtshuk *et al.*, 1992). It began to be considered that defining phylogenetic groups on phenotypic characteristics was subjective and could lead to misrepresentation of the different types of known bacteria and a misunderstanding of the evolution of bacteria (Ash *et al.*, 1991). Woese (1987) argued that genotypic information was superior to phenotypic information as a means of classifying bacteria, because it was easier to interpret and it gave more clues to the evolutionary background of the organism. Woese (1987) also suggested that when gene similarity is used as the determinant of a group of bacteria, the gene should be ubiquitous and essential to all bacteria so that comparisons with all sorts of bacteria can be made. The use of 16S rRNA sequence as a source of genotypic information for comparison of bacteria has been accepted by many taxonomists because it appears to be ubiquitous and essential to the organism and so the sequence changes very slowly over time (Woese, 1987).

Ash *et al*, (1991; 1993) regrouped the bacteria formerly in the genus *Bacillus* into five phylogenetic clusters based on 16S rRNA sequence similarity. This original grouping of five clusters in the genus *Bacillus* was re-grouped by other researchers into at least ten phylogenetic clusters, also based on 16S rRNA similarity (Shida *et al.*, 1997a). One of these clusters was comprised of the acidophilic species and was reclassified as the genus *Alicyclobacillus* (Wisotzkey *et al.*, 1992), the halophilic species cluster was reclassified into the genus *Halobacillus* and the group four cluster of Ash *et al.* (1991;

1993) was split and reclassified into two genera *Brevibacillus* and *Aneurinibacillus* (Shida *et al.*, 1997a). The bacteria in the group three cluster: *B. polymyxa*, *B. alvei*, *B. gordonae*, *B. larvae*, *B. pulvifaciens*, *B. macerans*, *B. azotofixans*, *B. pabuli*, *B. macquariensis*, *B. amylolyticus* and *B. validus*, were reclassified by Ash *et al.* (1993) and placed in the new genus *Paenibacillus* (from the Latin, paene, meaning almost). Emendments to this original compilation of *Paenibacillus* have since been reported; *P. gordonae* and *P. validus* were found to be the same species and *P. pulvifaciens* was determined to be a subspecies of *P. larvae* (Shida *et al.*, 1997a). The following bacterial species have also been added to the genus *Paenibacillus*: *B. lautus*, *B. peoriae*, *B. alginolyticus*, *B. chondroitinus*, *B. curdlanolyticus*, *B. glucanolyticus*, *B. kobensis* and *B. thiaminolyticus* (Heyndrickx *et al.*, 1996; Shida *et al.*, 1997a; 1997b; Achouak *et al.*, 1999). *Clostridium duram*, which had been moved to the genus *Paenibacillus*, has since been reclassified as a strain of *P. azotofixans* (Rosado *et al.*, 1997). Two new species, previously considered to be members of *B. circulans*, have been described and named *P. illinoisensis* and *P. chibensis* (Shida *et al.*, 1997b). Another new species was also introduced to the genus as *P. apiarius* (Shida *et al.*, 1997a). The species within the genus *Paenibacillus* have 16S rRNA sequences with at least 89.6% similarity.

The use of 16S rRNA comparison, based on sequences derived from PCR-amplified 16S rRNA genes, to determine clusters of similar bacteria within a diverse collection of bacteria, appears to have been accepted by the scientific community, however its use in determining similarity between closely related species has been questioned. Nubel *et al.* (1996) amplified a 347 bp fragment containing variable regions V6 and V8 of 16S rRNA from the genomes of four strains of *P. polymyxa*. The researchers analysed the PCR products from each strain by temperature gradient gel electrophoresis, which can separate DNA fragments based on sequence differences. Each *P. polymyxa* strain had multiple PCR products amplified from its genome. The PCR products from one of the strains were cloned into a plasmid and sequenced. Six different PCR products, of the same length but differing at ten nucleotides, were found within this one strain of *P. polymyxa*. These six PCR products were radiolabelled and hybridized to the digested genome, and were found to be scattered throughout the chromosome. It was determined that *P. polymyxa* strains tend to carry many 16S rRNA genes with different sequences throughout their genomes. The researchers suggested that 16S rRNA sequence heterogenicity should be taken into account when assessing the similarity of closely related species and designing rRNA-specific probes.

Paenibacillus species share the following traits: rod-shape, development of subterminal ellipsoidal spores in swollen sporangia, motility by peritrichous flagella, optimum growth at pH 7.0, growth inhibition by 10% NaCl, no pigment production on nutrient agar, no prototrophs, no production of hydrogen sulfide, and anteiso-$C_{15:0}$ as the major cellular fatty acid. Variable traits in this genus are: Gram stain response (Gram positive, negative or variable), faculatively anaerobic or strictly aerobic, catalase activity (all but two subspecies; *P. larvae* subsp. *larvae* and subsp. *pulvifaciens*, are catalase positive), oxidase activity, indole production, nitrate reduction to nitrite, polysaccharide degradation, starch, casein or urea hydrolysis, the optimum growth temperature range (optimum temperature for all but one species, *P. macquariensis*, is 28-30°C), gas production (four of the species; *P. polymyxa, P. peoriae, P. azotofixans* and *P. macerans* produce gas from certain sugars), and the G+C content ranges from 45-54 mol % (Ash *et al*, 1991; 1993; Shida *et al.*, 1997a; 1997b).

P. polymyxa, is a Gram positive organism and as the species name suggests (poly-myxa is from the Greek, meaning much-slime), produces a large amount of extracellular levan (polysaccharide) in liquid and on solid media. The colonies are opaque, tan and glistening, with an entire edge and contoured surface. This species tends to have complex nutrient requirements and so are uncommon in poor nutrient soils, favouring growth on rotting plant material or in association with plant roots in the soil (Sonenshein, 1993).

There have been no reports of *P. polymyxa* infection in humans or animals, although it can cause spoilage of canned fruits due to its ability to grow in low pH and remain dormant as endospores. *P. polymyxa* was also isolated, along with other spore forming bacteria, from paperboard that is used to make cartons for packaging of food-stuff liquids such as milk and milk products (Pirttijarvi *et al.*, 1996). However, *P. polymyxa* was not found to contaminate the food itself. This bacterial species has also been found in medicated creams, lotions, antacids and toothpaste (Parry *et al.*, 1983).

I.1.2 Life cycle of endospore-forming bacteria

P. polymyxa has a vegetative (growth) stage and a spore (dormant) stage in its life cycle (Figure I.1.2.; Doi and McGloughlin, 1992). The bacterium grows in a vegetative state until environmental signals such as nutrient deficiency or cell density trigger the cells to form endospores. Endospores are spores that are formed within a

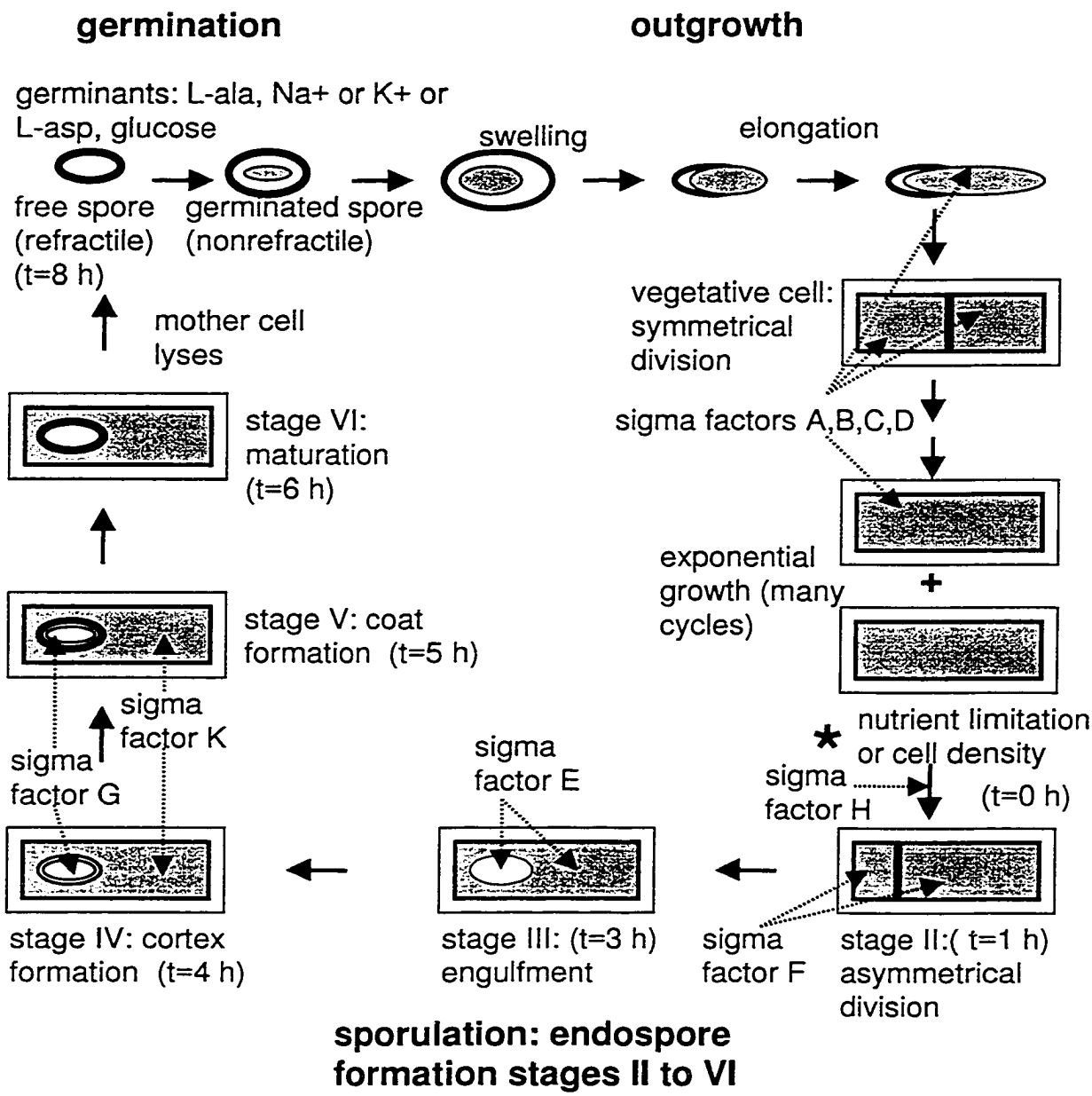


Figure I.1.2. Life cycle of an endospore forming bacterium such as *P. polymyxa*. The bacteria can go through many cycles or one cycle of exponential growth before forming spores again. * At this point, just before onset of sporulation, secondary metabolites such as antibiotics are produced. Generalized times (t=n) are given for the onset and duration of sporulation. The sigma factors and the genes they activate are: A, housekeeping genes; B and C, unknown; D, flagellar genes; H, initiation of sporulation; F, differentiation of prespore and mother cell; E, engulfment of forespore; G, forespore genes; and K, mother cell genes (Doi and McGloughlin, 1992).

mother cell and released after lysis of the mother cell surrounding the spore. When environmental conditions are favourable for vegetative growth, the spores develop into vegetative cells. Spores are resistant to UV radiation, heat and dessication and can remain in the spore stage for extended periods of time while retaining the capability to return to the vegetative stage.

Researchers have studied the regulation of cell cycle events in *B. subtilis* as a model endospore-forming organism (Cosmina *et al.*, 1993; Spiegelman *et al.*, 1995). Regulation of antibiotic production (gramicidin S and tyrocidine) has also been studied in *B. brevis* (Spiegelman *et al.*, 1995; Mootz and Marahiel, 1997). The regulatory proteins and systems of regulation in these bacteria have been seen to be very similar. It is generally considered that the regulation of cellular systems observed in these organisms can be extended to other endospore forming bacteria, such as *P. polymyxa*.

The cell cycle events in endospore-forming bacteria are highly regulated to ensure that the cell will not commit to sporulation, which is energetically expensive, unless there is a need to do so. It is speculated that if *Bacillus* cells in nature are usually in a nutrient-limited or a nutrient-variable state, then they are constantly in a 'boundary' phase where they are ready to either return to growth or start sporulation (Spiegelman *et al.*, 1995). Central to this hypothesis is the ability of the cell to take in and process external signals such as an increase or decrease in the concentrations of nutrients or intercellular communicators.

The transition from exponential phase to sporulation (stationary phase) is a metabolically active phase for endospore-forming bacteria. Bacteria become highly motile, develop competence for DNA uptake, secrete enzymes such as amylases and proteases and often produce secondary metabolites such as antibiotics (O'Reilly and Devine, 1997).

Extracellular signals must be very important to endospore forming bacteria so that they can react to environmental fluctuations such as carbon, nitrogen and phosphorus levels and to their own cell density (Marahiel *et al.*, 1993; Cosby *et al.*, 1998). The transcription of genes that produce gene products associated with onset of sporulation, such as peptide synthetase enzymes that are directly related to sporulation at certain stages, can be reduced or inhibited by glucose, glutamine and acidic pH. Schaeffer *et al.* (1965) determined that glucose containing medium allowed for the highest growth rate of *B. subtilis* culture but did not allow for the most sporulation, and increasing the glucose concentration repressed sporulation. These authors also

determined that growth of *B. subtilis* in NH_4^+ containing medium allowed for an increased intracellular pool of glutamine than growth of the bacterium in other nitrogen sources such as NO_3^- or N_2, and that under those conditions sporulation was also repressed. Cosby *et al.*, (1998) found that surfactin production in *B. subtilis* was induced in low cell density, glucose and glutamine containing cultures when the pH of the culture broth was increased to neutral pH. The authors suggested that a putative extracellular factor that induces surfactin production may be present in low cell density, glucose and glutamine containing cultures, but it is not active at acidic pH.

Bacillus spores germinate when the environmental conditions are favourable for growth. Even though endospores have a nearly impermeable wall, needed to protect the bacterial DNA from environmental damage, they are still able to sense environmental signals. The rate of germination of a spore suspension is decreased, even in nutrient rich conditions, if the OD_{600} of the spore suspension is 0.5 OD units or higher (Doi and McGloughlin, 1992). This effect is brought about by a racemase in the spore, that is triggered by high spore density to convert L-ala in the spore to D-ala, which inhibits protein formation. Some of the compounds that trigger germination of *Bacillus* spores (germinants) are L-ala with Na^+ or K^+, L-ala analogues and L-asp with glucose. Germination of some bacterial spores is triggered by specialized germinants; for instance *B. thuringiensis* spores will germinate in the presence of larval gut fluid (Doi and McGloughlin, 1992; Harwood and Cutting, 1990).

I.1.3 Industrial and commercial uses of *P. polymyxa*

P. polymyxa as a plant growth promoting rhizosphere bacterium has the potential to be commercially useful in agriculture (Section A.5), and many of its cellular products also have commercial uses. Some examples of compounds made by *P. polymyxa* that have commercial value are; 2,3-butanediol, levans, colistins/polymyxins, α- and β-amylases, and pentosanases (xylanases; Doi and McGloughlin, 1992).

The alcohol 2,3-butanediol is produced by *P. polymyxa* as a by-product of fermentation. This fermentation process is termed diol fermentation. Pyruvate (2 mol) is converted to 1 mol 2,3-butanediol to reoxidize NADH to NAD^+, so glycolysis can occur. This reaction cannot recycle all the NADH needed by the cell, therefore other metabolites, such as ethanol, acetoin, lactic acid and acetic acid are also generated. The production of 2,3-butanediol by growth of *P. polymyxa* on five-carbon sugars such as

xylose has been optimized so that lignocellulosic wastes can be utilized in the culture. This is both a good and economic source of xylose. Even though 2,3-butanediol is formed by an anaerobic process., some aeration of the culture by stirring or shaking can increase production of the chemical. This is thought to be caused by the agitation of the culture allowing CO_2 to disperse from the medium and exposing new substrate to the bacterial population (Garg and Jain, 1995). 2,3-Butanediol is a colourless, odourless chemical with a high boiling point (184°C) and a low freezing point (-60°C). It is produced by *P. polymyxa* in a 98% optically pure form when the culture is grown under specific conditions (Nakashimada *et al.*., 1998). This chemical has a wide variety of uses; antifreeze agent, high-octane fuel additive, aviation fuel, explosives, food additive as a flavouring agent (when converted to diacetyl), precursor of polyurethane foams for use in drugs, cosmetics and lotions, antiseptic agent (0.1% solution can kill many bacteria), printing inks, perfumes, fumigants and as a solvent for resins and laquers (Garg and Jain, 1995).

Levans are extracellular polysaccharides made up of D-fructose, linked with a β-(2 to 6) linkage, that can consist of up to 3 million residues. The levan made by *P. polymyxa* is closely related in chemical structure to a plant produced polyfructan (inulin) that has the D-fructose units linked by a β-(2 to 1) linkage. *P. polymyxa* produces three times more levans than other known polysaccharide producers when grown on an 8% sucrose medium. It has been suggested that *P. polymyxa* produces and secretes levans so that they can be used as a 'glue' to stick the bacteria to the roots of certain plants. *P. polymyxa* has been found on the roots of spring wheat, where it acts as a PGPR (Section A.5) bacterium of wheat.. As well, the spring wheat roots excrete sucrose that *P. polymyxa* can use as a carbon and energy source, and for the production of levan (Bezzate *et al.*, 1994). Levan is extracted from producing organisms for use as low-cost gums for food, cosmetic and industrial purposes (Han, 1989).

Extracellular α- and β-amylases are produced by *P. polymyxa* (Doi and McGloughlin, 1992). These enzymes degrade amylose, a glucose polymer consisting of α-1,4-glucoside and α-1,6-glucoside linked glucose molecules, that is a part of starch. The α-amylases are endoenzymes; they cleave internal α-1,4 linkages, whereas the β-amylases are exoenzymes and cleave the same linkage from the end of the sugar chain (Doi and McGloughlin, 1992). The α-amylases are used in syrup, adhesives, sewage

treatment and detergent-producing industries and β-amylases are used in brewing, distilling and baking industries (Doi and McGloughlin, 1992).

P. polymyxa produces extracellular xylanases that can hydrolyse xylan, a polysaccharide made up of the pentose xylose in β-(1, 4) linkages. Xylans are present in the cell wall of all land plants; they are the major polysaccharide (or hemicellulose) of monocots and can represent from 7 to 30% of the total dry weight of wood (Morales *et al.*, 1993). Xylanases are used by the food and feed industries in many applications that require the breakdown of xylan (Doi and McGloughlin, 1992).

I.1.4 Antibiotics produced by *P. polymyxa*

P. polymyxa is known to produce several peptide antibiotics (Table I.1.4). A major family of peptide antibiotics produced by this species is the polymyxin/colistin/circulin family (Figure A.6; Umezawa *et al.*, 1978; Shoji *et al.*, 1977). Other peptide antibiotics produced by *P. polymyxa* are: polypeptins (Figure I.1.4; Doi and McGloughlin, 1992), jolipeptin (Ito and Koyama, 1972a; 1972b), gatavalin (Nakajima *et al.*, 1972; Nakajima *et al.*, 1975), gavaserin, saltavidin (Pichard *et al.*, 1995), LI-F03, -F05, -F07, –F08 (Kurusu and Ohiba, 1987), fusaricidins A,B,C and D (Figure I.1.4; Kajimura and Kaneda, 1996; 1997) and polyxin (Piuri *et al.*, 1998). There are also numerous reports in the literature of *P. polymyxa* species exhibiting antimicrobial and or antifungal activity, but the nature of the antibiotic activity was not determined.

The first polymyxins were discovered by researchers in 1947; afterward many more polymyxin-like antibiotics were found, including colistin A and B (which turned out to be identical to polymyxins E_1 and E_2; Storm *et al.*, 1977; Volger and Studer, 1966). Colistins A and B (polymyxins E_1 and E_2) were originally isolated from a bacterial strain called *Bacillus colistinus*. This species was later found to be a subspecies of *P. polymyxa*, now called *P. polymyxa* subsp. *colistinus koyama* (Nakajima *et al.*, 1972). Polymyxin F was originally thought to be produced by *B. circulans* subsp. *jordan*, but was later determined to be *P. polymyxa* (ATCC). Polymyxins with the same letter designation, such as E_1 and E_2, were originally reported as one antibiotic compound but were later found to consist of two separable components (Volger and Studer, 1966). The polymyxin group of antibiotics are all polycationic, contain five or six 2,4-diaminobutyric acid (DAB) residues each and a fatty acid component. They are decapeptides made of a seven membered ring structure and a three membered side chain with the fatty acid

Table I.1.4 Antibiotics discovered from *P. polymyxa*.

name	composition[a]	structure[b]	properties	activity[c]	reference
polymyxins B_1, B_2 C (P), D_1, D_2, E_1, (colA), E_2, (colB), M (A), S, T	refer to Figure A.6	yes	stable in pH 2 to 7 with heat, unstable at 23°C after 36 h, active after pepsin, trypsin, pancreatin, soluble: methanol, water, insoluble: ether, MW~1280 Da	Gram + and – bacteria; alter membrane structure	Shoji *et al.*,1977; Umezawa *et al.*, 1978
polypeptins A,B	ile (1) D-phe (1) thr (1) D-val (1) leu (2) DAB[d] (3) R (1)= A: HMHA[e] B: HMPA[f]	yes	MW~1145 D	Gram + and – bacteria; alter membrane structure	Umezawa *et al.*, 1978; Doi and McGloughlin, 1992
jolipeptin[g]	glu (1) gly (1) ala (2) DAB (2) ser (2) val (2)	no	soluble: methanol, n-butanol, isopropanol, insoluble: methyl acetate, butyl acetate, diethyl ether, petroleum ether, benzene, chloroform, active after pH 2-11, 100°C, 10 min, active after proteinase, pos. ninhydrin, MW~800 Da	Gram+ and – bacteria; alters membrane structure	Ito and Koyama, 1972a;1972b
gavaserin[g]	ala (1) glu (1) ser (1) val (2) DAB (3) R (1)=octanate	no	insoluble in: butanol, ethyl acetate, ethanol, isopropanol, methanol soluble in: water, formic and acetic acids, positive ninhydrin test, active after pronase, trypsin, alkaline protease, active after heating to 110°, 30 min, MW=911 Da	microbial phytopathogens of cauliflower, *Xanthomonas campestris* pv. *campestris*	Pichard *et al.*, 1995
saltavalin[g]	ala (1) ser (1) val (1) DAB (2) leu (2) thr (2)	no	same as gavaserin except MW=903 Da	microbial phytopathogens of cauliflower, *X. campestris* pv. *campestris*	Pichard *et al.*, 1995

Table I.1.4 continued

name	composition[a]	structure[b]	chemical characteristics	activity[c]	reference
gatavalin[g] (LI-F04)	asx (1.1) glx (1.0) ala (2.2) thr (3.8) val (3.8)	no	soluble: DMSO[i], acetic acid, methanol, ethanol, slightly soluble: water, insoluble: ethylether, acetone, butylacetate, neg. ninhydrin, active: 10 min at pH3-100°C, inactive in alkali, MW LI-F04: 897 Da (100)[j] and 883 Da (90)[j]	Gram + bacteria, mycobacteria, yeasts, molds	Nakajima *et al.*, 1972; Nakajima *et al.*, 1975; Kurusu and Ohba, 1987
LI-F03	asx (1.0) glx (1.0) ala (2.0) tyr (1.7) val (2.2) thr (3.8)	no	slightly soluble: water, methanol, acetonitrile, insoluble: chloroform, acetone, ethyl acetate, diethyl ether, n-hexane, neg. ninhydrin test, MW=961 Da (89)[j] and 947 Da (100)[j]	Gram + bacteria, fungi, yeasts	Kurusu and Ohba, 1987
LI-F05	asx (1.0) glx (1.0) ile (0.9) X[h] (0.7) ala (2.0) val (1.6) thr (3.6)	no	same as LI-F03 MW=911 Da (100)[j] and 897 Da (87)[j]	Gram + bacteria, fungi, yeasts	Kurusu and Ohba, 1987
LI-F07	asx (1.3) glx (1.0) phe (1.5) ala (2.4) val (2.2) thr (4.3)	no	same as LI-F03 MW=945 Da (100)[j] and 931 Da (94)[j]	Gram + bacteria, fungi, yeasts	Kurusu and Ohba, 1987
LI-F08	asx (0.9) glx (1.0) ile (1.5) X[h] (1.5) ala (2.0) thr (3.5)	no	same as LI-F03 MW=925 Da (100)[j] and 911 Da (96)[j]	Gram + bacteria, fungi, yeasts	Kurusu and Ohba, 1987

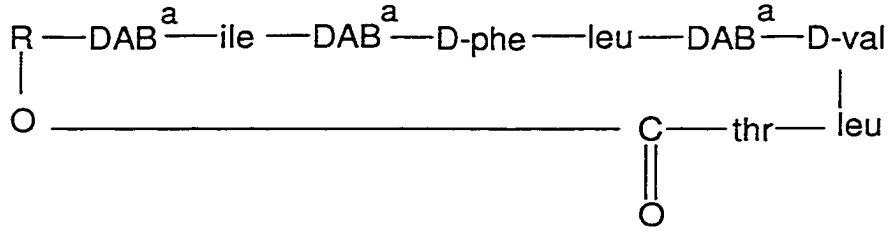
Table I.1.4 continued

name	composition[a]	structure[b]	chemical characteristics	activity[c]	reference
fusaricidin A	ala (1) asn (1) val (2) thr (2) R (1)=GHPD[j]	yes	negative ninhydrin test, soluble inmethanol, DMSO, insoluble in chloroform, n-hexane. MW 883	Gram + bacteria, fungi	Kajimura and Kaneda, 1996
fusaricidin B	ala (1) gln (1) val (2) thr (2) R (1)=GHPD[j]	yes	negative ninhydrin test, soluble inmethanol, DMSO, insoluble in chloroform, n-hexane. MW 897	Gram + bacteria, fungi	Kajimura and Kaneda, 1997
fusaricidin C	ala (1) asn (1) tyr (1) val (1) thr (2) R (1)=GHPD[j]	yes	negative ninhydrin test, soluble inmethanol, DMSO, insoluble in chloroform, n-hexane. MW 947	Gram + bacteria, fungi	Kajimura and Kaneda, 1997
fusaricidin D	ala (1) gln (1) tyr (1) val (1) thr (2) R (1)=GHPD[j]	yes	negative ninhydrin test, soluble inmethanol, DMSO, insoluble in chloroform, n-hexane. MW 961	Gram + bacteria, fungi	Kajimura and Kaneda, 1997
polyxin	unknown	no	inactivated by pronase E, trypsin, proteinase K, inactive in alkaline conditions (pH 9 to 10), active at pH 2 to 8, decreased activity after heating to 100°C for 10 min, MW less than 10kDa	bacteriocidal against Gram +, bacteriostatic against Gram -	Piuri *et al.*, 1998

[a] amino acids are in the L conformation unless otherwise stated.
[b] if the peptide structure has been elucidated, it is depicted in Figure I.1.4 or in Figure A.6 as indicated.
[c] mode of action is listed if known.
[d] DAB, 2,4-diaminobutyric acid.

[e] HMHA, 3-hydroxy-4,6-dimethylheptanoic acid
[f] HMPA, 3-hydroxy-4-methyl-pentanoic acid.
[g] the conformation of the amino acids in these antibiotics was not determined.
[h] X, unidentified amino acid.
[i] DMSO, dimethylsulfoxide.
[j] relative intensities of these parent ions from FAB-MS (m/z).
[j] GHPD, 15-guanidino-3-hydroxypentadecanoic acid

A

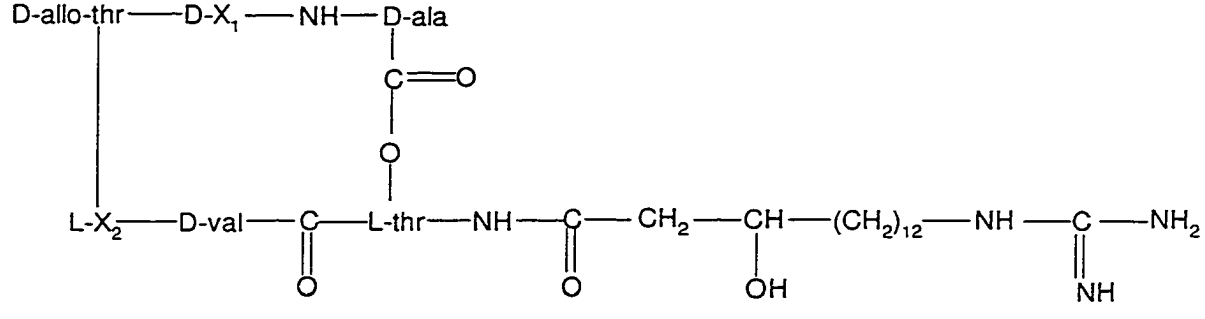


Polypeptin A: R=3-hydroxy-4,6-dimethylheptanoic acid

Polypeptin B: R=3-hydroxy-4-methylpentanoic acid

[a] DAB: 2,4-diaminobutyric acid

B



fusaricidin	$D\text{-}X_1$	$L\text{-}X_2$
A	D-asn	L-val
B	D-gln	L-val
C	D-asn	tyr
D	D-gln	tyr

Figure I.1.4. The structures of A. polypeptin A and B and of B. fusaricidin A, B, C, and D. Adapted from Umezawa *et al.*, 1978 and Kajimura and Kaneda, 1996 and 1997.

bound to the end of the chain by an amide bond to the α-amino group of DAB (Paulus and Gray, 1964; Volger and Studer, 1966). Cyclization of the ring structure occurs by an amide bond involving the γ-amino group of the third (or second for polymyxins C, D_1 and S) DAB and the α-carboxyl group of the last threonine (Volger and Studer, 1966). The various polymyxins differ at three amino acid sites in the structure, and by the nature of the fatty acid component (Figure A.6). The different polymyxins are all effective inhibitors of Gram negative bacteria and moderate inhibitors of some Gram positive bacteria, though the 6-methyloctanoic acid containing polymyxins tend to be more active than the polymyxins with 6-methylheptanoic acid (Storm *et al.*, 1977). The mode of action is by alteration of bacterial membrane structure. This is thought to occur by insertion of the fatty acid side chain into the hydrophobic domain of the cell membrane phospholipid bilayer, putting the polycationic ring structure in a position to displace the Mg^{2+} and Ca^{2+} ions that normally interact with the negatively charged polar heads of the phospholipid (Storm *et al.*, 1977). Many of the uses for polymyxins in medicine are limited because they can cause proteinuria and histopathological lesions in the kidneys (nephrotoxicity) of animals (Korzybski *et al.*, 1978). Polymyxins B, E_1 and E_2 are the least nephrotoxic so are used internally to treat Gram negative bacterial infections in humans caused by: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *E. coli*, and *Shigella* sp. and topically to treat *P. aeruginosa* infections of burns patients (Korzybski *et al.*, 1978; Parry *et al.*, 1983). Polymyxins have also been used as molecular tools to study structure and function of bacterial membranes in the laboratory (Storm *et al.*, 1977). Another family of antibiotics, the octapeptins isolated from *B. circulans*, are similar to polymyxins in that they all contain five DAB residues and are cyclic with an amino acid side chain and an attached fatty acid component. They differ from polymyxins in that they are comprised of eight amino acids instead of ten, and the fatty acid component can be 3-hydroxy-8-methyldecanoic acid, 3-hydroxy-8-methylnonanoic acid, 3-hydroxydecanoic acid or 3-hydroxy-6-methyloctanoic acid, whereas the polymyxins carry either 6-methyloctanoic acid or 6-methylheptanoic acid (Umezawa *et al.*, 1978).

Polypeptins A and B were originally isolated from *B. circulans* (Korzybski *et al.*, 1978), but have also been described as being produced by *P. polymyxa* (Doi and McGloughlin, 1992). These two antibiotics are similar to the polymyxins in that they contain three DAB residues and a fatty acid component, but differ in most other aspects (Umezawa *et al.*, 1978). Polypeptins A and B differ from each other by the fatty acid component; polypeptin A carries 3-hydroxy-4,6-dimethylheptanoic acid and polypeptin B

carries 3-hydroxy-4-methylpentanoic acid. The antibiotic structures contain a nine amino acid peptide that is cyclized by the formation of an ester bond between threonine and the fatty acid (Umezawa *et al.*, 1978). These antibiotics are active against Gram negative and some Gram positive bacteria. They appear to have the same mode of action as the polymyxins (Storm *et al.*, 1977).

Jolipeptin is an antibiotic isolated from *P. polymyxa* subsp. *colistinus* that is active against Gram negative organisms such as *E. coli* and *P. aeruginosa*, and Gram positive organisms such as *B. subtilis*. This antibiotic differs from polymyxins E_1 and E_2, which are also produced by *P. polymyxa* subsp. *colistinus*, not only in composition and structure but also by their distribution within the producing culture. Jolipeptin was isolated from the ribosomal fraction after ultracentrifugation of lysed cells, whereas polymyxins E1 and E2 were isolated from culture broth after the bacterial culture was acidified to pH 3 and heated to 80°C for 10 min (Nakajima *et al.*, 1972). Jolipeptin production within the cell occurred concomitantly with the end of the exponential growth phase, as with other peptide antibiotics (Ito and Koyama, 1972a). Jolipeptin is a decapeptide containing two DAB compounds, similar to polymyxins, but it does not carry a fatty acid component. The structure of this antibiotic is not known (Koryzybski *et al.*, 1978). Jolipeptin appears to inhibit bacterial cells by altering the permeability of the cellular membrane (Ito and Koyama, 1972b).

Gatavalin is another peptide antibiotic produced by *P. polymyxa* subsp. *colistinus*. This antibiotic is different from the other peptide antibiotics discussed so far in that it contains no DAB residues and is active against Gram positive bacteria, yeasts and fungi, but not Gram negative bacteria (Nakajima *et al.*, 1972). Some of the Gram positive bacteria gatavalin is active against are: *B. subtilis*, *B. cereus* and *Staphylococcus aureus*, and some of the fungi are: *Candida pseudotropicalis*, *S. cerevisiae*, *Aspergillus niger*, *Penicillium expansum* and *Fusarium* sp. (Nakajima *et al.*, 1972). Gatavalin is found in the culture broth of *P. polymyxa* subsp. *colistinus* along with polymyxins E_1 and E_2, after they are released from the bacteria by acidification to pH 3 and heating to 80°C for 10 min (Korzybski *et al.*, 1978). The structure is not known, though from the molar ratios of the amino acids detected by compositional analysis, it appears to be a decapeptide (Nakajima *et al.*, 1975).

The antibiotics gavaserin and saltavadin were isolated from a strain of *P. polymyxa* found on cauliflower seeds (Pichard *et al.*, 1995). This strain of *P. polymyxa* had an inhibitory effect on microbial pathogens of cauliflower such as *Xanthomonas*

campestris pv. *campestris.* Inhibition was caused by gavaserin and saltavidin. The structures of these two antibiotics are not known, but amino acid compositional analysis showed that they are similar to polymyxins in that they both contain DAB residues; gavaserin has three and saltavidin has two. As well, gavaserin has a fatty acid component, octanoic acid, whereas saltavidin does not (Pichard *et al.*, 1995). These two antibiotics could be isolated from the culture broth from 12 h before sporulation until stationary phase (Pichard *et al.*, 1995).

Five antibiotics named LI-F03, LI-F04, LI-F05, LI-F07 and LI-F08 were isolated from the culture broth of *P. polymyxa* strain L-1129 and contained within a sample that was active against the indicator organism, *S. aureus.* This active sample was resolved by reverse phase HPLC into five fractions active against *S. aureus.* The five fractions were analyzed by fast atom bombardment mass spectroscopy (FAB-MS), where it was determined that each fraction was still a mixture of two components that differed in MW by 14. The amino acid composition of these peptide antibiotics was determined; none contain DAB or carry a fatty acid side chain like the polymyxins. LI-F03, LI-F04, LI-F05, LI-F07 and LI-F08 were all similar to each other in that they all contain the same number of aspartic acid, glutamic acid, alanine and threonine amino acid residues (except LI-F04 contains one less threonine than the rest) with additional amino acid residues unique to each (Kurusu and Ohba, 1987). It was also determined that the amino acid compositions determined for LI-F04 and for gatavalin were identical, suggesting that these two peptides are the same antibiotic (Nakajima *et al.*, 1975; Kurusu and Ohba, 1987).

The antibiotics fusaricidin A,B,C and D were isolated from the culture broth *of P. polymyxa* KT-8, with fusaricidin A as the major component of the active fraction (Kajimura and Kaneda, 1996; 1997). This bacterium was found in the rhizosphere of a garlic plant that was infected with *Fusarium oxysporum*, a fungus that causes basal rot of plants. These antibiotics are active against Gram positive bacteria and fungi. Fusaricidin A,B,C and D are hexa-depsipeptides that all carry a 15-guanidino-3-hydroxypentadecanoic acid moiety. Fusaricidins A and B were separated from the active mixture but C and D were not separable (Kajimura and Kaneda, 1997).

A strain of *P. polymyxa* that was able to inhibit many Gram positive and Gram negative bacteria, including the food-contaminating pathogens: *B. cereus* and *E. coli* and non-pathogens such as lactic acid bacterial strains, was isolated from Argentinian regional fermented sausages (Piuri *et al.*, 1998). The antimicrobial activity was found in the culture broth during stationary phase when the *P. polymyxa* strain was grown under

culture conditions where only 0.1% of the cells had sporulated. No antimicrobial activity against the indicator organism, *Lactobacillus plantarum*, was found when *P. polymyxa* was grown on a different medium, where 10% of the cells had sporulated. The crude active fraction was sensitive to proteolytic enzymes and was not dialysable through 12 kD cut-off dialysis tubing. Based on these findings the inhibitory agent was thought to have bacteriocin-like characteristics, unlike any other antibiotic known to be produced by *P. polymyxa*, and so was named polyxin (Piuri *et al.*, 1998).

There are many reports in the literature on the antimicrobial and antifungal activities of *P. polymyxa* by undefined inhibitory agents (Rosado and Seldin, 1993; Smid *et al.*, 1993; Oedjijono *et al.*, 1993; Mavingui and Heulin, 1994; Liang *et al.*, 1996; Dijksterhuis *et al.*, 1999; Seldin *et al.*, 1999). The authors of the reports either indicated that they were in the process of determining the nature of the activity or that the focus of the research was to characterize the producer strain instead.

Two of these reports (Rosado and Seldin, 1993; Seldin *et al.*, 1999) are continuing studies on *P. polymyxa* strain SCE2 that shows inhibitory activity against Gram positive bacteria, Gram negative bacteria and fungi. The authors concentrated on the inhibitory activity of this strain against human pathogenic organisms, including 28 multi-drug resistant *S. aureus* strains, all inhibited by *P. polymyxa* SCE2. This range of inhibition is different than the other antibiotics known to be produced by *P. polymyxa* to date, which tend to be active against Gram negative and Gram positive bacteria (but not fungi) or against Gram positive bacteria and fungi (but not Gram negative bacteria). This activity spectrum suggests that *P. polymyxa* SCE2 produces two or more different antibiotics that together can inhibit a wide range of organisms.

Other reports concerning the production of antibiotics by *P. polymyxa* are concentrated on strains isolated from plant rhizospheres, and their ability to inhibit plant pathogenic fungi (Section A.5). In many of these cases the identification of the inhibitory agent is either not addressed or is in progress.

I.1.5 Research design

This chapter will focus on the characterization of a bacterial strain isolated from canola stubble that was inhibitory against *L. maculans*. Analyses such as cellular and colonial morphology, biochemical tests and fatty acid methyl ester (FAME) were initially done to determine the identity of the environmental isolate as *P. polymyxa*. The 16S

rRNA gene was amplified from the bacterial genome, sequenced and compared to known 16S rRNA genes in the DNA databases to search for those most similar to the isolate. The growth cycle and antibiotic production of the *P. polymyxa* isolate was characterized and the growth conditions were modified to optimize antibiotic production. The growth and antibiotic production of other strains of *P. polymyxa* were characterized and compared to the *P. polymyxa* strain isolated from canola stubble.

I.2 Materials and Methods

I.2.1 Strains of bacteria and fungi and other reagents and supplies

Paenibacillus polymyxa PKB1 was from P. Kharbanda at the Alberta Research Council (ARC). A strain of *Leptosphaeria maculans*, that was highly virulent in the formation of blackleg on canola, was also obtained from P. Kharbanda. *Bacillus polymyxa* subsp. *koyama* was obtained from the American Type Culture Collection (ATCC No. 21830; Rockville, Maryland), *Paenibacillus polymyxa* SCE2 was obtained from V. Clementino, Department of Biological Sciences, University of Alberta, *B. polymyxa* NCIMB 8648 was obtained from T. Hantos, Department of Biological Sciences, University of Alberta,

Taq DNA polymerase used for amplification of DNA in the polymerase chain reaction (PCR) was obtained from A. Hashimoto, Department of Biological Sciences, University of Alberta.

All chemicals were reagent grade, obtained from Sigma (St. Louis, Missouri) or Fisher Chemical (Fairlawn, New Jersey) unless otherwise stated.

I.2.2 *P. polymyxa* storage conditions

P. polymyxa was maintained as 20% glycerol spore stocks stored at -70°C or as a lyophilized stock (Section I.2.3.2) at -20°C.

I.2.3 Media used in optimization of growth and antibiotic production studies

All values are given as percent weight/volume unless otherwise stated.

Brain heart infusion (BHI) broth was purchased from Difco (Detroit, Michigan) and made according to manufacturer's instructions.

Katznelson and Lochhead (KL) medium (Paulus and Gray, 1964) contained 0.15% NH_4SO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 0.01%$CaCl_2$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $ZnSO_4$, 0.001% $MnSO_4 \cdot H_2O$ and 8.7% K_2HPO_4. Filter sterilized biotin (trace amount; New England Biolabs), 0.15% filter sterilized glucose, 0.001% dithiothreitol (DTT) and vitamin free, enzyme hydrolyzed 0.25% filter sterilized casein were added

after the medium was autoclaved. In modified KL medium (KLM1) 0.5% dextrin was substituted for 0.15% glucose, and DTT, NaCl and casein were omitted.

Nutrient broth (NB) contained 0.3% beef extract and 0.5% peptone; the pH was adjusted to 7.3.

Potato dextrose broth (PDB) was purchased from Difco and made according to manufacturer's instructions. PDB optimized media No. 1 (PDB1) contained 2.4% PDB, 0.2% $NaNO_3$, 0.14% KH_2PO_4, 0.014% Na_2SO_4 and 0.0006% $MgCl_2$. PDB optimized media No.2 (PDB2) contained the same ingredients as PDB1 but $NaNO_3$ was omitted. PDB No. 3 (PDB3) contained 2.4% PDB and 0.2% $NaNO_3$. PDB No.4 (PDB4) contained 2.4% PDB and 1.1% $NaNO_3$. PDB No. 5 (PDB5) contained 2.4% PDB and 2.1% $NaNO_3$. PDB No.6 (PDB6) contained 2.4% PDB and 1.1% urea.

Potato extract and maltose (PEM) contained 2.2% potato extract (Difco) and 0.22% maltose.

Starch dextrin peptone broth (SDP) contained 2.2% soluble starch, 0.22% dextrin (Difco) and 0.3% peptone (Difco).

Stansly's medium (Paulus and Gray, 1964) contained 1% glucose, 2% NH_4SO_4, 0.5% YE, 0.3% K_2HPO_4, 0.3% $MgSO_4 \cdot 7H_2O$, 0.005% NaCl and 0.001% $FeSO_4 \cdot 7H_2O$. Modified Stansly's No.1 (S1) contained the same ingredients as Stansly's medium except 0.5% dextrin was substituted for 1.0% glucose, and K_2HPO_4 and NaCl were omitted. Modified Stansly's No.2 (S2) contained 1% glucose, 1.05% $NaNO_3$, 0.5% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.001% $FeSO_4 \cdot 7H_2O$. Modified Stansly's No.3 (S3) contained the same ingredients as S2 except 0.5% YE was replaced with 0.5% soluble starch (Difco). Modified Stansly's No.4 (S4) contained the same ingredients as S3 but 0.15% KH_2PO_4 and 0.1% NH_4SO_4 were added. Modified Stansly's No.5 (S5) contained the same ingredients as S2 but 0.15% KH_2PO_4 and 0.1% NH_4SO_4 were added. For all the modified Stansly media, glucose, dextrin and KH_2PO_4 were autoclaved separately and added to the broth after it was autoclaved.

Trypticase soy with starch medium (TCSS) contained 3% trypticase soy (Difco) and 1% soluble starch. TCSS1 contained the same ingredients as TCSS with the addition of 0.5% NaCl, 1.7% filter sterilized casein, 0.25% K_2HPO_4 and 0.25% dextrose. The last three ingredients were added to the medium after it had been autoclaved, as sterile components.

The volume of the medium in the culture flasks, aeration and the growth temperature were all varied in separate experiments. The extent of growth was

monitored by measuring the OD_{600} of a 1/10 diluted sample of the culture using a UV/VIS Philips Model PU 8740 scanning spectrophotometer. The production of antifungal antibiotic by *P. polymyxa* was monitored using a method to extract a small quantity of antifungal material from cultures as described in Section I.2.3.3. The pH of the culture was monitored by measurement of the culture broth that was discarded in the mini-extraction method.

I.2.3.1 Standard production conditions for growth of *P. polymyxa*

Standard growth conditions used to grow various strains of *P. polymyxa* were: 1% (v/v) starter culture inoculum into 200 ml PDB in a 500 ml Erlenmeyer flask, shaken at 200 rpm and incubated at 28°C. The starter culture was also standardized to: 1% (v/v) inoculation from a 20% glycerol stock into 25 ml TCSS medium in a 125 ml Erlenmeyer flask, shaken at 200 rpm and incubated at 28°C.

I.2.3.2 Growth conditions for bioreactor cultures and lyophilization of biomass

The bioreactor cultures were grown and harvested by R. Mah, Department of Biological Sciences, University of Alberta. Bioreactor tanks with total volume capacities of 14 and 75 L, were used to grow 10 and 50 L of *P. polymyxa*, respectively. The 14 L vessel was 210 mm in diameter and 450 mm in height. Three impellers of 74 mm diameter were used to mix the culture. The 75 L vessel 310 mm in diameter and 925 mm in height. Three impellers of 100 mm in diameter were used to mix the culture. PDB was used as the medium and 1% (v/v) polypropylene glycol was added as an antifoam agent. The air flow was set to 15 L/min and the temperature was maintained at 26°C. *P. polymyxa* was added as a 1% inoculum in the 10 L bioreactor culture and a 5% inoculum in the 50 L bioreactor culture, from a TCSS starter culture grown for 24 h at 28°C and shaken at 250 rpm.

Lyophilized *P. polymyxa* stocks were generated using cells/spores from a 10 L or 30 L bioreactor that were pelleted by centrifugation at 15 300 X g for 10 min. The endospore pellet was resuspended as a slurry in distilled H_2O (18 g endospore/L distilled H_2O) and freeze dried in 200 ml portions each in 500 ml round bottom flasks on a Freeze Mobile 24 freeze dryer (Virtis Co, Inc., Gardiner, New York).

I.2.3.3 Mini-extraction method to monitor production of antifungal antibiotic

An aliquot, 1.5 ml, of the culture was removed from the rest of the culture and the cells or spores were pelleted by centrifugation in a microfuge at 16 000 X G for 5 min. The pellet was resuspended in 200 µl methanol and incubated at room temperature for 20 min. The cell or spore mass was pelleted again by centrifugation in a microfuge for 5 min and 75 µl of the methanol supernatant was used in a well bioassay (Section II.2.4.1).

I.2.4 Identification of *P. polymyxa* PKB1

I.2.4.1 Cell stains

Gram stains were done using the Bacto 3-step Gram stain kit (Difco). This kit works in a similar manner to regular Gram staining procedure, except that no water wash step is required. Cells were taken from both liquid culture and agar plates for Gram staining.

Flagellar stains were carried out by M. Hicks, Department of Biological Sciences, University of Alberta, following a modified version of the Gray staining method (Mayfield and Innis, 1977).

I.2.4.2 Biochemical tests and growth on selective and differential media

The API 20E and API 20NE identification tests (bioMérieux, Marcy-l'Etoile, FR) were done on *P. polymyxa* PKB1 by A. Greene, Department of Biological Sciences, University of Alberta. Pure colonies were prepared for the tests by incubation for approximately 1 wk on LB agar. Both tests were inoculated according to the instructions provided with the kits. In all cases, no growth occurred until 48 h after inoculation.

Both selective and differential media were prepared to test the bacterial isolate *P. polymyxa* PKB1 by A. Greene. These media were all prepared using the instructions given in the Difco Manual (1984); lactose broth, MacConkey agar, sheep blood agar (provided by A. Szenthe, Department of Biological Sciences, University of Alberta), mannitol salt agar, starch agar and triple sugar iron slant agar. Interpretations of the results were also carried out using the Difco Manual as a reference. When the use of a certain compound as the sole carbon source by the bacterium was tested, it was added

to B+N8P medium (Kropp *et al.*,1994). Each medium was inoculated with *P. polymyxa* PKB1 and incubated at room temperature. Positive control medium for growth of *P. polymyxa* PKB1 was LB agar.

I.2.4.3 Fatty Acid Methyl Ester (FAME) analysis of *P. polymyxa* PKB1

FAME anaysis of the *P. polymyxa* PKB1 isolate versus a type culture collection strain of *P. polymyxa*, NCIB 8648 was conducted by J. Germida, University of Saskatoon, Sask. Both strains of bacteria were grown on TCSS agar slants, under the same conditions.

I.2.4.4 Chitinase Assay of *P. polymyxa* PKB1

P. polymyxa PKB1 was tested for the ability to degrade chitin using the assay procedure outlined by O'Brien and Colwell (1987). The substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide was purchased from Sigma.

I.2.5 Sequence of 16S rRNA

I.2.5.1 Primer design

All oligonucleotide sequences designed for this thesis research were synthesized by P. Murray, DNA synthesis and sequencing service, University of Alberta. Stock primers were dissolved into 200 μl distilled H_2O and the concentration determined by measuring the A_{260} of the solution with a UV spectrophotomer (Section III.2.5). The concentration of the oligonucleotide was calculated assuming that an A_{260} value of 1 represented 33 μg/ml of oligonucleotide.

Two primers were designed for generating a PCR fragment that encompassed most of the known 16S rRNA sequence for *E. coli*, these primers were based on primers designed by Hauben *et al.* (1997). These primers were also used for the initial portion of the sequencing reaction. The primers that were used for amplification and sequencing are listed in Table I.2.5.1. The forward primer, 8^F, encompassed the forward amplification primer sequence 16F27 from Hauben *et al.* (1997): $^{5'}$AGAGTTTGATC ATGGCTCAG$^{3'}$ and a sequence that contained sites for the restriction enzymes *Sac*1,

Table I.2.5.1. Amplification and sequencing primers used to determine the 16S rRNA sequence of *P. polymyxa* PKB1.

primer name	primer sequence[a]	application
8^F	$^{5'}$GAGCTCTAGAATTCAGAGTTTGATCATGGCTCAG$^{3'}$	amplification
704^F	$^{5'}$TGTGTAGCGGTGAAATGCGTAGA$^{3'}$	sequencing
1176^F	$^{5'}$AGGAAGGGGGGGAGGAGGT$^{3'}$	sequencing
358^R	$^{5'}$CCCACTGGTGCCTCCCGTA$^{3'}$	sequencing
1106^R	$^{5'}$CGCCCTTTTCGGGACTTAACCC$^{3'}$	sequencing
1403^R	$^{5'}$TCGAGCTCTAGAATTCGGGCGGTGTGTACAAGGC$^{3'}$	amplification

[a] primers were based on similar sequences reported by Hauben *et al.* (1997).

*Eco*RI and *Xba* I: $^{5'}$GAGCTCTAGAATTC$^{3'}$. The reverse primer sequence (1403^R) encompassed the reverse amplification primer sequence 1387R from Marchesi *et al.* (1998): $^{5'}$CGGAACATGTGAGGCGGG$^{3'}$ and the reverse of the restriction enzyme site sequence. The primer sequences were compared with those for the *E. coli* 16S rRNA sequence (Carbon *et al.*, 1979); the primers described above extend from nucleotides 8 to 27 in a forward direction and from nucleotides 1403 to 1386 as a reverse complement to the reported sequence of *E. coli*, that is 1541 nucleotides long. Other sequencing primers were designed based on a series of ten primers described by Hauben *et al.* (1997). Modifications to the suggested primer sequences were made based on the sequences for *P. polymyxa* PKB1 and a Gram negative isolate WPW-S28 (Greene, 1999) which were obtained by sequencing from the amplification primers.

I.2.5.2 Generation of 16S rDNA PCR fragment from *P. polymyxa* PKB1 genomic DNA

A PCR protocol that produced the maximum amount of 16S rDNA from *P. polymyxa* PKB1 genomic DNA, using the primers 8^F and 1403^R (Table I.2.5.2), was developed. The concentration of genomic DNA was determined spectrophotometrically as described in Section III.2.5. PCR reactions were carried out in an MJ Research Minicycler. The temperature program was designed based on those of Hauben *et al.* (1997) and Marchesi *et al.* (1998), with modifications to improve *P. polymyxa* PKB1 and WPW-S28 DNA amplification, and is shown in Table I.2.5.2.

The reaction components for generation of the 8^F-1403^R PCR fragment for sequencing contained: 1.2 µl of 25 ng/µl *P. polymyxa* PKB1 genomic DNA, 0.67 µl each of 30 pmol/µl primer 8^F and 30 pmol/µl primer 1403^R, 0.4 µl of 25mM dNTP mix (25 mM each of dATP, dCTP, dGTP and dTTP, Boeringher Mannheim), 5 µl of 10X PCR buffer (0.5 g KCl, 8.48 g tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.8, 1 ml of 1% Triton X-100 per 100 ml, in distilled H_2O), 0.75 µl of 0.1 M $MgCl_2$, 28.25 µl of distilled H_2O and 1 µl of 5 units/µl Taq DNA polymerase. After preparation, a small amount of sterile mineral oil (Fisher Scientific) was put in each tube and the tubes were centrifuged at 16 000 X g for 5 s. The Taq DNA polymerase was added to each reaction mix once the PCR thermocycler had heated the samples to 94°C for approximately 2 min. For PCR reactions used to test the ability of the sequencing primers to interact with *P. polymyxa* PKB1 genomic DNA, the primers of interest were substituted for primers 8^F

Table I.2.5.2. Temperature program for amplification of the 16S rDNA fragments of *P. polymyxa* PKB1 by PCR.

step	protocol
1	5 min 94°C
2	30 s 94°C denaturation of DNA primers and template
3	30 s 55°C annealing of DNA primers and templates
4	1.5 min 72°C extension of new DNA strands
5	repeat steps 2 through 4, 29 times
6	5 min 72°C extension of new DNA strands
7	4°C stop PCR reaction and refrigerate DNA product
8	end

and 1403^R, at the same concentrations as these primers. Each test reaction was run with the sequencing primer of interest acting as the amplification primer in one direction, and either the primer 8^F or the primer 1403^R as the second direction amplification primer in the test reaction.

I.2.5.3 DNA sequencing

DNA fragments were sequenced by P. Murray at the DNA Sequencing Facility, Department of Biological Sciences, University of Alberta. The DNA was sequenced using the automated 373A Sequencer with Stretch Upgrade and Amersham Thermosequenase II kit. Samples submitted as templates for sequencing were either gel purified (Section III.2.4) PCR products or plasmids with inserts of interest. The DNA for sequencing was supplied as 5 µl of a 200 µg/ml solution in distilled H_2O. DNA concentrations were determined as described in Section III.2.5. The primers used to initiate sequencing were either provided at a concentration of 3 pmol/µL in distilled H_2O if they were specific for a certain portion of the template DNA, or universal forward and reverse primers for pUC118 (New England Biolabs) were used to sequence plasmid inserts.

I.2.5.4 Computer analysis of DNA sequences

I.2.5.4.1 Basic local alignment search tool (BLAST)

BLASTn and BLASTp were used to search for DNA and amino acid sequences, respectively, in the databases available to the search tool, with similarity to DNA or amino acid sequences generated in this study. This search tool was developed by the National Center for Biotechnology Information (USA). BLAST provides a sequence database for DNA, RNA and proteins (Peruski and Peruski, 1997). The web site address is http://www.ncbi.nlm.nih.gov, the reference for its use is Altschul *et al.* (1997). An advanced ungapped BLAST search of the nucleic acid databases was performed.

I.2.5.4.2 Ribosomal Database Project (RDP) search and analysis tool

The *P. polymyxa* PKB1 16S rRNA sequence was analysed using tools developed by the RDP for sequence alignment and for generation of a phylogenetic tree with other 16S rRNA sequences that are in the database (Maidak *et al.*, 1997). The web site addresses for the RDP are http://rdpwww.life.uiuc.edu/ and http://www.cme.msu.edu/RDP.

I.3 Results and Discussion: Characterization of a Bacterial Environmental Isolate as *Paenibacillus polymyxa* and Determination of the Optimal Conditions for Production of Antifungal Antibiotic.

The antifungal antibiotic producing environmental bacterium isolated from canola stubble was initially characterized as *B. macerans* in preliminary studies conducted at the Alberta Environmental Centre. *B. macerans* has since been re-classified as *P. macerans* (Ash *et al.*, 1991; 1993). Phenotypically, *P. macerans* and *P. polymyxa* are similar to each other and to a third species: *B. circulans* (Parry *et al.*, 1983). With the use of 16S rRNA similarity to group bacteria into genotyptically defined clusters instead of phenotypic clusters, many strains of *B. circulans* have been re-classified as *Paenibacillus* spp. (Shida *et al.*, 1997b). The *B. circulans* strain that produces polymyxin F was re-named as *P. polymyxa* (Parker *et al.*, 1978). Further characterization of the environmental isolate using the Voges-Proskauer test (VP test) suggested that it was *B. polymyxa* (re-classified as *P. polymyxa*; Ash *et al.*, 1991; 1993). The VP test, which detects the production of acetoin from glucose, illustrates one main biochemical difference between *P. polymyxa* and the other two species (Holt *et al.*, 1994). Over 90% of the culture collection strains of *P. polymyxa* are positive for acetoin production, while over 90% of the culture collection strains of *P. macerans* and *B. circulans* are negative for acetoin production (Holt *et al.*, 1994).

Based on these initial results, the environmental isolate was deemed a putative *P. polymyxa* strain pending further identification tests. The results of both biochemical tests and 16S rRNA sequence similarity are outlined within this chapter. Based on this information, the putative *P. polymyxa* isolate was confirmed as *P. polymyxa* and was given the strain designation PKB1. It has been deposited into the American type culture collection (ATCC) as number 202127.

I.3.1 Cellular and colony morphology of the putative *P. polymyxa* environmental isolate

The cellular morphology of the environmental bacterial isolate was observed at 400 X magnification with a phase contrast microscope. The cells in the vegetative state were Gram positive, rod-shaped, approximately 2 to 4 μm long and 1 to 1.5 μm wide, in comparison to *E. coli* rods that are 2 to 6 μm long and 1 to 1.5 μm wide (Holt *et al.*,

1994). A modified version of the Gray flagellar stain (Mayfield and Innis, 1977) demonstrated peritrichous flagella on the cells. The spores in the dormant state were ellipsoid and refractile. During lag phase before culture doubling, the cells were single and motile; in exponential growth the cells appeared as doublets and were motile, and at the end of exponential growth the cells were single and highly motile. At the onset of stationary phase the cells clumped together in large groupings, enmeshed in a polysaccharide-like substance. A very small number (less than 1%) of cells at this time were single and highly motile. The cells in clumps developed ellipsoid, refractile endospores that were subterminal in the mother cell. Approximately 24 h after spores appeared, the mother cell surrounding each endospore was shed, releasing the spore into the spent culture medium. The spores appeared singly.

The colony morphology of the putative *P. polymyxa* isolate grown on a solid medium, such as potato dextrose agar, was 2 to 4 mm in diameter, opaque, tan and glistening, with an entire edge and wrinkled surface. When grown on high sucrose media, colonies would develop gas bubbles that would expand and break, releasing the trapped gas and letting the surface fall onto itself, causing a wrinkled appearance. The colonies produced extracellular polysaccharide that encapsulated the colony in a non-runny, white, shiny mucus layer. Solid medium containing starch caused colonies to produce the most polysaccharide.

The cellular and colony morphology of the putative *P. polymyxa* isolate was very similar to that described for the type culture, *P. polymyxa* ATCC 842 and other culture collection strains (Parry *et al.*, 1983; Holt *et al.*, 1994).

I.3.2 Physiological tests for identification of the putative *P. polymyxa* isolate

Biochemical tests (API 20E and API 20NE) were performed on the putative *P. polymyxa* isolate, and growth characteristics on a variety of media were determined to gather further evidence of the identity of the organism.

I.3.2.1 Identification of the putative *P. polymyxa* isolate using API 20E and API 20NE tests

Although both the API 20E (enteric) and API 20NE (non-enteric) kits contain biochemical tests designed for identification of *Enterobacteria* in particular, and Gram

negative bacilli in general, they were used to aid in characterization of the Gram positive putative *P. polymyxa* isolate. The kits provided a readily available source of biochemical tests that could highlight some important physiological characteristics of *Paenibacillus* spp. (Section I.1.1) including: production of hydrogen sulfide, production of catalase, production of indole, reduction of nitrate to nitrite, use of citrate as the sole C source, hydrolysis of urea, hydrolysis of gelatin, production of acetoin from glucose (VP test) and use of glucose, arabinose, mannitol and maltose. The ability of the putative *P. polymyxa* to hydrolyze chitin was also determined using the rapid chitobiose test (not an API test) by O'Brien and Colwell (1987).

Table I.3.2.1 lists the results of the biochemical tests with the putative *P. polymyxa* isolate and the reported results from these tests on known *P. polymyxa* culture collection strains. The results of the biochemical tests for the putative *P. polymyxa* isolate were consistent with the reported biochemistry of known *P. polymyxa* strains for all but two of the tests. Utilization of glucose as a C source aerobically and anaerobically by the putative *P. polymyxa* were both negative when tested in the API 20NE kit. Holt *et al.* (1994) reported that over 90% of *P. polymyxa* strains were positive for use of glucose as a C source both aerobically and anaerobically. The discrepancies among the glucose test results are probably due to poor inoculation of the test ampules in the API 20NE kit. *P. polymyxa* is well known for use of glucose as a C source anaerobically via the Diol-fermentative pathway (Section I.1.3; Garg and Jain, 1995). As well, the putative *P. polymyxa* grew well and developed yellow halos on minimal salts agar containing glucose as the sole C source and the pH indicator bromthymol blue (Section I.3.2.2). The positive acetoin production from glucose illustrates again that this putative *P. polymyxa* is not *B. circulans* or *P. macerans.*

P. polymyxa strains are well represented as utilizing arabinose and mannitol; for instance *P. polymyxa* ATCC 842 is the control strain for the determination of a positive result of acid production from arabinose and mannitol (Holt *et al.*,1994). *P. polymyxa* is known to use many types of carbohydrates as C sources, as indicated by growth of the putative *P. polymyxa* isolate on maltose and mannose in the API 20NE kit.

Chitin is a polysaccharide consisting of (1-4)-β-linked glucosamine residues that are mostly N-acetylated. It is abundant in nature; for instance chitin is found in fungal cell walls. Chitin is hydrolyzed by the enzyme chitinase yielding chitobiose, disaccharide subunits of chitin. Chitobiose is further hydrolyzed to N-acetylglucosamine with the enzyme chitobiase, which is an N-acetyl-β-D-glucosamidase (O'Brien and Colwell 1987).

Table I.3.2.1. API 20E and API 20NE test results for the putative *P. polymyxa* isolate.

API 20E test		API 20NE test[a]	
biochemical test	+ or − result[b,c]	biochemical test	+ or − result[b,c]
acetoin from glucose; VP test	+ (+, 90%)	esculin hydrolysis	+
catalase producer	+ (+, 90%)	β-galactosidase; lactose to glucose and galactose	+
gelatin hydrolysis; by proteases	+ (+, 90%)	glucose fermentation	- (+, 90%)
H_2S from thiosulfate	- (-, 90%)	nitrate reduction (NO_3^- to NO_2^-)	+ (+, 90%)
lysine decarboxylase; lys into cadaverine	-	N-acetylglucosamine; C source	-
ornithine decarboxylase; orn into putrescine	-	arabinose; C source	+ (+, 90%)
tryptophan deaminase; trp into indole-pyruvic acid	- (-, 90%)	glucose; C source	- (+, 90%)
urease; release of NH_4^+ from urea	- (-, 90%)	maltose; C source	+
citrate; C source	- (-, 90%)	mannitol; C source	+ (+, 90%)
chitinase test[d]	+	mannose; C source	weak+

[a] tests that were repetitive in the two kits are listed once.
[b] A + (positive) result for a substrate indicates utilization or production of the substrate within 48 h of inoculation in the test strip.
[c] the values in brackets are the results of these tests on known *P. polymyxa* strains (Parry *et al.*, 1983; Holt *et al.*, 1994).
[d] chitinase test by O'Brien and Colwell (1987) is not an API test.

The putative *P. polymyxa* is positive for chitobiase and chitinase activity according to the rapid chitinase test of O'Brien and Colwell (1987). The test measures microbial chitobiase activity, assuming that chitobiase activity indicates chitinase activity. Chitinase activity has been reported for many plant growth promoting rhizosphere bacteria, including *P. polymyxa* (Section A.5; Mavingui and Heulin, 1994). The chitinase activity of the putative *P. polymyxa* suggests that production of the antifungal antibiotic is not the only method of fungal antagonism that this strain exhibits.

The putative *P. polymyxa* strain tested negative for use of N-acetyl-glucosamine (GlcNAc) as a C source. Although use of GlcNAc as a C source is not an indicative characteristic of *P. polymyxa*, it was expected that this strain would be able to use the compound because it is able to hydrolyze chitin and chitobiose into GlcNAc units.

I.3.2.2 Growth of the putative *P. polymyxa* on selective and differential media

Selective and differential media were used to assist in the identification of *P. polymyxa* PKB1 (Table I.3.2.2). Growth of the putative *P. polymyxa* was seen on each of glucose-, glycerol-, and sucrose-containing agar with the concomitant formation of acidic metabolites. Lactose was also utilized as a sole C source by the putative *P. polymyxa* in a broth culture; gas was produced. Growth of the isolate on blood agar showed β-hemolysis of the red blood cells in the medium, as seen by clear colourless zones around the colonies. The putative *P. polymyxa* isolate grew well on starch agar; the colonies were surrounded by clear zones, indicating hydrolysis of the starch by amylases produced by the bacterium. Aerobic and anaerobic growth was seen when the putative *P. polymyxa* grew on and within a triple sugar iron agar slant. Acidic metabolites were produced by the putative *P. polymyxa* aerobically, and alkaline metabolites were produced by the bacterium anaerobically. A large amount of gas was produced during growth on this medium. Growth of the putative *P. polymyxa* isolate was inhibited by 7.5% salt in mannitol containing agar. These growth characteristics are all common traits of known *P. polymyxa* strains (Parry *et al.*, 1983; Holt *et al.*, 1994).

The putative *P. polymyxa* isolate was inoculated on potato dextrose agar (PDA) and MacConkey's agar as positive and negative indicators of growth, respectively. MacConkey's agar is a selective medium for Gram negative enterobacteria due to the addition of crystal violet and bile salts; these additives are inhibitory to Gram positive

Table I.3.2.2. Growth of putative *P. polymyxa* isolate on different types of media.

medium[a]	growth, reaction or characteristics	interpretation	known *P. polymyxa*
blood agar	good growth, clear colourless zones in medium	β-hemolysis was observed	+[b]
glucose	growth	acidic products (detected by pH indicator in medium)	+[c]
glycerol	growth	acidic products (detected by pH indicator in medium)	---
lactose broth	good growth, gas production	utilizes lactose as a carbon and energy source	---
MacConkey agar	no growth	inhibited by bile salts, not enterobacteria; inhibited crystal violet, not Gram negative	---
mannitol salt (7.5%) agar	no growth	inhibited by 7.5% salt	-[c]
potato dextrose agar	good growth, round, mucoid, tan, entire edge, opaque colonies	able to use dextrose and starch as carbon sources	+[c]
starch agar	clear zones after flooding plate with iodine	starch hydrolysis, amylases produced	+[c]
sucrose	growth	acidic products (detected by pH indicator in medium)	---
triple sugar iron slant (glucose, lactose, sucrose)	good growth on slope and in butt, yellow colonies on slope; red colonies in butt; agar was split and bubbled; no black precipitate formed;	aerobic and anaerobic growth, anaerobic or micro-aerobic growth, large amount of gas produced from sugar utilization, no thiosulfate reduction to sulfide	+; anaerobic[c] +; gas from glucose[c]

[a] Where only a substrate name is indicated, agar plates consisted of B+N8P medium with the substrate as a sole carbon source and bromthymol blue as a pH indicator.
[b] data from Parry *et al.*, (1983).
[c] data from Holt *et al.*, (1994).

microbes such as *P. polymyxa*. The putative *P. polymyxa* isolate grew well on PDA, as expected since it can hydrolyse starch.

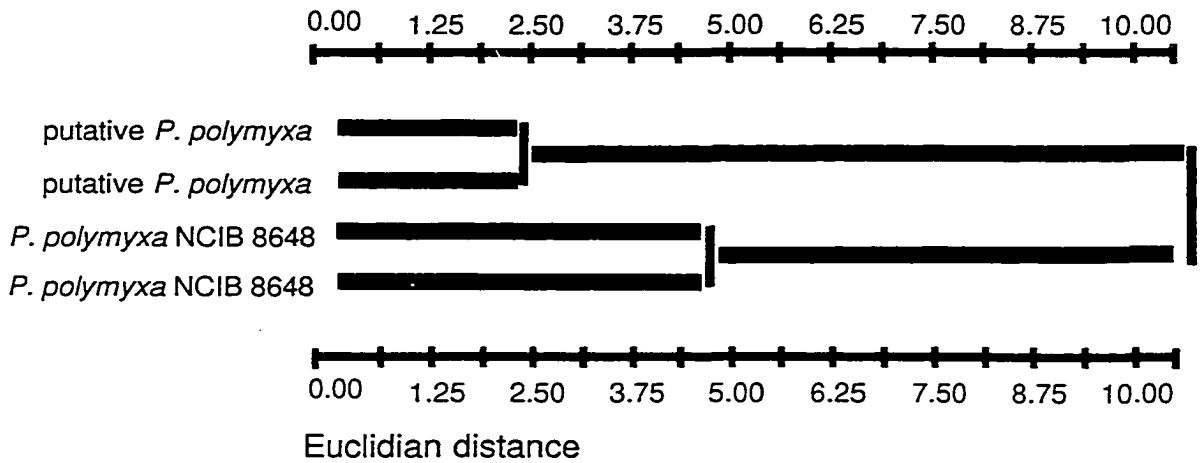
I.3.3 Fatty acid methyl ester (FAME) analysis

Comparison of fatty acid profiles of whole bacterial cell envelopes is a common method to determine the relatedness of bacteria. The data from FAME, in the form of fatty acid retention times and percentages from gas-liquid chromatography, is depicted as a dendogram. The dendogram is a graphical comparison of the fatty acid profiles of the tested organisms. It shows the relatedness of the organisms measured in Euclidian distances; a distance of 25 or less indicates relatedness at the genus level, approximately 10 or less indicates relatedness at the species level, approximately 6 or less indicates relatedness at the subspecies level and approximately 2 or less indicates the same strain.

FAME analysis was done on the putative *P. polymyxa* isolate to measure its relatedness to the *P. polymyxa* type culture NCIMB 8648 (Figure I.3.3). The isolate was within 3 Euclidian distances of the type culture, which suggests that it is similar to *P. polymyxa* NCIMB 8648 at both the genus and species levels. The major fatty acid found in both the *P. polymyxa* environmental isolate and the NCIMB 8648 strain was 15:0 anteiso, which has been reported as characteristic of *Paenibacillus* spp. (Shida *et al.*, 1997a; 1997b). The fatty acid profiles determined for the putative *P. polymyxa* isolate and the NCIMB 8648 strain were compared to the MIDI microbial identification system library of reference bacterial fatty acid profiles; they both showed high similarity indexes (a perfect match would be an index of 1) to the fatty acid profile of the *P. polymyxa* reference strain in the MIDI library. The NCIMB 8648 strain gave an average similarity index of 0.896 to the reference strain and the environmental *P. polymyxa* isolate gave an average similarity index of 0.721.

I.3.4 Identification of putative *P. polymyxa* PKB1 by 16S rRNA sequence

It is difficult to determine the species identity of an environmental isolate that appears to be a *Bacillus* based on phenotype. This is due to the similarity of phenotypes of these species, especially between *P. polymyxa*, *P. macerans* and *B. circulans* (Parry *et al.*, 1983). The antifungal antibiotic producing isolate was identified as *P. polymyxa*



putative *P. polymyxa* fatty acid profile

fatty acid	average percent
14:0 iso	1.23
14:0	1.67
15:0 iso	6.94
15:0 anteiso	**63.72**
15:0	0.63
16:1 ω7c alcohol	0.60
16:0 iso	5.53
16:1 ω11c	2.85
16:0	5.09
17:1 iso ω10c	0.72
17:1 anteiso	0.52
17:0 iso	2.67
17:0 anteiso	7.87

P. polymyxa NCIB 8648 fatty acid profile

fatty acid	average percent
14:0 iso	1.35
14:0	1.50
15:0 iso	7.31
15:0 anteiso	**62.35**
15:0	0.44
16:1 ω7c alcohol	0
16:0 iso	6.43
16:1 ω11c	1.30
16:0	6.38
17:1 iso ω10c	0
17:1 anteiso	0
17:0 iso	3.96
17:0 anteiso	9.20

Figure I.3.3. FAME analysis of the putative *P. polymyxa* isolate and *P. polymyxa* NCIB 8648. The Euclidian distance is outlined above and below the dendogram. The bacteria were analyzed in duplicate. The fatty acid marked in bold is the major fatty acid found in *Paenibacillus* spp.

based on the results of biochemical tests and FAME analysis. Genotypic information was sought to confirm the identification. The genus *Bacillus* had also undergone re-organization based on 16S rRNA sequence similarity, therefore 16S rRNA DNA sequence was used to confirm the identity of the putative *P. polymyxa* isolate.

Genomic DNA from *P. polymyxa* PKB1 was isolated, the 16S rRNA DNA was amplified by PCR, then sequenced. The sequencing and amplification primers were derived from Hauben *et al.* (1995) and are listed in Table I.2.5.1. The primer sequences suggested by Hauben *et al.* (1995) were altered to ensure optimum annealing in the sequencing reactions. These alterations were based on sequences already obtained from *P. polymyxa* PKB1 using the amplification primers for sequencing. The primer length was changed to yield a T_m of at least 55°C, since the T_m of some primers suggested by Hauben *et al.* (1995) was too low for use in sequence determination.

The structure of 16S rRNA is complex; the entire molecule is involved in a series of stem loops. This type of structure can prevent the primers from binding to template DNA during the sequencing reactions. Nucleotide substitutions and additions to the primers of Hauben *et al.* (1995) were therefore made with reference to the folded structure of 16S rRNA given in Woese (1987), which showed where stem loop structures occurred in *E. coli* 16S rRNA. Primers were selected in regions of DNA where the sequences of *E. coli* and *P. polymyxa* PKB1 were similar; these were likely to be highly conserved regions. DNA amplification and purification was partly done by A. Greene (Dept. of Biological Sciences, University of Alberta, Edmonton, AB).

The sequence of the *P. polymyxa* PKB1 16S rRNA DNA is shown in Figure I.3.4. Highly conserved bases that are used as a probe to distinguish *P. polymyxa* from *P. macerans* (Jurtshuk *et al.*, 1992) are marked in bold on the putative *P. polymyxa* 16S rRNA sequence. The entire *P. polymyxa* probe sequence designed by Jurtshuk *et al.* (1992) is present in the 16S rRNA derived from the putative *P. polymyxa* isolate. Highly conserved bases that are used to distinguish eubacteria from archaebacteria (Woese, 1987), are marked by asterisks above the sequence. Only 19 of the *P. polymyxa* PKB1 bases were different from the 569 conserved bases (Woese, 1987), representing a 3.3% divergence from the expected sequence for eubacteria. Low G+C and high G+C mol % Gram positive bacteria have signature nucleotides that differentiate the two groups (Woese, 1987). The signature nucleotides are marked on the 16S rRNA sequence by a double underline. This signature is comprised of seven nucleotides that are found throughout the 16S rRNA. Low G+C mol % bacteria have G (81%) base 168, G (92%)

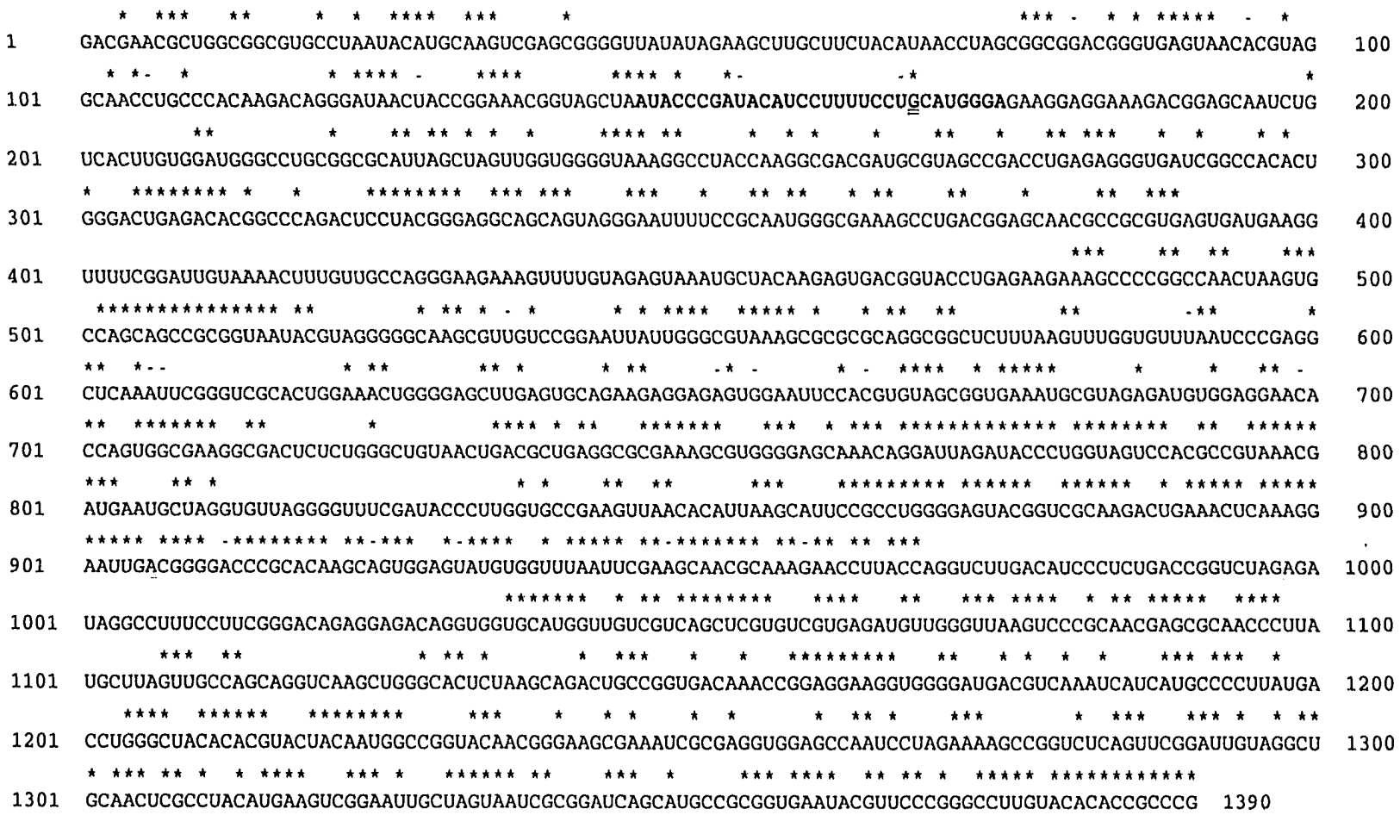


Figure I.3.4. 16S rRNA sequence for *P. polymyxa* PKB1. Bases that are highly conserved among all eubacteria are marked above with an asterisk (Woese, 1987). The bases that are marked above with a line represent sequence that did not match the highly conserved bases. The nucleotides in bold make up the *P. polymyxa* determining probe sequence (Jurtshuk *et al.*, 1992). Nucleotides marked with either one or two lines underneath are the signature sequence for Gram positive bacteria (Woese, 1987).

base 906, U (99%) base 955, C (88%) base 998, U (95%) base 1116, A (62%) base 1167 and A (94%) base 1410 (Woese, 1987). *Paenibacillus* are low G+C mol% bacteria, however the sequence of the putative *P. polymyxa* isolate has the following nucleotides in the signature base positions (listed in the same order as above); G, A, C, A, C, G, the last nucleotide in the signature is not known for the putative *P. polymyxa* because the 16S rRNA sequence was not determined past position 1390. The nucleotide in the first position, G, reflects the major nucleotide of low G+C mol % bacteria, however the rest of the nucleotides in the putative *P. polymyxa* signature are the major nucleotides found at these positions for high G+C mol% bacteria. The 16S rRNA sequences derived from other *P. polymyxa* strains, such as for *P. polymyxa* IAM 13419, have the same nucleotides in those positions as the putative *P. polymyxa* strain, indicating that the sequence differences are not due to contamination of the DNA with a high G+C mol% bacterial genome. Woese (1987) described the signature sequence of low G+C mol% bacteria as the ancestral composition and the signature sequence for high G+C mol% bacteria as evidence of rapid evolutionary change. Perhaps now that there is more 16S rRNA sequence information from species of the genus *Paenibacillus*, their ancestral background can be better assessed.

The 16S rRNA sequence from the putative *P. polymyxa* was submitted to the Basic Local Alignment Search Tool (BLAST) and the Ribosomal Database Project (RDP) to determine the identity of *P. polymyxa* PKB1 by comparison with the 16S rRNA DNA of known microorganisms.

I.3.4.1 Identification of putative *P. polymyxa* from BLAST search

A BLASTn search (Altschul *et al.*, 1997, Peruski and Peruski, 1997) for the identities of DNA sequences most similar to the *P. polymyxa* PKB1 16S rRNA gene sequence was carried out. The sequence for *P. polymyxa* PKB1 16S rRNA was most similar to *P. polymyxa* (accession number x60632) 16S rRNA (Ash *et al.*, 1991). This sequence was 1406 nucleotides long.

I.3.4.2 Identification of putative *P. polymyxa* from the Ribosomal Database Project (RDP)

The putative *P. polymyxa* 16S rRNA sequence was submitted to the RDP to determine its relatedness to other bacteria and to construct a phylogenetic tree (Figure

I.3.4.2.A). The program was set so that only 16S rRNA sequences of 1000 nucleotides or more were included in the comparison. The RDP uses a gapped sequence alignment protocol to identify the organisms with the most similar 16S rRNA sequence to the gene sequence submitted (Maidak *et al.*, 1997). The sequence alignment of the putative *P. polymyxa* 16S rRNA sequence and the sequence from the most closely similar bacterium; *P. polymyxa* IAM 13419, is shown in Figure I.3.4.2.B. Sixteen of the 1390 nucleotides sequenced for the putative *P. polymyxa* isolate were different from the most similar 16S rRNA sequence. This is a genetic divergence of only 1.2% between these two 16S rRNA sequences.

I.3.5 Optimization of *P. polymyxa* PKB1 growth and antibiotic production in broth culture

The *P. polymyxa* environmental isolate was observed to inhibit *L. maculans* when grown on PDA. This type of medium has a pH of 5 and is used frequently for growth of fungi. Most bacteria grow better in less acidic conditions, so a search for a medium that allowed optimum growth and antibiotic production was undertaken. Many different kinds of both complex and defined media were used as growth substrates for the *P. polymyxa* environmental isolate.

The growth rates, growth characteristics and antibiotic production levels of the *P. polymyxa* isolate grown in complex, chemically undefined media such as beef heart infusion broth (BHI), potato dextrose broth (PDB) and trypticase soy with starch medium (TCSS; plus modifications of these; PDB1-6, PEM, SDP, TCSS1) and chemically defined media such as Katznelson and Lochhead (KL) and Stansly's medium (plus modifications of these; KLM1, S1) were measured.

Growth temperature and aeration rate were varied in order to determine the optimal growth conditions. Scale-up cultures of 10 L and 50 L for use in 15 and 75 L bioreactors were tested, using the conditions for growth outlined in Section I.2.3.2, to determine whether the organism could produce antifungal antibiotic in large quantities.

Growth of the bacterium in broth culture was measured by determining the OD_{600} of the culture. The growth of *P. polymyxa* PKB1 before the onset of sporulation is exponential. The specific growth rate (k) was calculated for the exponential growth phase by graphing time versus $\log OD_{600}$ and determining the slope of the graph before the graph levels out, which is when endospores are seen in the *P. polymyxa* PKB1

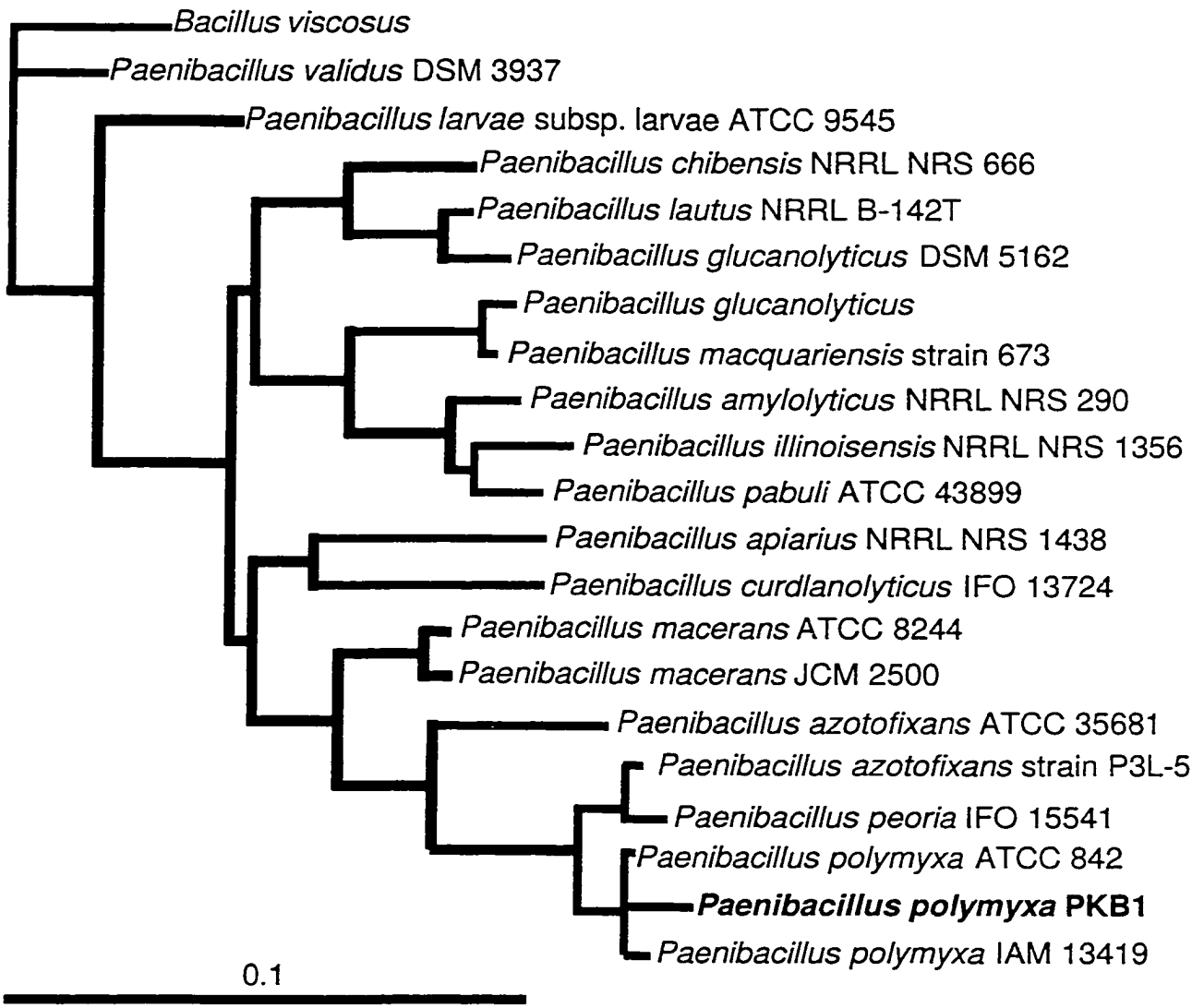


Figure I.3.4.2.A. Phylogenetic tree, determined by the maximum likelihood approach, showing the similarity of *P. polymyxa* PKB1 to other bacteria based on 16S rRNA DNA. The similarity was measured using the RDP (Maidek *et al.*, 1997) and the information was depicted in this tree format using the TreeView program. The measurement of a Euclidian distance of 0.1 is shown.

Figure I.3.4.2.B. Alignment of *P. polymyxa* PKB1 16S rRNA with *P. polymyxa* IAM 13419 using the RDP (Maidek *et al.*, 1997). Differences between the alignments are depicted with a cross (+), identical nucleotides are represented with a dash (-).

```
P. polymyxa IAM 13419 GACGAACGCUGGCGGCGUGCCUAAUACAUGCAAGUCGAGCGGGGUUAAUU   50
P. polymyxa PKB1       GACGAACGCUGGCGGCGUGCCUAAUACAUGCAAGUCGAGCGGGGUUAUAU   50
P. polymyxa IAM 13419 AGAAGCUUGCUUCUAACUAACCUAGCGGCGGACGGGUGAGUAACACGUAG  100
P. polymyxa PKB1       AGAAGCUUGCUUCUACAUAACCUAGCGGCGGACGGGUGAGUAACACGUAG  100
P. polymyxa IAM 13419 GCAACCUGCCCACAAGACUGGGAUAACUACCGGAAACGGUAGCUAAUACC  150
P. polymyxa PKB1       GCAACCUGCCCACAAGACAGGGAUAACUACCGGAAACGGUAGCUAAUACC  150
P. polymyxa IAM 13419 CGAUGCCUCCUUUUCCUGCAUGGGAGAAGGAGGAAAGGCGGAGCAAUCUG  200
P. polymyxa PKB1       CGAUACAUCCUUUUCCUGCAUGGGAGAAGGAGGAAAGACGGAGCAAUCUG  200
P. polymyxa IAM 13419 UCACUUGUGGAUGGGCCUGCGGCGCAUUAGCUAGUUGGUGGGGUAAAGGC  250
P. polymyxa PKB1       UCACUUGUGGAUGGGCCUGCGGCGCAUUAGCUAGUUGGUGGGGUAAAGGC  250
P. polymyxa IAM 13419 CUACCAAGGCGACGAUGCGUAGCCGACCUGAGAGGGUGAUCGGCCACACU  300
P. polymyxa PKB1       CUACCAAGGCGACGAUGCGUAGCCGACCUGAGAGGGUGAUCGGCCACACU  300
P. polymyxa IAM 13419 GGGACUGAGACACGGCCCAGACUCCUACGGGAGGCAGCAGUAGGGAAUCU  350
P. polymyxa PKB1       GGGACUGAGACACGGCCCAGACUCCUACGGGAGGCAGCAGUAGGGAAUUU  350
P. polymyxa IAM 13419 UCCGCAAUGGGCGAAAGCCUGACGGAGCAACGCCGCGUGAGUGAUGAAGG  400
P. polymyxa PKB1       UCCGCAAUGGGCGAAAGCCUGACGGAGCAACGCCGCGUGAGUGAUGAAGG  400
P. polymyxa IAM 13419 UUUUCGGAUCGUAAAGCUCUGUUGCCAGGGAAGAACGUCUUGUAGAGUAA  450
P. polymyxa PKB1       UUUUCGGAUUGUAAAACUUUGUUGCCAGGGAAGAAAGUUUUGUAGAGUAA  450
P. polymyxa IAM 13419 CUGCUACAAGAGUGACGGUACCUGAGAAGAAAGCCCCGGCUAACUACGUG  500
P. polymyxa PKB1       AUGCUACAAGAGUGACGGUACCUGAGAAGAAAGCCCCGGCCAACUAAGUG  500
P. polymyxa IAM 13419 CCAGCAGCCGCGGUAAUACGUAGGGGGCAAGCGUUGUCCGGAAUUAUUGG  550
P. polymyxa PKB1       CCAGCAGCCGCGGUAAUACGUAGGGGGCAAGCGUUGUCCGGAAUUAUUGG  550
P. polymyxa IAM 13419 GCGUAAAGCGCGCGCAGGCGGCUCUUUAAGUCUGGUGUUUAAUCCCGAGG  600
P. polymyxa PKB1       GCGUAAAGCGCGCGCAGGCGGCUCUUUAAGUUUGGUGUUUAAUCCCGAGG  600
P. polymyxa IAM 13419 CUCAACUUCGGGUCGCACUGGAAACUGGGGAGCUUGAGUGCAGAAGAGGA  650
P. polymyxa PKB1       CUCAAAUUCGGGUCGCACUGGAAACUGGGGAGCUUGAGUGCAGAAGAGGA  650
P. polymyxa IAM 13419 GAGUGGAAUUCCACGUGUAGCGGUGAAAUGCGUAGAGAUGUGGAGGAACA  700
P. polymyxa PKB1       GAGUGGAAUUCCACGUGUAGCGGUGAAAUGCGUAGAGAUGUGGAGGAACA  700
P. polymyxa IAM 13419 CCAGUGGCGAAGGCGACUCUCUGGGCUGUAACUGACGCUGAGGCGCGAAA  750
P. polymyxa PKB1       CCAGUGGCGAAGGCGACUCUCUGGGCUGUAACUGACGCUGAGGCGCGAAA  750
P. polymyxa IAM 13419 GCGUGGGGAGCAAACAGGAUUAGAUACCCUGGUAGUCCACGCCGUAAACG  800
P. polymyxa PKB1       GCGUGGGGAGCAAACAGGAUUAGAUACCCUGGUAGUCCACGCCGUAAACG  800
P. polymyxa IAM 13419 AUGAAUGCUAGGUGUUAGGGGUUUCGAUACCCUUGGUGCCGAAGUUAACA  850
P. polymyxa PKB1       AUGAAUGCUAGGUGUUAGGGGUUUCGAUACCCUUGGUGCCGAAGUUAACA  850
P. polymyxa IAM 13419 CAUUAAGCAUUCCGCCUGGGGAGUACGGUCGCAAGACUGAAACUCAAAGG  900
P. polymyxa PKB1       CAUUAAGCAUUCCGCCUGGGGAGUACGGUCGCAAGACUGAAACUCAAAGG  900
P. polymyxa IAM 13419 AAUUGACGGGGACCCGCACAAGCAGUGGAGUAUGUGGUUUAAUUCGAAGC  950
P. polymyxa PKB1       AAUUGACGGGGACCCGCACAAGCAGUGGAGUAUGUGGUUUAAUUCGAAGC  950
P. polymyxa IAM 13419 AACGCGAAGAACCUUACCAGGUCUUGACAUCCCUCUGACCGGUCUAGAGA 1000
P. polymyxa PKB1       AACGCAAAGAACCUUACCAGGUCUUGACAUCCCUCUGACCGGUCUAGAGA 1000
P. polymyxa IAM 13419 UAGGACCUUUCCUUCGGGACAGAGGAGACAGGUGGUGCAUGGUUGUCGUC 1050
P. polymyxa PKB1       UAGG-CCUUUCCUUCGGGACAGAGGAGACAGGUGGUGCAUGGUUGUCGUC 1049
P. polymyxa IAM 13419 AGCUCGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCGCAACCCUU 1100
P. polymyxa PKB1      AGCUCGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCGCAACCCUU 1099
P. polymyxa IAM 13419 AUGCUUAGUUGCCAGCAGGUCAAGCUGGGCACUCUAAGCAGACUGCCGGU 1150
P. polymyxa PKB1       AUGCUUAGUUGCCAGCAGGUCAAGCUGGGCACUCUAAGCAGACUGCCGGU 1149
P. polymyxa IAM 13419 GACAAACCGGAGGAAGGUGGGGAUGACGUCAAAUCAUCAUGCCCCUUAUG 1200
P. polymyxa PKB1       GACAAACCGGAGGAAGGUGGGGAUGACGUCAAAUCAUCAUGCCCCUUAUG 1199
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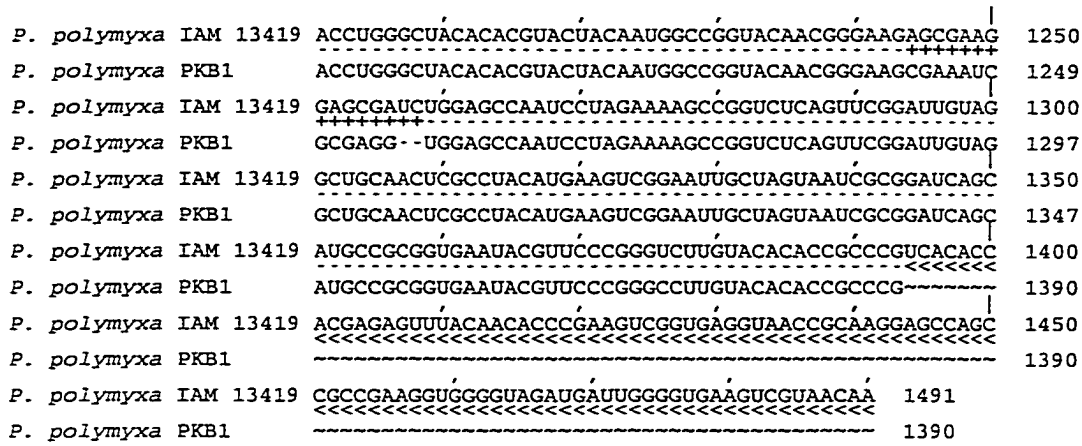


culture. The generation time (g) was calculated from k and the reciprocal of this number gave the generations per h (μ). The μ for each growth culture was determined to allow comparison of the growth rate of *P. polymyxa* PKB1 in the different media or in different growth conditions.

Antibiotic production by the culture was measured by the mini-extraction method and well bioassay using *L. maculans* as an indicator organism. The amount of antifungal antibiotic produced by *P. polymyxa* PKB1 when grown in various media and conditions were compared to each other by comparing the maximum zone of inhibition (ZOI; mm) obtained from that growth condition.

Some conditions for increased growth and antifungal antibiotic production were determined by T. Palmer-Stone working as a project and summer student in the Department of Biological Sciences at the University of Alberta. Based on these studies, the optimum inoculum size was determined to be 1% of the growth medium. Antifungal antibiotic production was found to be optimal in media at a pH range of 5.5 to 6.

I.3.5.1 Optimization of starter culture media

Different media were used to grow *P. polymyxa* to determine the optimal starter culture media based on the maximum μ and lack of sporulation (Table I.3.5.1). The medium had to support exponential growth during a short time period and not allow sporulation to occur to ensure that the inoculum from this culture to the production medium would be vegetative rods. *P. polymyxa* grown in TCSS gave the maximum μ, at 0.39 h^{-1} and no sporulation and so was used as the starter culture medium.

I.3.5.2 Optimization of production culture media

Variations in nutrient components of the media used to culture *P. polymyxa* PKB1 were tested to determine the nutrients required for optimum antifungal antibiotic production. The medium used for antifungal antibiotic production was referred to as the production medium. In order to optimize antifungal antibiotic production, factors such as generation time of the exponentially growing culture and sporulation of the cells were investigated.

A medium that allows for a rapid generation time does not necessarily produce the most, or any, antifungal antibiotic. This was observed when *P. polymyxa* PKB1 was

Table I.3.5.1. Determination of starter culture medium for optimization of antifungal antibiotic production by *P. polymyxa* PKB1.

medium	μ^a (h^{-1})	comments
LB	0.19	no sporulation
MYM	0.27	sporulation
NB	0.22	sporulation
PDB	0.29	sporulation
Stansly's	0.34	sporulation
TCSS	0.39	no sporulation

[a] μ: generations per h of *P. polymyxa* during exponential growth.

grown in complex, undefined medium such as BHI and TCSS1 (Table I.3.5.2); the rate and extent of growth was high but antifungal antibiotic production was not detected nor did the bacteria sporulate in these media.

Non-ribosomal peptide antibiotic production appears to be associated with the onset of sporulation. The nutrient requirements and conditions for onset of sporulation by endospore forming bacteria such as *P. polymyxa* are governed by many regulatory pathways and are therefore complex. Antibiotic production and sporulation of bacteria are typically repressed by the presence of certain nutritional components in the medium such as glucose and certain nitrogen (N) sources, and activated by the lack of other nutritional components in the medium. Schaeffer *et al.* (1965) suggested that the probability of an endospore forming cell committing to sporulation was related to the components of the growth medium, specifically the C and N sources. The authors determined that; the highest growth rate, from glucose as the C source, did not correlate with the most sporulation, increasing the concentration of glucose did not affect the growth rate but did repress sporulation, use of histidine as a N source allowed for sporulation but casein as a N source repressed sporulation, and the addition of amino acids to the medium slightly increased the growth rate but repressed sporulation.

The medium PDB provided for moderate growth of *P. polymyxa* and production of the most antifungal antibiotic in comparison to the media originally tested: BHI, KL, Stansly's and TCSS, in a reproducible manner. The media KL and Stansly's were tested for use as production media because they were chemically defined and were reported as media used to grow *P. polymyxa* in other studies (Paulus and Gray, 1964; Kanamori *et al.*, 1987). Attempts to optimize antifungal antibiotic production by *P. polymyxa* PKB1 were carried out by modifying PDB, KL and Stansly's media. The type and concentration of C and N sources were varied within the media. Nitrate, phosphate, sulphate and magnesium were added to PDB and starch or dextrin was added to the defined media such as KL and Stansly's to attempt to determine which combinations of nutrients would optimize antifungal antibiotic production.

Some media that were tested as production media for *P. polymyxa* PKB1; KL, KLM1, Stansly's and S1, allowed for sporulation without detectable antifungal antibiotic production, whereas there were no media that allowed for detectable antifungal antibiotic production without sporulation. The media KL, KLM1, Stansly's and S1, were similar in that they all contained glucose as the C source and NH_4^+ as the N source.

Table I.3.5.2. Comparison[a] of *P. polymyxa* PKB1 growth and antifungal antibiotic levels in various production media to those observed in PDB.

medium	μ^b h^{-1}	antifungal antibiotic (ZOI max.)	comments: sporulation, carbon (C) and nitrogen (N) sources, the other components are also listed for the PDB based media
PDB	**0.29**	**+++**	sporulation, starch and dextrose, undefined N
BHI	0.33	-	no sporulation, undefined C, N
KL	0.11	-	sporulation, 0.15% glucose, 0.15% NH_4^+, 0.25% casein
KLM1	0.20	-	sporulation, 0.5% dextrin, 0.15% NH_4^+,
PDB1	0.11	v	variable sporulation, starch and dextrose, 0.2% NO_3^-, KH_2PO_4, Na_2SO_4, $MgCl_2$
PDB2	0.26	++	sporulation, starch and dextrose, undefined N source, KH_2PO_4, Na_2SO_4, $MgCl_2$
PBD3	0.32	+++	sporulation, starch and dextrose, 0.2% NO_3^-
PDB4	0.32	++	sporulation, starch and dextrose, 1.1% NO_3^-
PDB5	0.32	+++	sporulation, starch and dextrose, 2.1% NO_3^-
PDB6	0.29	+	sporulation, starch and dextrose, 1.1% urea,
PEM	0.25	+	sporulation, starch and 0.22% maltose, undefined N
SDP	0.73	+	sporulation, starch and 0.22% dextrin, undefined N
Stansly's	0.34	-	sporulation, 1% glucose, 2.0% NH_4^+
S1	0.28	-	sporulation, 0.5% dextrin, 2.0% NH_4^+
TCSS1	0.38	-	no sporulation, starch and 0.25% dextrose, 1.7% casein (nitrogen source)
PDB 10 L[d]	0.22	++	sporulation
PDB 50 L[d]	0.46	++++	sporulation

[a] same growth conditions used for all media (Section I.2.3.1).

[b] μ· generations per h of *P. polymyxa* during exponential growth.

[c] measured by the mini-extraction method (Section I.2.3.5.1) and well bioassay (Section II.2.4.1). The ZOI max. from antifungal material produced by *P. polymyxa* grown in PDB was 25 mm, indicated as +++ antifungal antibiotic. The ZOI max. from *P. polymyxa* grown in the other media is compared to growth in PDB; v is variable, - is none, + is much less than, ++ is less than and ++++ is larger than antifungal antibiotic production in PDB.

[d] *P. polymyxa* PKB1 grown in bioreactors at the volume indicated (Section II.2.3).

The media that allowed for antifungal antibiotic production and sporulation were; PDB, PDB2 through to PDB6, PEM and SDP. These media were similar in that they all contained starch as an undefined C source, a carbohydrate such as dextrose or maltose as another C source and none contained added NH_4^+ as an N source. The N sources in these media were either undefined, NO_3^- or urea.

Kanamori *et al.* (1987) studied ammonia assimilation and the resulting growth rate of *P. polymyxa* when the cells were grown in KL medium with either NH_4^+, NO_3^- or N_2 as the sole N sources. The authors found that the growth rate was the highest with NH_4^+, followed by NO_3^- and N_2.

Antifungal antibiotic was detected in cultures grown using starch and glucose as C sources and either an undefined N or NO_3^- as the N source, but not when NH_4^+ was the N source, even though *P. polymyxa* could sporulate with NH_4^+ as an N source. It is possible that NH_4^+ represses antifungal antibiotic production by the cells. The effect of NO_3^- concentration on antifungal antibiotic production by *P. polymyxa* was also measured. The isolate was grown in the media series PDB3 to 5 which varied in NO_3^- concentration from 0.2%, 1% and 2%, respectively. None of these concentrations increased the antifungal antibiotic production of *P. polymyxa* in a reproducible manner as compared to production in PDB without added NO_3^-.

Cosby *et al.* (1998) studied production of surfactin by *B. subtilis*. The authors showed that if the pH of the culture medium was increased to neutrality; surfactin production increased. During the present study, for those media which supported antifungal antibiotic production the pH of the culture media was found to increase from pH 5 to pH 7 as the culture progressed from vegetative growth to dormant spores.

Based on these results, antifungal antibiotic production and sporulation appeared to be repressed in media that supported rapid and extensive growth of *P. polymyxa* and antifungal antibiotic was only detected in cultures that sporulated. However, *P. polymyxa* PKB1 appeared to produce antifungal antibiotic and sporulate in a nutrient dependent manner not a growth rate dependent manner in liquid culture since slow growth alone was not enough to ensure antibiotic production. There was no defined C or N source that increased the production of the antifungal antibiotic by *P. polymyxa* in a reproducible manner to a higher level than that detected when the isolate was grown in the undefined medium PDB. The medium PDB was therefore used as the production medium in flask and bioreactor cultures (Section I.2.3.1).

I.3.5.3 Effect of aeration on antifungal antibiotic production

The effect of aeration on the production of the antifungal antibiotic by *P. polymyxa* in PDB, was analyzed using varying volumes of the medium in both 125 ml and 500 ml Erlenmeyer flasks, shaken at 200 rpm. The growth rate and ability to sporulate for each culture were similar regardless of the volume (Figure I.3.5.3). The one exception to this observation was with the culture grown at a v/v ratio of 1:1.25 in both sized flasks that showed a reduced growth rate in comparison to the others. Antifungal antibiotic production by these *P. polymyxa* cultures varied with aeration (Table I.3.5.3). Antifungal antibiotic was not detected in cultures grown in both sized flasks at a v/v ratio of 1:12.5 or for culture grown in a 500 ml flask at a v/v ratio of 1:25. Antifungal antibiotic was detected in the v/v ratio of 1:5, 1:2.5 and 1:1.25 in both sized flasks. The *P. polymyxa* culture at the v/v ratio of 1:2.5 in both sized flasks produced the highest amount of detectable antifungal antibiotic by well bioassay for the longest period of time. At a v/v ratio of 1:5 and 1:1.25 the antifungal antibiotic was detectable at 50.5 h whereas it was detectable from 31 to 50.5 h from the v/v ratio of 1:2.5 culture. The v/v ratio of 1:2.5 culture to Erlenmeyer flask was used to grow *P. polymyxa* in the production medium, shaken at 200 rpm, for all subsequent shake flask studies.

Han (1989) noted that *P. polymyxa* produced less levan in highly aerated cultures. Levan is a contaminant of the antifungal antibiotic samples in the purification of the antifungal antibiotic from *P. polymyxa* cultures. The effect of aeration on growth, antifungal antibiotic production and levan production was measured by growing *P. polymyxa* in production medium at a v/v ratio of 1:2.5 with springs in the flask to increase aeration of the culture, shaken at 200 rpm. This culture was compared to *P. polymyxa* grown in production medium at a v/v ratio of 1:2.5 without springs, shaken at 200 rpm and also an identical culture that was not shaken (still culture; left on counter for entire growth period; Table I.3.5.3). The increased aeration did not affect the growth rate or antifungal antibiotic production as compared to the culture shaken at 200 rpm without springs, and the production of levan was not reproducibly decreased in the high aeration cultures. The non-shaken culture produced much more levan than the other cultures and also produced less antifungal antibiotic, however some of the antibiotic may have been lost when the levan was fractionated from the cell/spore sample. The non-shaken culture had a 25 h lag phase to exponential growth and the growth rate was 5.8 X slower than that of *P. polymyxa* PKB1 shaken at 200 rpm.

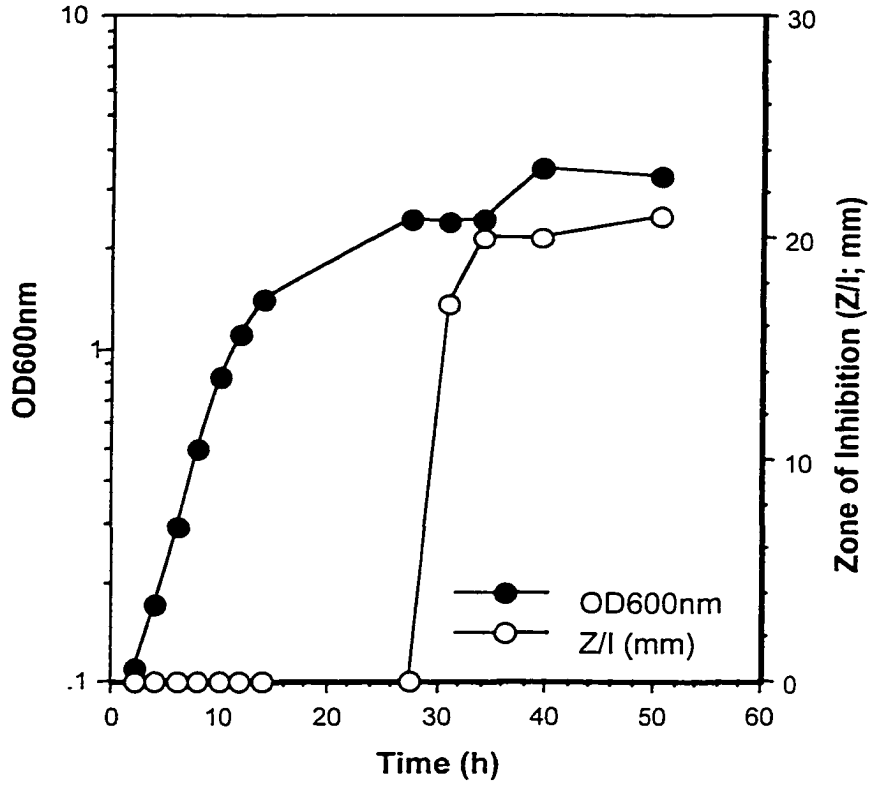


Figure I.3.5.3 Growth and antibiotic production of *P. polymyxa* PKB1 in PDB at 1:2.5 v/v culture to Erlenmeyer flask ratio. This growth curve is indicative of the growth of *P. polymyxa* PKB1 at all tested volumes. The antifungal antibiotic production was not the same at all volumes.

Table I.3.5.3. Effect of aeration on growth, production of antifungal antibiotic[a] and sporulation by *P. polymyxa* PKB1.

(v/v) ratio	μ, generations per hour (h^{-1})	antifungal antibiotic ZOI max. (mm)	onset of antifungal antibiotic production (h)	sporulation
125 ml flasks				
1:25	0.32	15	50.5	yes
1:12.5	0.32	0	none	yes
1:5	0.32	20	50.5	yes
1:2.5	0.31	22	50.5	yes
1:1.25	0.30	17	50.5	yes
500 ml flasks				
1:25	0.32	0	none	yes
1:12.5	0.33	halo[b]	50.5	yes
1:5	0.33	17	50.5	yes
1:2.5	0.31	22	31	yes
1:1.25	0.28	20	50.5	yes
still[c]	0.05	21	80	yes

[a] antifungal antibiotic production was monitored until: 50.5 h growth for all flasks except the still culture which was monitored until 128.5 h of growth.
[b] halo, barely detectable zone of inhibition.
[c] still culture was left motionless for the duration of the growth of *P. polymyxa* PKB1, growth conditions of the still culture were PDB at a (v/v) ratio of 2:5 and 28°C incubation.

I.3.5.4 Temperature range and growth of *P. polymyxa*

P. polymyxa in PDB at 40% culture volume was grown at temperatures ranging from 10°C to 30°C. The optimum temperature for growth was 28°C. Growth of *P. polymyxa* PKB1 at 10°C exhibited a lag time to exponential growth of approximately 29 h (Figure I.3.5.4) as compared to no lag time at 28°C. The exponential growth rate at 10°C was 5.8 X slower than for growth at 28°C. The antifungal antibiotic was detected in the culture just prior to the onset of sporulation as it is when *P. polymyxa* is grown at 28°C. The antifungal antibiotic production increased, in a reproducible manner, when compared to production at 28°C. The ZOI max was 36 mm for *P. polymyxa* PKB1 grown at 10°C versus the ZOI max of 24 mm for *P. polymyxa* PKB1 grown at 28°C. The much slower growth rate of *P. polymyxa* PKB1 at 10°C argued against use of this temperature for routine studies but indicated that production of antifungal antibiotic in cold soils may be favoured.

I.3.5.5 Growth and antifungal antibiotic production of *P. polymyxa* in bioreactors

P. polymyxa PKB1 was grown in bioreactors at 10 and 50 L volumes in PDB. The bioreactor cultures produced much more levan than the smaller scale flask cultures and the 50 L bioreactor culture produced more levan than the 10 L bioreactor culture. Levan production by *P. polymyxa* increases with decreased aeration and increased sucrose. Since the same medium was used for both the bioreactor cultures and the flask cultures, the increase in levan may have been due to uneven aeration in the bioreactors and more so in the 50 L bioreactor as compared to the 10 L bioreactor. This result appears different from the effect of aeration on shake flask cultures, however, it should be noted that direct comparisons between the shake flask cultures and the bioreactor cultures can not be made due to many other variables that can affect the growth and antifungal antibiotic production of the culture in these two situations.

The growth stage induced pH change of the culture medium that is measured in *P. polymyxa* cultures grown in PDB in shake flasks is not as prominent in *P. polymyxa* culture grown in bioreactors. The average pH change in bioreactors was from pH 5.1 at onset of exponential growth to pH 5.9 at sporulation.

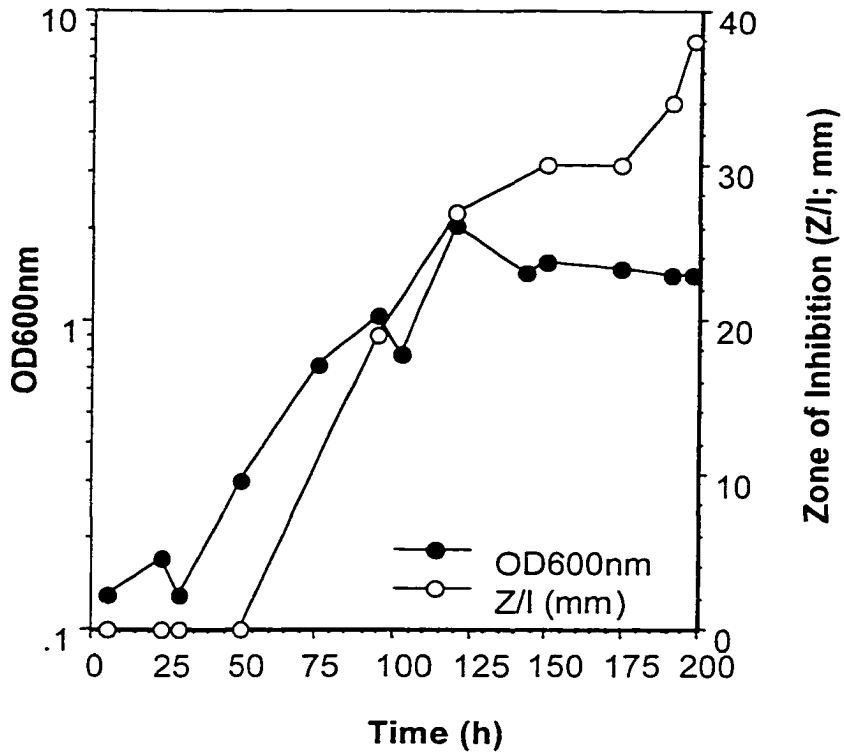


Figure I.3.5.4 *P. polymyxa* PKB1 grown at 10°C in 200 ml PDB per 500 ml Erlenmeyer flasks.

Antifungal antibiotic was detected in the bioreactor culture after endospore formation, the ZOI max due to the antifungal antibiotic was 25 mm from the 10 L bioreactor culture and 32 mm from the 50 L bioreactor culture.

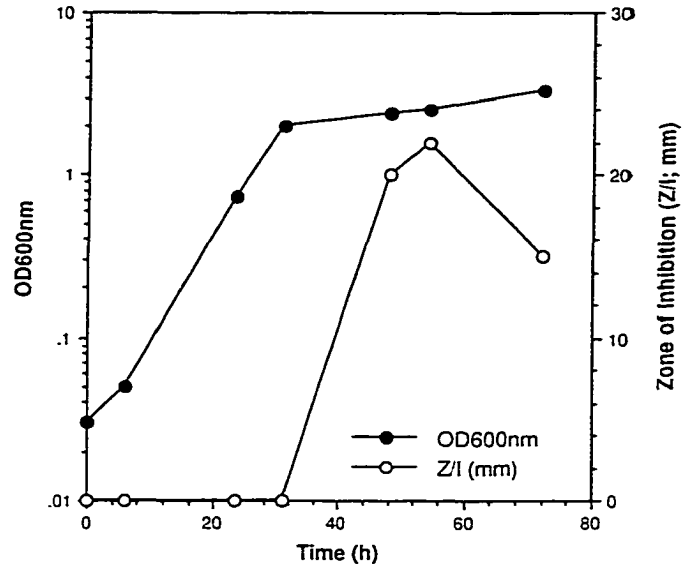
P. polymyxa PKB1 biomass grown in these bioreactors was harvested, washed and resuspended in water and lyophilized for use in routine studies of antifungal antibiotic production (Section i.2.3.2). The concentration of lyophilized cells and spores was determined to be 2.4 X 10^8 CFU/g of lyophilized material.

The growth rate of *P. polymyxa* during exponential growth, in 10 L of PDB grown in the bioreactor was 5.8 X slower than the growth rate of *P. polymyxa* grown in PDB under standard production conditions (Figure I.3.5.5; Section I.2.3.1), whereas the growth rate of *P. polymyxa* in 50 L of PDB grown in the bioreactor was 1.5 X faster than *P. polymyxa* grown under standard production conditions (Table I.3.5.2). The faster growth rate of *P. polymyxa* PKB1 in the 50 L culture in comparison to the 10 L culture may be due to an apparent increase in the OD_{600} of the 50 L culture due to the higher levan production observed.

I.3.6 Comparison of growth and antibiotic production of *P. polymyxa* PKB1 with other *P. polymyxa* strains

The growth rates, characteristics and ability to inhibit *L. maculans* of three other *P. polymyxa* strains were compared to *P. polymyxa* PKB1 (Figure I.3.6). One strain is *P. polymyxa* SCE2; isolated from soil in Brazil, the other is *P. polymyxa* subsp. *colistinus koyama*; (ATCC strain 21830), isolated from soil in Date City, Japan and the third is a culture collection strain, *P. polymyxa* NCIMB 8648. *P. polymyxa* SCE2 can inhibit Gram negative and Gram positive bacteria and fungi by uncharacterized means (Section I.1.4), *P. polymyxa* subsp. *colistinus koyama* produces the antibiotics colistin, jolipeptin and gatavalin (Section I.1.4), and *P. polymyxa* NCIMB 8648 is not known to be able to inhibit fungi or Gram positive bacteria. The three strains have very similar colony and cellular morphologies to that of *P. polymyxa* PKB1. They all show the same growth characteristics (polysaccharide production, endospore formation) as that of *P. polymyxa* PKB1 when grown on PDB in the same production conditions. The *P. polymyxa* strains SCE2, *colistinus koyama*, and NCIMB 8648 all exhibit a lower exponential growth rate than PKB1, at 1.6 X, 2.6 X, and 5.8 X slower, respectively. The spores of *colistinus koyama* tend to clump together, unlike the spores of SCE2, NCIMB 8648 or PKB1. Both SCE2 and *colistinus koyama* produced antifungal antibiotic material that was extracted

A



B

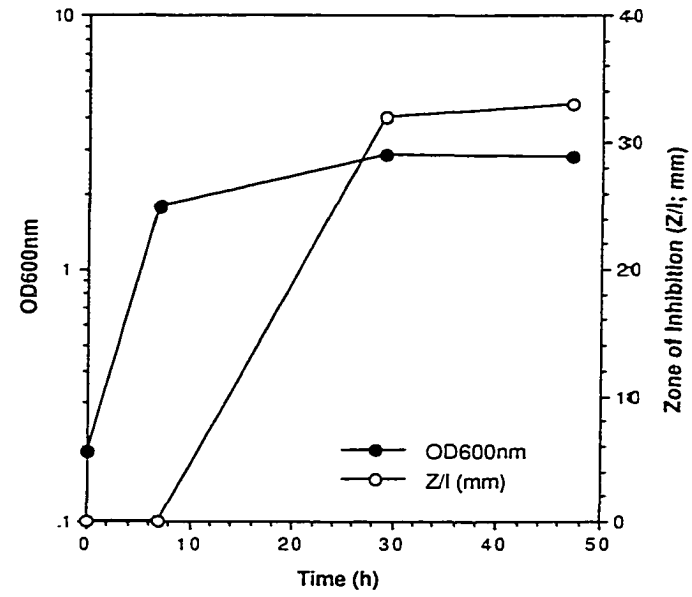


Figure I.3.5.5 Growth and antibiotic production of *P. polymyxa* PKB1 in two different bioreactor volumes at 26°C. A. Growth in 10 L PDB in a 15 L bioreactor. B. Growth in 50 L in a 75 L bioreactor.

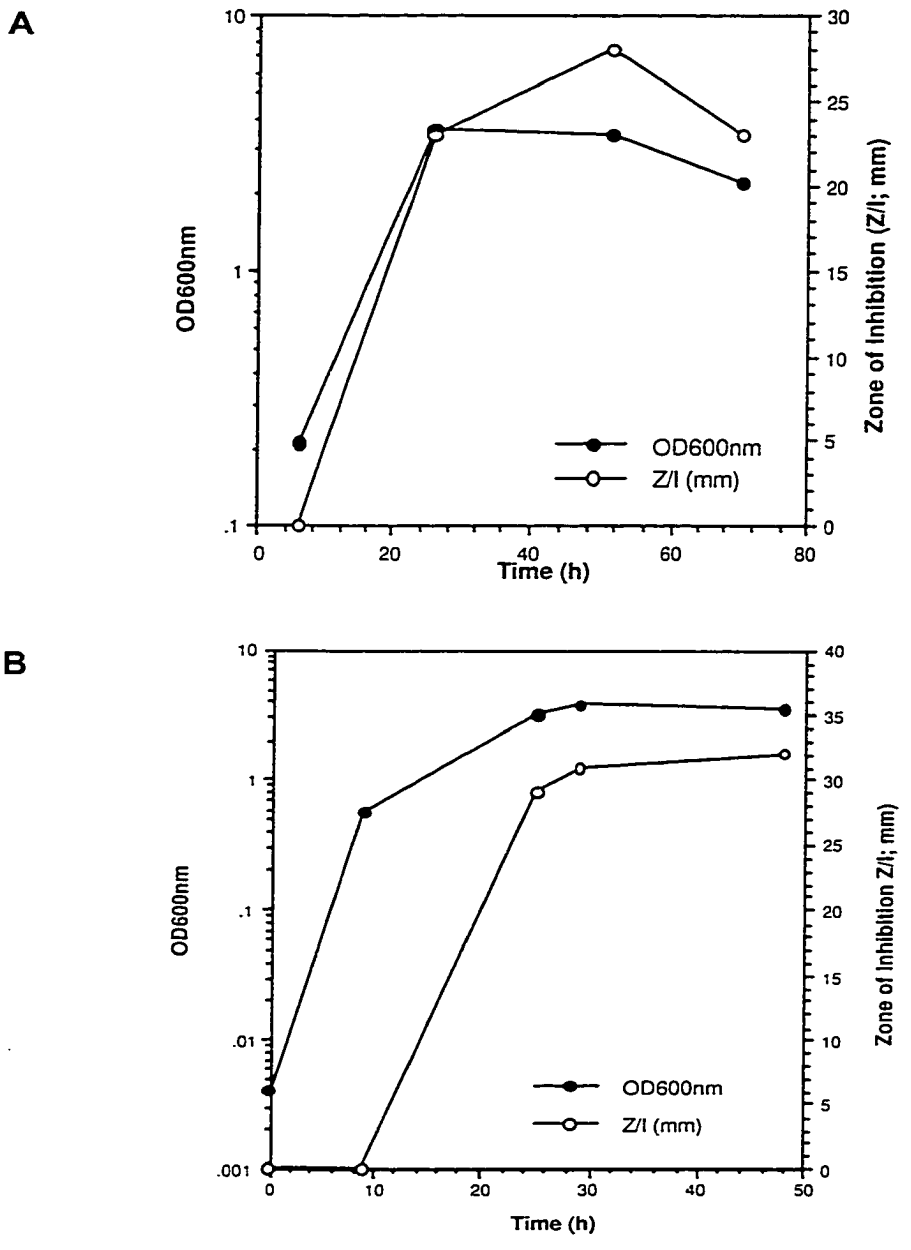


Figure I.3.6 Growth and antibiotic production of A. *P. polymyxa* subsp. *colistinus koyama* and B. *P. polymyxa* SCE2. Growth conditions were the same for both of these and for the *P. polymyxa* PKB1 (Figure I.3.5.3) cultures.

from the cultures by the mini-extraction method. This antifungal antibiotic material from SCE2 and *colistinus koyama* inhibited the growth of *L. maculans* in a well bioassay. *P. polymyxa* NCIMB 8648 did not produce antifungal antibiotic. No activity was seen against *L. maculans* from either the mini-extraction method or from methanol extraction of the NCIMB 8648 cell/spore pellet from a 200 ml culture. The antifungal antibiotic from *P. polymyxa* SCE2 and *P. polymyxa* subsp. *colistinus koyama* was detected in both cultures at the same time (endospore formation) as for *P. polymyxa* PKB1; the ZOI max was 32 mm from SCE2 and 28 mm from *colistinus koyama.*

Based on these results these three strains appear to have similar growth characteristics and *P. polymyxa* SCE2 and *P. polymyxa* subsp. *colistinus koyama* can produce methanol soluble antifungal material that is inhibitory to *L. maculans.*

I.3.7 Inhibitory spectrum of antifungal antibiotic produced by *P. polymyxa* PKB1

Crude methanol extracts of antibiotic material that were obtained from *P. polymyxa* PKB1 using the mini-extraction method were tested against many fungi and some bacteria (Table I.3.7). The antibiotic material was found to be active against a variety of fungi, including ones responsible for costly agricultural crop diseases. The number of bacteria tested was limited, but the antibiotic was found to inhibit (ZOI max = 30 mm) the Gram positive *Micrococcus luteus.* It was also able to marginally inhibit (ZOI max = 17) a Gram negative, *E. coli* ESS. This strain of *E. coli* is an antibiotic sensitive strain, which may be why the antifungal antibiotic was able to inhibit it. The antifungal material was not able to inhibit *E. coli* XL1B.

This range of activity; against Gram positives and fungi, is similar to that of the known *P. polymyxa* antibiotics gatavalin (LI-F04), LI-F03, LI-F05, LI-F07, LI-F08, and fusaricidins A, B, C and D (Section I.1.4). It is not similar to the inhibition range of the polymyxins, polypeptins A, B, jolipeptin, gavaserin, saltavalin or polyxins (Section I.1.4).

Table I.3.7. Spectrum of inhibition of the *P. polymyxa* PKB1 antifungal antibiotic.

inhibition against fungi	inhibition against bacteria
Alternaria brassicae[a]	Gram positive bacteria
Aspergillus niger[b]	*M. luteus*
Fusarium avenaceum[a]	*S. clavuligerus*
L. maculans[a]	
Marasmius oreades[a]	Gram negative bacteria
Neurospora crassa	*E. coli* ESS (slightly)
Penicillium chrysogenum[b]	
P. roquefortii[b]	
Rhizoctonia solania[a]	
Sclerotinia sclerotiorum[a]	
Sporobolomyces[b]	
Trichoderma 85[b]	

[a] as determined by P. Kharbanda, Alberta Environmental Centre, Vegreville, Alberta
[b] as determined by T. Palmer-Stone, Department of Biological Sciences, University of Alberta

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II.1 Introduction: Purification and Partial Characterization of the Antifungal Antibiotic from *P. polymyxa* PKB1

P. polymyxa PKB1 is an environmental bacterium isolated from canola stubble. It was able to inhibit *L. maculans*, a fungus that causes a disease of canola called blackleg. *P. polymyxa* is a spore-forming bacterium. The spore-formers are well known for the production of peptide structures just prior to, or at the onset of sporulation, that exhibit antibiotic characteristics. These peptides tend to be produced by the cell nonribosomally, by peptide synthetase (PS) multienzyme complexes. Strains of *P. polymyxa* are known to produce many different peptide antibiotics including the polymyxins, gatavalin and jolipeptin. The nature of the antifungal antibiotic produced by *P. polymyxa* PKB1 was investigated.

II.1.1 Unusual amino acids and modifications of peptide antibiotics

Peptide antibiotics produced by sporeforming bacteria tend to contain certain amino acids and other residues not common to proteins. The peptides may also be linear, branched or cyclic and may have bonds other than peptide bonds within the structure of the peptide. Some of the unusual amino acids and other residues of peptides produced by strains of *P. polymyxa* include: D-amino acids, allo-amino acids, 2,4-diaminobutryic acid, 3-hydroxy-4,6-dimethylheptanoic acid, 3-hydroxy-4-methylpentanoic acid and 15-guanidino-3-hydroxypentadecanoic acid (Table I.1.4; Bodanszky and Perlman, 1969; Shoji *et al*, 1977; Kajimura and Kaneda, 1996; 1997). Some of the peptide antibiotics isolated from *P. polymyxa* contain unusual amino acids that remain uncharacterized (Kurusu and Ohba, 1987).

The number of unusual amino acids and modifications found within peptide antibiotics from producing bacteria and fungi are in the hundreds. These unusual amino acids or modifications include: dehydrobutyrine, dehydroalanine, δ-aminopentanoic acid, 2-aminohexenoic acid, α, β-diaminopropionic acid, sarcosine, ornithine, methylation, acetylation, formylation, hydroxy acids (D-α-hydroxyisovaleric acid), sugars and amines (cadaverine; Bodanszky and Perlman, 1969)

II.1.2 Typical methods for purification, detection and analysis of peptide antibiotics

Many of the antibiotics isolated from *P. polymyxa* have been characterized as peptide antibiotics (Section I.1.4). Most of the peptide antibiotics isolated from bacteria and fungi are less than 3000 Da (Umezawa *et al.*, 1978). Of the known peptide antibiotics from *P. polymyxa*, the polymyxins have the highest molecular weight, approximately 1280 Da. Despite their peptide nature, different methods of purification, detection and analysis are generally employed for the purification of low MW peptides in comparison to the methods used for higher MW molecules like proteins. Several such methods which were employed in the current study are described in more detail here.

II.1.2.1 Size exclusion chromatography; purification using Sephadex LH-20

Size exclusion (or gel filtration) chromatography separates molecules by differences in molecular size and by their steric hindrance through a gel matrix. (cross-linked dextran in the case of Sephadex LH-20). Larger molecules have less access to the porous matrix of the column bed than smaller molecules and elute first. Smaller molecules enter the column bed and travel through the matrix, which slows their elution. There are many types of matrices that can be used in size exclusion chromatography that differ in component exclusion limit depending on porosity (Snyder and Kirkland, 1979).

Sephadex LH-20 is a type of matrix that is used to fractionate low MW, polar molecules. The matrix is made of spherical, porous, cross-linked dextran beads that are coated with hydroxypropylate to allow polar organic solvents to be used as the mobile phase. The range of bead size when slurried in an organic solvent like methanol is 27 to 163 μm and the exclusion limit of peptides is approximately 4000 Da (Amersham Pharmacia wesite; http://www.apbiotech.com).

II.1.2.2 Reverse phase high performance liquid chromatography

RP-HPLC is frequently used to separate peptides because organic solvents can be used in the mobile phase and many peptides are soluble in polar organic solvents. RP-HPLC separates on the basis of the interaction of the sample molecule; such as a

peptide, with the mobile phase; dependent on solvent polarity, and the stationary phase of the column. If the mobile phase is a gradient, then two solvent solutions; one of higher polarity than the other, are mixed so the gradient begins as most polar and changes to least polar during the chromatography. This will affect the retention of the peptide within the column and therefore the elution.

The presence of the ion-pairing reagents, trifluoroacetic acid (TFA; hydrophobic) or orthophosphate (hydrophilic), in the RP-HPLC solvent system can also alter the retention time of a sample molecule such as a peptide (Guo *et al.*, 1987). By the addition of an ion-pairing reagent to the solvent system, the hydrophilicity of the peptide is reduced which has the effect of increasing its' retention time on the column.

Other factors that affect the retention time of a sample molecule include column length and diameter, pore size of the column matrix, gradient steepness, flow rate of the mobile phase and the temperature of the system (Guo *et al.*, 1987).

II.1.2.3 Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel elctrophoresis (SDS-PAGE) systems were mainly designed to separate medium to high MW proteins (Schagger and von Jagow, 1987). These types of SDS-PAGE cannot resolve molecules of 10 kDa or less.

Schagger and von Jagow (1987) optimized SDS-PAGE to allow low MW molecules to concentrate efficiently in the stacking gel; separated from the SDS so as to reduce streaking or smearing of the molecule through the separating gel. The authors replaced glycine with tricine as the trailing ion in the cathode buffer. Tricine optimizes the stacking of low MW molecules because it has a similar effective mobility, unlike glycine, that allows it to trail after the faster migrating components of the sample. Efficient stacking of molecules of 1 to 20 kDa in the stacking gel is achieved with 16.5% acylamide tricine SDS-PAGE (Schagger and von Jagow, 1987).

II.1.2.4 Analysis of peptides by mass spectroscopy

Mass spectroscopy (MS) is a powerful tool that allows rapid, sensitive and accurate determination of the mass of the molecular ion of the component under study. Fragmentation of the molecular ion can provide information regarding the nature of the molecule.

Mass spectrometers are made up of three basic components: an ion source, a mass analyzer and a detector. Once the sample is ionized, the mass analyzer separates the ions based on their mass to charge (m/z) ratio, the separated ions are detected and the information plotted as a spectrum of percent relative abundance versus m/z (Silverstein *et al.*, 1991).

Many different types of ionization methods have been used to analyze peptides such as; fast atom bombardment (FAB), plasma-desorption, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). These are soft ionization methods that allow the molecular ion $[M+H]^{+1}$ of large, polar, nonvolatile molecules, such as peptides of even few amino acids, to be singly charged (protonated) and detected as intact structures (Papayannopoulos, 1995; Chanda, 1998). The molecular ion can also be doubly charged by two protons $[M+2H]^{+2}$ or by a proton and a sodium ion $[M+Na+H]^{+2}$, if it contains amino acids or other components that can be protonated, a free N-terminus, or if the peptide is over 1000 Da in size. The molecular ion peak masses are also subject to the mass of the isotopes of the elements present in the sample. The ion peak that is the sum of the most abundant isotopes of the elements present is called the monoisotopic peak. The percentages of the naturally abundant isotopes of C, hydrogen (H), N, and oxygen (O), elements found in amino acids, are very high. For a peptide of MW less than 2000 Da, the monoisotopic peak is the most abundant peak of the parent molecular ion cluster followed by the peak representing the average mass values of the isotopes present in the peptide (Chanda, 1998).

There are different types of mass analyzers, such as the quadrupole mass filter and the time of flight (TOF) analyzer. The quadrupole mass filter is commonly used with ESI and the TOF analyzer is commonly used with MALDI. The ESI and MALDI ionization methods are used to ionize peptide samples for analysis because the sensitivity of these methods can be in the subpicomole range and a large MW range (up to 150 kDa for ESI and 350 kDa for MALDI) of peptides and proteins can be analyzed. MALDI-TOF is used frequently to analyse biological components like peptide antibiotics because the samples can contain phosphates or alkali metal salts and still be analysed with accuracy.

The ESI-quadrupole and the MALDI-TOF systems can be modified so that a particular molecular ion from a peptide is mass selected, fragmented either by inducement or spontaneously, and the resulting fragment ions analysed separately from the rest of the ions in the sample. This technique is called tandem MS when used with ESI-quadrupole because it is two MS instruments linked together, and post-source

decay (PSD; also called metastable decay) with MALDI-TOF because after the molecular ion is formed at the ionization source, it can fragment (or decay) into ions called metastable ions, in the flight tube (the TOF mass analyzer) on the way to the detector (Chanda, 1998). The use of MS in the characterization of peptide antibiotics has become prevalent with the advent of tandem MS and PSD. These techniques allow the mass of the molecular ion of the peptide to be determined as well as the amino acids comprising the peptide, in a sequential manner (Payapannopoulos, 1995). This can be done even if the peptide is present in a complex mixture, because one ion is selected from the ions generated by the first MS and passed to the second MS for further fragmentation (Payapannopoulos, 1995; Biemann, 1986; Chanda, 1998).

The fragmentation patterns of linear peptides have been well described (Biemann, 1986), however the fragmentation of cyclic peptides can be difficult to interpret because the formation of the ions does not necessarily follow the known fragmentation patterns for linear peptides.

As mentioned in Section II.3.1.1, cyclic peptides may contain only amide bonds linking the amino acids to form a homomeric structure or they may contain both amide and ester bonds linking the amino acids forming a depsipeptide structure. They may also carry side chains or unusual amino acids or other components in the ring structure. All of these characteristics will affect the fragmentation of a cyclic peptide in MS (Eckart, 1994).

The cyclic peptide structure must be cleaved before fragmentation of the ring can occur. If the cyclic peptide is homomeric then the protonated peptide could potentially cleave at any of the amide bonds resulting in linear peptides with different sequences but the same mass. The ions that are formed can be acylium-amino acid residue(s) or immonium-amino acid residue(s) ions (Falick *et al.*, 1993; Eckart, 1994; Papayannopoulos, 1995). If the cyclic peptide is a depsipeptide then cleavage is favoured at the ester bond(s), resulting in one linear peptide if there is one ester bond in the structure or potentially more than one linear peptide with different sequences and masses. Cleavage at an ester bond generally results in an ion fragment with a C-terminal carboxylic acid group. Further fragmentation of the peptide fragment ion by loss of a neutral amino acid, can aid in sequence determination of the peptide.

Acylium and immonium ions can fragment further so that the peak associated with the generated ion is represented on the spectrum as a satellite peak. The acylium-amino acid residue ions can lose the acylium ion (CO^+), seen by a loss of 28 mass units

(mu). The immonium-amino acid residue ions can lose the ammonium ion (NH_3^+), seen by a loss of 17 mu (Payapannopoulos, 1995). Non-sequence specific ions can also form from side chain losses or as single amino acid residue immonium ions. (Biemann, 1986; Biemann *et al.*, 1986; Payapannopoulos, 1995).

Arrendale *et al* (1988) used gas chromatography/MS (GS/MS) to characterize iturin type peptides that were isolated from the culture broth of a strain of *B. subtilis* able to inhibit *Monilinia fructicola*; a peach brown rot fungus. The researchers hydrolysed the antifungal antibiotic sample and analyzed the resulting amino acids. MS determined that the antifungal antibiotic sample was a mixture of two peptides differing by the structure of the β-amino aliphatic acid. Fragmentation of both β-amino aliphatic acids revealed that they differed by one methylene unit; their parent ions and other key ion peaks had mass differences of 14 Da.

Ishikawa and Niwa (1990) sequenced a mixture of two unknown, cyclic peptide antibiotics from *B. subtilis* that were either intact or partially acid hydrolysed, using FAB-MS. The FAB-MS of intact peptides produced immonium ions that allowed for the correct identification of asn/asp and gln/glu amino acids in the peptide. The authors used collision-induced dissociation (CID) of the peptide ion fragments to determine the connectivity of the amino acids in the peptide.

Erhard *et al.* (1997) used MALDI-TOF MS to characterize certain strains of bacteria, using the whole bacteria as the sample. The researchers were interested in whether the mass spectra of the whole bacteria could be used to type pathogenic strains. The researchers found that the MALDI-TOF spectra of the whole cells mainly showed the secondary metabolites that the organism produced. The secondary metabolites appeared to have been preferentially extracted from the whole cells while suspended in the sample ionization matrix. The authors also investigated an unknown secondary metabolite from the cyanobacterium, *Microcystis aeruginosa* using PSD and MALDI-TOF. The metabolite was a cyclic peptide that was determined to be similar in structure to anabaenopeptolin B, a cyclic peptide produced by another cyanobacterium; *Anabaena flosaquae.* The unknown peptide was named anabaenopeptolin C. The two peptides differ by one amino acid; anabaenopeptolin B contains a valine that is exchanged for an isoleucine in anabaenopeptolin C.

Bonsall *et al.* (Tobias, 1998) used HPLC-MS to identify antibiotics produced by various fluorescent pseudomonads that were able to inhibit *G. graminis* var. *tritici*, the fungus that infects wheat with take-all disease. The researchers extracted antibiotics

directly from wheat root-rhizosphere soil samples and analyzed them in an automated process that involved separation of the biological components such as antibiotics by HPLC, mass analysis of the separated components by MS and potential identification of the component by matching the MS information to a library of MS data of natural and synthesized peptides.

II.1.3 Amino acid compositional analysis and Edman degradation sequencing

Quantitative amino acid compositional analysis of picomole levels of peptides involves: complete acid hydrolysis of the peptide to free amino acids, derivitization of the free amino-group of the amino acids with phenylisothiocyanate (PITC) to form phenylthiocarbamyl-amino acid derivatives (PTC-AA) for detection and analysis of the deriviatized amino acids by a RP-HPLC system calibrated with amino acid standards.

The acid hydrolysis step damages some of the amino acids found in proteins; cysteine and tryptophan are destroyed, serine and threonine are partially damaged, valine and isoleucine are slow to cleave and the side chains of asparagine and glutamine become deaminated and indistinguishable from aspartic acid and glutamic acid.

The amino acid sequence of a peptide can be determined by Edman degradation if there is a free N-terminal amino acid. PITC reacts with the free amino group of the N-termial amino acid forming a PTC derivitized amino acid. The peptide is treated to mild acidic conditions that cyclizes the PTC-AA and releases it from the rest of the intact peptide. If the N-terminus of the peptide is blocked by chemical modification or cyclization, Edman degradation cannot be done (Stryer, 1988).

II.1.4 Purification methods used with some peptide antibiotics

Methods of purification which have been applied to some of the peptide antibiotics from *P. polymyxa* are described below.

Nakajima *et al.* (1972; 1975) isolated gatavalin by acidifying and heating a sporulating culture and extracting it from the broth with n-butanol. Gatavalin was precipitated from n-butanol with ethylether and the crude powder was dissolved in a mixture of methanol-dilute hdyrocholoric acid (HCl). The antibiotic solution was passed through activated alumina by elution with methanol-dilute HCl and the effluent was

mixed with n-butanol again to extract gatavalin from the methanol. Gatavalin was precipitated from the n-butanol with ethylether and the powder was dissolved in a methanol-water solution and passed through a Sephadex LH-20 column as a final purification step.

Ito and Koyama (1972a; 1972b) precipitated jolipeptin and other biological components from disrupted cells in alkaline broth, by the addition of benzaldehyde. The precipitate was mixed with acidic water-saturated n-butanol to extract jolipeptin, which was then precipitated from the n-butanol phase with ethylether. The crude powder was dissolved in methanol and insoluble material was removed. Jolipeptin was reprecipitated from the methanol with ethylether, dried, redissolved in methanol and passed through a Sephadex LH-20 column in methanol as a final purification step.

Parker *et al.* (1977; 1978) isolated polymyxin F by acidifying the culture broth. The cells were pelleted and polymyxin F was extracted from the acidic supernatant with n-butanol. The concentrated n-butanol extract was diluted with methanol and acetone to precipitate polymyxin F. The crude polymyxin F powder was adsorbed on a IRC-50 resin, eluted with equal parts methanol-dilute HCl and extracted with n-butanol. The n-butanol extracted material was further purified by carboxymethyl-cellulose chromatography to yield the hydrochloride form of polymixin F.

Kurusu and Ohba (1987) isolated the peptide antibiotic series LI-F03, -F04, -F05, -F07 and –F08 from cell-free culture broth. The broth was passed through an Amberlite XAD-2 column and eluted with methanol. The methanol eluate was dried, dissolved in concentrated acid and extracted with n-butanol. The n-butanol extracted material was further purified on a silica gel column. Analytical HPLC showed that the material consisted of over ten active components, five of these components could be separated from each other by RP-HPLC with an acetonitrile:water:dilute trifluoroacetic acid (TFA) mobile phase. These five components (LI-F03, -F04, -F05, -F07 and –F08) were analyzed by FAB-MS and each was found to still be a mixture of two components.

Pichard *et al.* (1995) isolated gavaserin and saltavalin from cell free culture broth that was heat treated and lyophilized. The lyophilized broth was dissolved in water and purified by semi-preparative and analytical RP-HPLC with a gradient mobile phase of dilute TFA and acetonitrile.

Kajimura and Kaneda (1996; 1997) isolated fusaricidins A, B, C and D from cell-free culture broth by extraction of the supernatant with n-butanol and purification on a silica gel column. Active fractions were purified by chromatography on a Sephadex LH-

20 column. The antibiotics were further purified using a preparative RP-HPLC column with a gradient mobile phase of acetonitrile and dilute TFA that separated the sample into two peaks. One peak was further separated by a second round of RP-HPLC to yield fusaricidin A and B. The other peak consisted of fusaricidins C and D that could not be separated by further passes through a RP-HPLC column under various conditions.

Piuri *et al.* (1998) isolated polyxin from cell-free culture broth that was filter sterilized and precipitated with dilute TFA to give a crude polyxin preparation used in characterization studies.

II.1.5 Research design

This chapter will focus on the purification strategies and characterization of the antifungal antibiotic from *P. polymyxa* PKB1. The antifungal antibiotic was extracted from lyophilized *P. polymyxa* spores with methanol, and from acidified and heated *P. polymyxa* culture broth with n-butanol. Further purification of the crude methanol extract was achieved using Sephadex LH-20, C_{18} RP-Sep-pak and semi-preparative C_{18} RP-HPLC. The acidic butanol extract was purified with C_{18} RP-Sep-pak and either semi-preparative C_{18} RP-HPLC or preparative C_4 RP-HPLC. Antifungal antibiotic was detected throughout the purification using a well bioassay, thin layer chromatography with bioautography or tricine SDS-PAGE with either coomassie blue, silver stain or bioautography. The antifungal antibiotic was characterized with regard to stability, solubility, ninhydrin reaction and reactivity with degradative enzymes and chemicals. The composition and structure of the antifungal antibiotic was investigated with amino acid compositional analysis and mass spectroscopy using a variety of ionization and fragmentation methods.

The antifungal antibiotic was traced through the purification process using the following methods.

II.2.4.1 Well bioassay

Square 245 mm petri dishes (Corning Costar Corporation; Cambridge, Massachessetts) were filled with 100 ml of molten PDA, which was allowed to harden. Between 500 to 800 µl of *L. maculans* glycerol stock was spread onto the agar surface, then wells were punched out of the agar, to the bottom of the plate, with the large end of a sterile 1000 µl pipet tip (13 mm in diameter) and 75 µl of sample to be bioassayed was placed into each well. Methanol (75 µl) was used as a control. Plates were incubated at room temperature in the light, right side up. After the fungus had grown (48 to 72 h), the diameter of the zone of inhibition around each well, was measured.

II.2.4.2 Tricine SDS PAGE with Coomassie blue stain, silver stain or bioautography

Two different sized tricine SDS-PAGE systems were used: a small gel (8.5 cm wide) with a 5 cm high separating gel and a 2 cm high stacking gel, and a large gel (14.5 cm wide) with a 8.5 cm high separating gel, a 3 cm high spacer gel and a 2 cm high stacking gel. Both sizes of gels were made using the same components. The gels were made according to Schagger and von Jagow (1987), with a 4% polymerizing solution stacking gel, 10% polymerizing solution spacer gel and a 16.5% polymerizing solution separator gel. The polymerizing solution was 48% (w/v) acrylamide and 1.5% (w/v) bisacrylamide to make a 49.5% final solution. Stacking gels contained 1 ml 49.5% polymerizing solution, 3.1 ml gel buffer (3.0 M Tris-HCl, 0.3% SDS, pH 8.5)and 8.4 ml distilled H_2O. Spacer gels contained 6.1 ml 49.5% polymerizing solution, 10 ml gel buffer and 14 ml distilled H_2O. Separator gels contained 10 ml 49.5% polymerizing solution, 10 ml gel buffer, 8 ml 50% glycerol and 2 ml distilled H_2O. Each gel solution was polymerized separately with 100 µl 10% ammonium persulfate (Bio Rad) and 10 µl N,N,N', N'-tetramethylethyl-enediamine, (TEMED; Bio Rad). The gels were electrophoresed in a dual running buffer system at 100 V (11.8 V/cm) for the small gels and 150 V (10 V/cm) for the large gels. The anode (bottom) buffer was 0.2 M Tris-HCl pH 8.9 and the cathode (top) buffer was 0.1 M Tris, 0.2% SDS and 0.1 M Tricine (Sigma). Coloured protein molecular weight markers (MW range 46000 to 2350,

II.2 Materials and Methods

II.2.1 Chemicals and reagents

Chemicals used in media, electrophoresis gels or in chemical assays were reagent grade, purchased from Aldrich (Milwaukee, Wisconsin), Bio Rad (Hercules, California), Sigma (St. Louis, Missouri), ICN (Aurora, Ohio), BDH (Toronto, Ontario), Amersham Pharmacia Biotech (Arlington Heights, Illinois) or Fisher (Fair Lawn, NJ) unless otherwise indicated.

Agar and pre-mixed solid media were obtained from Difco (Detroit, Michigan) and agarose was from Amersham Pharmacia Biotech.

Solvents used were obtained from the following sources: HPLC grade acetonitrile from Fisher Scientific, 99.7% analytical reagent grade chloroform, spectro grade methanol, 100% glycerol and glass distilled ethyl acetate from Caledon Laboratories LTD (Georgetown, ON), and laboratory reagent grade phenol from Anachemia (Montreal, PQ).

II.2.2 Biological culture preparation and storage

Lyophilized *P. polymyxa* stocks were generated as described in Section I.2.3.2.

L. maculans was stored at -70°C as a 20% glycerol stock of pycnidiospores at 2 X 10^7 spores/ml. Spore stocks were made by growing a lawn of *L. maculans* on PDA for 48 h in the light. The lawn of fungus growing in a 100 X 15 mm petri dish was flooded with 5 ml 20% sterile glycerol and the pycnidiospores were scraped off the mat of growth with a sterile spatula. This mixture was filtered through sterile cotton fluff and collected in 1.5 ml Eppendorf tubes.

II.2.3 Media and growth conditions for bioreactors

The media and growth conditions for bioreactor cultures are described in Section I.2.3.2, as is the lyophilization procedure for the harvested biomass.

II.2.4 Detection of antifungal activity

Rainbow™, Amersham Pharmacia Biotech Life Science), were used to determine an approximate size of the proteins and peptides in the samples. The peptide samples that were electrophoresed in these gels were dissolved in 20 to 50 µl sample buffer (50 mM Tris-HCl, 4% (w/v) SDS, 12% (w/v) glycerol, 2% (v/v) mercaptoethanol, 0.01% Serva blue G (Sigma), pH 6.8).

The peptide bands were visualized by one of three methods: staining with Coomassie blue dye as described by Blakesley and Boezi (1977), silver staining (Oakley *et. al.*, 1980) or using a bioautography method adapted from Hash (1975). For a bioautograph, the gel was immersed in a freshly made fixer solution (50% methanol, 10% acetic acid) for 30 min with gentle agitation, then washed in distilled H_2O for 1.5 h twice. The gel was then placed onto PDA (100 ml) in a 15 X 15 cm petri dish and overlaid with 100 ml of molten PDA seeded with 800 µl of *L. maculans* glycerol stock. The plate was incubated right side up at room temperature in the light and the zones of inhibition were measured after the fungus had grown (48 to 72 h).

II.2.4.3 Thin layer chromatography and bioautography

Antibiotic activity was also detected using bioautography of thin layer chromatograms. Chromatograms were prepared by spotting 10 µl of samples with antifungal activity onto cellulose plates (Eastman Kodak Company; Rockford, New York) that had been presoaked in the mobile phase, then dried. The chromatograms were developed using a mobile phase of either 3:1:1 (v/v/v) n-butanol: acetic acid: sterile water or 70% n-propanol. The developed chromatogram was allowed to completely dry, then was placed onto PDA (100 ml) in a square 245 mm petri dish that had previously been inoculated by spreading with 800 µl of a *L. maculans* spore stock. The chromatogram was left on the agar for 30 to 60 min, the borders of the chromatogram and the solvent front were marked on the outside of the petri dish and the chromatograph was removed. The plate was incubated at room temperature in the light and the zones of inhibition were measured after the fungus had grown (48 to 72 h).

II.2.4.4 Bicinchoninic acid (BCA) protein assay

The protein content of samples taken at various steps in the antibiotic purification process was quantified, using a BSA standard. The test tube method in the BCA protein

assay kit from Pierce (U.S. Patent No. 4839295, Rockford, Illinois) was used as described by the manufacturer.

II.2.5 Antifungal antibiotic extraction and purification

Two different protocols were employed to extract the antifungal activity from the spores of *P. polymyxa* PKB1. The first was developed as a part of this thesis research and the second was adapted from Nakajima *et al.* (1975). For all of the subsequent purification and characterization studies, the methanol extraction procedure described below was used for the initial extraction of antifungal material, unless otherwise stated. Three different columns were used in the purification process as described in Sections II.2.5.1-3.

II.2.5.2 Methanol extraction of antifungal material

Lyophilized *P. polymyxa* spores (1 g) were washed twice by resuspending in 100 ml distilled H_2O then pelleting by centrifugation at 15 300 X g for 20 min. The washed spore pellet was extracted twice by resuspending in 60 ml 100% methanol, incubating for 1 h and pelleting by centrifugation at 10 000 X g for 15 min. The methanol supernatants were pooled and reduced to between 10 and 12 ml by rotoevaporation under vacuum, in a 60°C water bath. The resulting crude methanol extract was centrifuged in a clinical centrifuge at 890 X g for 10 min to sediment cellular debris and polysaccharide. The crude methanol extract was further reduced to between 3 and 6 ml by rotoevaporation or under a stream of nitrogen. The antifungal activity of the crude methanol extract was determined by well bioassay (Section II.2.4.1). This crude methanol extract was purified by the protocols described in Sections II.2.5.4 to 6.

II.2.5.3 Extraction of antifungal material as adapted from Nakajima *et al.* (1975)

The antifungal material was initially extracted from between 400 ml and 4 L of a *P. polymyxa* culture grown in PDB, after the cells had developed into endospores, by one of two methods. The first method involved acidifying the culture to a pH of 3 with 5 M HCl, heating to 80°C for 15 min, then centrifuging at 15 300 X g for 15 min to obtain the supernatant containing the antifungal material. The second method involved

centrifuging the culture at 15 300 X g for 15 min, resuspending the spore pellet in a volume of distilled H_2O equal to the original culture volume, acidifying the suspended spores to a pH of 3 with 5 M HCl, heating at 80°C for 15 min, then centrifuging at 15 300 X g for 15 min to obtain the supernatant containing the antifungal material.

The crude acidic extracts from these two extraction methods were further purified by mixing with n-butanol in a 3:1 ratio in a separatory funnel. The mixture was shaken and the immiscible solvents were allowed to separate; the antifungal material was extracted into the n-butanol phase. The n-butanol phase was reduced by rotoevaporation under vacuum, in a 37°C water bath to between 30 and 50 ml.

The antifungal material was precipitated from the n-butanol by the addition of 3 volumes of ethyl acetate. The solid antifungal material was dissolved in 4 ml methanol and purified using a C_{18} Sep-pak column and RP-HPLC, with both the C_{18} and C_4 columns as described in Sections II.2.5.2 and II.2.5.3.

II.2.5.4 Sephadex LH-20: size exclusion chromatography

Sephadex LH-20 beads, in methanol range in diameter from 27 to 163 μm; they are coated with hydroxypropylate which allows these beads to be used in an organic solvent. A Sephadex LH-20 column was prepared by forming a slurry of 10 g LH-20 gel beads (Amersham Pharmacia Biotech) in 100 ml 100% methanol. The slurry was placed at 4°C and the beads were left to swell for 1 h. The slurry was then poured into a 2.0 X 24 cm glass column to a height of 15 cm and left to equilibrate at 4°C, overnight, with a methanol mobile phase running through it at 0.2 ml/min. The column was used at 4°C in the purification procedure.

The crude methanol extract (3 to 6 ml) was layered onto the Sephadex LH-20 column in 3 ml aliquots. The mobile phase was 100% methanol, rate of flow was 0.2 ml/min. The detector was a LKB Bromma Z238 Uvicord SII spectrophotomer (Bromma, Sweden) set to measure the A_{280} of the eluted samples; chart speed was 0.2 mm/min. Fractions were collected using a LKB Bromma 7000 Ultrorac II fraction collector (Bromma, Sweden), and collection time was 10 min/fraction. Antifungal activity of the fractions was determined by well bioassay (Section II.2.4.1). Fractions containing antifungal material from the Sephadex LH-20 column were pooled and reduced from between 10 and 12 ml to 4 ml under a stream of nitrogen. These samples were termed Sephadex partially pure antifungal material.

II.2.5.5 Reverse phase (RP) C_{18} Sep-pak (Waters; Milford, Massachessetts)

The Sephadex partially pure antifungal material was purified further by passage through a C_{18} Sep-pak Classic Short Body column. This is a disposable mini-column purchased from Waters Associates. The packing material of the column is a hydrophobic, non-polar, C_{18} component, with a pore size of 125 Angstrom and a particle size of 80 μm, bound to a silica surface. The column was first equilibrated with 2 ml 100% methanol then 5 ml distilled H_2O, at a flow rate of 5 ml/min. An equal volume of distilled H_2O was added to the 4 ml sample in 100% methanol to make the sample in 50% methanol. This 8 ml sample was loaded onto the C_{18} Sep-pak column at a flow rate of 5 ml/min. After the sample was loaded, the column was washed with 2 ml distilled H_2O, then the active component was eluted from the column in 100% methanol at 1.2 ml/min; 1 ml fractions were collected. The activity of the fractions was determined using the well bioassay (Section II.2.4.1). The active fractions were pooled and reduced to dryness under a stream of nitrogen. The active fraction from this step was termed Sep-pak partially pure antifungal material.

II.2.5.6 Reverse phase high performance liquid chromatography (RP-HPLC)

A C_{18} μBondapak™ radially compressed 8 X 100 mm RP-HPLC (micro-preparative) column was used to further purify samples that had been purified by Sephadex and Sep-pak chromatography (Water Associates, Milford, Massachusetts). The packing material of the column is a C_{18} component, with a pore size of 125 Angstrom and a particle size of 10 μm, bound to a highly activated silica surface. The active samples that were dried after elution from the Sep-pak column were dissolved in 1 ml of the mobile phase that was to be used in the RP-HPLC. If the RP-HPLC elution protocol involved two mobile phases in a gradient then the first mobile phase (solvent A) was used to dissolve the active component. Samples were loaded on the column in 200 μl aliquots. A variety of linear gradients, mobile phases, and flow rates were employed; these are listed in Table II.2.5.6.A. Nine different RP-HPLC protocols were designed for use with the C_{18} column. The distilled H_2O used in all HPLC procedures was purified using a Milli-Q Water Purification System (Millipore Corporation, Milford, Massachesetts). All solvents were passed through a Type HV 0.45 μm filter (Millipore

Table II.2.5.6.A. Isocratic and gradient mobile phases used with C_{18} RP-HPLC to purify the antifungal material.

mobile solvent system number[c] (ms #N)	time (min)	% solvent A	% solvent B	flow rate (ml/min)
1. solvent A: methanol	60	100	none	2.0
2. solvent A: methanol, TFA[a]	60	100	none	2.0
3. solvent A: 2:1 acetonitrile:dH_20[d], TFA[a]	45	100	none	1.6
4. solvent A: 1:2 acetonitrile:dH_2O[d], TFA[a]	30	100	none	1.6
5. solvent A: 1:3	0	100	0	1.6
acetonitrile:dH_2O[d], phosphate[b]	5	40	60	
solvent B; acetonitrile	15	40	60	
	50	0	100	
	55	100	0	
6. solvent A: 1:3	0	100	0	1.0
acetonitrile:dH_2O[d], phosphate[b]	2.5	40	60	
solvent B: acetonitrile	10	40	60	
	50	0	100	
	55	100	0	
7. solvent A: 1:3	0	100	0	1.0
acetonitrile:dH_2O[d], phosphate[b]	2.5	40	60	
solvent B; acetonitrile	10	40	60	
	50	0	100	
	55	100	0	
	65	100	0	
8. solvent A: 1:3	0	100	0	1.0
acetonitrile:dH_2O[d], phosphate[b]	2.5	40	60	
solvent B: acetonitrile	10	40	60	
	50	0	100	
	60	0	100	
	65	100	0	
9. solvent A: 1:3	0	100	0	1.0
methanol:dH_2O[d], phosphate[b]	2.5	40	60	
solvent B: methanol	10	40	60	
	50	0	100	
	60	0	100	
	65	100	0	

[a] 0.1% (v/v) trifluoroacetic acid
[b] 0.1% (w/v) disodium orthophosphate
[c] All gradients were linear
[d] distilled H_2O

Corporation; Bedford, Massechessetts) before they were used in HPLC. The detector was a Lambda Max model 480 LC UV-Spectrophotometer (Waters, Milford, Massachusetts) set to measure the A_{214} of the eluted samples and fractions were collected with a Gilson model 201 fraction collector (Gilson, Villiers Le Bel, France).

A C_4 Delta-Pak™ radially compressed 2.5 X 10 cm RP-HPLC (preparative) column (Waters Associates) was added to the previously described RP-HPLC process. The packing material of the column is a C_4 component, with a pore size of 100 Angstrom and a particle size of 15 μm, bound to a silica surface. Dried active fractions eluted from the C_{18} RP-HPLC were dissolved in 200 μl 100% methanol and loaded on the C_4 column. A variety of linear gradients were used to elute the active fraction from the C_4 column, six different protocols were designed, these are outlined in Table II.2.5.6.B.

The activity of the fractions from the RP-HPLC were determined using the well bioassay (Section II.2.4.1). The active fractions were pooled and dried under a stream of nitrogen. The active fraction from the RP-HPLC was termed pure antifungal material.

II.2.6 Characterization of antifungal activity

Crude methanol extract, Sephadex partially pure antifungal material, Sep-pak partially pure antifungal material or pure antifungal material that was used for characterization studies was not quantified unless otherwise stated. Each antifungal preparation used in these tests was initially extracted from *P. polymyxa* spores using the methanol extraction of antifungal material method (Section II.2.5.2), unless otherwise stated. The antifungal activity of all the test samples was measured by the well bioassay (Section II.2.4.1).

II.2.6.1 Solubility of antifungal material

Solubility of the crude and pure antifungal material in distilled H_2O, culture broth, 50 mM sodium phosphate buffer (pH 8), 0.1% acetic acid, 50-100% methanol, 10-100% acetonitrile, acetone, ethyl acetate, 2-propanol, n-butanol, methylene chloride and n-hexane was determined by adding 1 ml of the solvent to be tested to a dried sample of antifungal material in a 1.5 ml Eppendorf tube. The dried antifungal material consisted of the pooled active fractions eluted from C_{18} RP-HPLC using gradient 1, Table II.2.5.6.A. Solution of the component in the test solvent was attempted by mixing with a vortex for 1

Table II.2.5.6.B. Gradient mobile phases used with the C_4 column in RP-HPLC to purify the antifungal antibiotic from *P. polymyxa* PKB1.

mobile solvent system number[bc]	time (min)	% solvent A	% solvent B	flow rate (ml/min)
10. A/B. solvent A: 1:3 methanol:dH_2O, phosphate[a] solvent B: methanol	0	100	0	4 #10A
	1.2	40	60	2 #10B
	3.6	40	60	
	23	0	100	
	27	100	0	
	30	100	0	
11. solvent A: 1:3 methanol:dH_2O, phosphate[a] solvent B: methanol	0	100	0	4
	1.2	40	60	
	2.5	40	60	
	30	0	100	
	38	0	100	
	40	100	0	
12. A/B. solvent A: 1:4:15 methanol: KH_2PO_4[d]:dH_2O solvent B: 1:1:1:2 dH_2O: KH_2PO_4 [d]:acetonitrile:methanol	0	100	0	4 #10A
	1.2	40	60	2 #10B
	2.5	40	60	
	30	0	100	
	38	0	100	
	40	100	0	
13. solvent A: 1:3 methanol:dH_2O, phosphate[a] solvent B: methanol	0	100	0	4
	1.2	20	80	
	2.5	20	80	
	35	0	100	
	38	0	100	
	40	100	0	
14. solvent A: 1:3 methanol:dH_2O, phosphate[a] solvent B: methanol	0	40	60	1
	1	40	60	
	3	30	70	
	5	30	70	
	7	15	85	
	10	15	85	
	14	12	88	
	18	12	88	
	22	10	90	
	27	10	90	
	31	5	95	
	35	5	95	
	38	0	100	
	40	100	0	
15. solvent A: 1:3 methanol:dH_2O, phosphate[a] solvent B: methanol	0	100	0	1
	1	40	60	
	3	40	60	
	5	25	75	
	7	25	75	
	10	13	87	
	14	13	87	
	18	10	90	
	22	10	90	
	27	5	95	
	31	5	95	
	35	0	100	
	38	0	100	
	40	100	0	

[a] 0.1% (w/v) disodium orthophosphate dissolved in solvent mixture.
[b] All gradients were linear
[c] Gradients 1 to 9 are listed in Table .2.5.3.A.
[d] 0.1 M KH_2PO_4 pH 4.5 was used in mobile phase.

min. A control well containing each solvent in the same amount was made on the same agar plate as each tested sample.

II.2.6.2 Stability of the antifungal material

The stability of the antifungal material was determined using Sep-pak partially pure antifungal material, which was reduced to dryness under a stream of nitrogen, then dissolved in either 200 µl distilled H_2O or 200 µl methanol. These solutions were treated at two temperature extremes: 90°C for 65 min or flash freezing by immersion in a dry ice, ethanol bath to -70°C for 65 min. The antifungal activity of the treated solutions versus the untreated solutions was determined.

II.2.6.3 Reactivity of antifungal material with stains, dyes and chemicals

The antifungal material was treated with stains, dyes and chemicals to detect certain chemical groups such as; reaction with ninhydrin (Sigma) to detect primary and secondary amine and iodine (Fisher Scientific) vapours to detect unsaturated fatty acids and also -HC=CH- linkages in other types of molecules (Zweig and Sherman, 1972). Thin layer chromatograms of active fractions (Section II.2.4.3) were sprayed with 2% ninhydrin in acetone and the formation of purple spots was recorded or the chromatograms were placed in a dessicator chamber with approximately 0.5 g iodine crystals. The formation of brown spots was recorded. Silver stain (Section II.2.4.2) was used to detect any lipids; with a blue colouration, glycoproteins; with a yellow-red colouration and peptides carrying free sulfhydryl and/or carboxyl groups; with a brown-black colouration, on tricine-SDS-PAGE (Section II.2.4.2). A stain to detect glycoproteins was also used on tricine SDS-PAGE as described by Rauchsen, (1979).

II.2.6.4 Size determination of antifungal material

The approximate size of the antifungal material was estimated by passing 60 ml of crude methanol extract (before reduction by rotoevaporation) through Diaflo® ultrafiltration membranes (Amicon, Beverly, Massechessetts) with molecular weight cutoffs of 10 000 (PM10 membrane), 3000 (YM3 membrane) and 1000 (YM2 membrane) according to the manufacturers' instructions. The fraction that passed

through the filter was termed the eluate. The upper-side of the membrane was washed with 5 to 10 ml of methanol and this liquid was termed the retentate. The resulting eluate and retentate fractions were analysed for antifungal material by tricine-SDS-PAGE and bioautography (Section II.2.4.2).

II.2.6.5 Susceptibility of antifungal material to enzymatic and chemical cleavage

Antifungal material of various purities was treated with degradative enzymes such as pronase, trypsin, carboxypeptidase, endoproteinase Asp-N and esterase. Chemical reactions such as acid hydrolysis and ammonia ester hydrolysis were also performed on the antifungal material.

II.2.6.5.1 Pronase reactivity

The pronase reaction was performed using 100 µl of crude methanol extract. This aliquot was reduced to dryness under a stream of nitrogen and 1 ml of pronase E (Sigma) solution at; 0.3, 0.5, 0.6 and 0.7 mg/ml in buffer was added to the antifungal samples. Reaction mixtures were incubated at room temperature for 2 h, then dried using a speed vacuum and the residue was resuspended in 100 µl 100% methanol. A 10 µl portion of the sample was spotted onto a thin layer chromatogram and developed (Section II.2.4.3); the remainder was used in a well bioassay (Section II.2.4.1).

II.2.6.5.2 Trypsin and carboxypeptidase reactivity

Sep-pak partially pure antifungal material (Section II.2.5.5), in a 100 µl volume, was used in three digestion reactions; the first with 75 µl 0.2% (w/v) trypsin (Boehringer Mannheim), the second with 40 µl of 1 mg/ml carboxypeptidase (Boehringer Mannheim) and the third with both proteases (same volumes). The reactions were repeated with 75 µl 1 mg/ml sample of bovine serum albumin (BSA; Bio-Rad) as a control substrate, without antifungal material. The three reactions were also set up without either substrate, with distilled H_2O substituted into the reactions. The reactions were carried out in 1 ml 0.1 M ammonium bicarbonate and incubated at room temperature for 30 min. For the reaction of both proteases against the peptide and BSA substrates, carboxypeptidase was added first and incubated for 30 min then trypsin was added and the reaction

mixture was incubated for another 30 min. The enzymes were heat inactivated at 70°C for 10 min. Samples were analysed using tricine-PAGE and bioautography (Section II.2.4.2) and C_{18} RP-HPLC using gradient no. 1 (Table II.2.5.6.A).

II.2.6.5.3 Endoproteinase Asp-N reactivity

Pure antifungal material, that was originally extracted from 400 ml *P. polymyxa* culture by the second crude acidic extraction method of the Nakajima (1975) purification protocol (Section II.2.5.2), was used in two series of cleavage reactions with endoproteinase Asp-N (Boehringer Mannheim). The first series of reactions was designed to cleave at aspartic acid residues, according to manufacturers' instructions. Approximately 5% of the pure antifungal material was dried and redissolved into 200 µl 50 mM sodium phosphate buffer, pH 8.0 to a concentration of 0.037 or 0.035 mg/ml. Aliquots (50 µl) of this sample were mixed with 0.0015 Units, 0.015 Units, 0.15 Units and 1.5 Units of enzymatic activity. These four reaction mixtures were incubated for 3 h at 37°C. The enzyme was heat inactivated at 80°C for 10 min. The second series of reactions with endoproteinase Asp-N was performed according to the manufacturers' instructions for the cleavage of glutamic acid residues. Approximately 5% of the pure antifungal material was dried and redissolved into 150 µl of 50 mM sodium phosphate, pH 8.0 with 10% acetonitrile, this volume was split into three 50 µl aliquots and 2.4 mUnits, 0.4 mUnits and 0.24 mUnits of enzyme was added. These three reactions were incubated at 37°C for 19 h. The enzyme was heat inactivated at 80°C for 10 min. A 4 µl aliquot of each reaction in the first series and a 10 µl aliquot each reaction in the second series were tested for antifungal activity using the well bioassay (Section II.2.4.1) and the rest of the mixture was sent to S. Keilland at the Micro-Sequencing laboratory at the University of Victoria (Victoria, B.C.) for amino acid sequencing.

II.2.6.5.4 Esterase Reactivity

Five ml of Sep-pak-partially pure antifungal material (Section II.2.5.5) was reduced to dryness under a stream of nitrogen and the dried antifungal material was treated with 100 units of esterase from pig liver (Boehringer Mannheim) in 1 ml sodium phosphate buffer at pH 8.0. One third of the sample was taken from the reaction vial at

30, 60 and 90 min and heat inactivated at 70°C for 10 min. The reaction mixtures were loaded onto a C_{18} Sep-Pak column and eluted as described in Section II.2.5.5, the fractions were tested for activity by the well bioassay (Section II.2.4.1), active fractions were pooled, dried under a stream of nitrogen and resuspended in 2:1 acetonitrile:distilled H_2O with 0.1% trifluoroacetic acid (TFA) and passed through the C_{18} RP-HPLC using protocol No. 3 (Table II.2.5.6.A).

II.2.6.5.5 Ester hydrolysis of antifungal material

An ester hydrolysis reaction was performed on 10 ml of pure antifungal material extracted by the methanol extraction procedure that was dried and resuspended in 1 ml of 3 M NH_4OH, using a vortex. The reaction was incubated on ice. After 30 min, 200 µl samples of the reaction were taken every 30 min, dried under a stream of nitrogen and resuspended in 2:1 acetonitrile:distilled H_2O with 0.1% trifluoroacetic acid (TFA) for analysis using C_{18} RP-HPLC gradient no. 3 (Table II.2.5.6.A) with fractions monitored using a well bioassay (Section II.2.4.1).

II.2.6.5.6 Acid hydrolysis of antifungal material

Acid hydrolysis was performed on a dried sample of crude methanol extract by treatment under vacuum with 6 N HCl, for 24 h at 100°C. After hydrolysis the sample was dried and the residue analysed by thin layer chromatography as described in Section II.2.4.3 and the chromatogram was treated with ninhydrin to visualize amino acids. This analysis was done by T. Palmer-Stone, Department of Biological Sciences, University of Alberta.

II.2.6.6 Amino acid composition and N-terminal sequence analysis

Amino acid compositional analysis and N-terminal sequence analysis of pure antifungal material extracted by the methanol extraction procedure and by the second acidic extraction procedure of Nakajima *et al* (1975) was done by S. Kielland. Antifungal material treated with Endoproteinase Asp-N (Section II.2.6.5.3) was used for attempts to determine the amino acid sequence of the peptide antibiotic.

II.2.6.7 Mass spectrometry

Different types of mass spectroscopic analyses were performed on dried C_{18} RP-HPLC pure antifungal material by different laboratories. M. McGillvray at the University of Victoria analysed the antifungal material after it was dissolved in a glycerol/thioglycerol matrix, by fast atom bombardment (FAB) and metastable ion analysis. Technicians at the University of Alberta Mass Spectroscopy laboratory used electrospray to ionize the antifungal material and analysed the ions by time of flight (TOF) tandem mass spectrometry (MS/MS). H. von Dohren at the Technische Universitat Berlin used matrix assisted laser desorption/ionization (MALDI) to ionize the antifungal material in either an alpha-cyano-4-hydroxycinnaminic acid (4HCCA) or a 2,5-dihydroxy-benzoic acid (DHB) matrix, then analyzed the ions by post source decay (PSD) with a TOF mass analyser. The Harvard Microchemistry protein sequencing laboratory used electrospray (ESI) to ionize the antifungal material and analyzed the ions by MS.

II.3 Results and Discussion: Purification and Partial Characterization of the Antifungal Antibiotic from *P. polymyxa* PKB1

II.3.1 Purification of antifungal antibiotic from *P. polymyxa* PKB1

Preliminary purification experiments by T. Palmer-Stone, Department of Biological Sciences, University of Alberta, showed that the antifungal antibiotic was associated with late exponential growth *P. polymyxa* PKB1 cells or spores rather than with culture broth. Based on these preliminary purification experiments, antifungal material was extracted from *P. polymyxa* PKB1 into 100% methanol or 0.1% acetic acid. However, extraction of the antifungal antibiotic was found to be most reproducible in methanol so it was used for routine studies of the antifungal material.

II.3.1.1 Preliminary characterization of antifungal material

Preliminary characterization of the antifungal material to determine its approximate MW was performed using ultrafiltration with membranes of MW cutoffs: 10 000, 3000 and 1000 Da. Whether a molecule passes through a filter is determined not only by size of the molecule in relation to the pore size of the filter but also by the steric characteristic of the molecule. If the molecular structure is bulky then it will appear bigger than it actually is by its performance in ultrafiltration. Activity was found in the 3000 Da eluant and the 1000 Da retentate, suggesting that the antifungal material is between 3000 to 2000 Da or that the antifungal material has some steric property that restricts its passage through the pores of the 1000 Da cut-off filter.

The antifungal material was determined to consist of amino acids by a preliminary experiment performed by T. Palmer-Stone, that involved acid hydrolysis of the antifungal material and subsequent analysis by TLC and ninhydrin detection. Ninhydrin reacts with primary (NH_3) and secondary (NH_2) amines, forming a coloured derivative (Zweig and Sherman, 1972), therefore any free amine groups in amino acids will react. Acid hydrolysis breaks the peptide bonds between amino acids in a peptide or protein. Hydrolyzed antifungal material showed ninhydrin positive spots, indicating free amino acids. This result, coupled with the ultrafiltration information that the material was of low MW, suggested that the antifungal material is a peptide. Spore-forming bacteria

such as species of *Bacillus* and *Paenibacillus* are known to produce many different peptide antibiotics.

The MW of the antifungal material was further estimated by electrophoresis on 16.5% acrylamide tricine SDS-PAG. The antifungal activity was found in a band corresponding to a standard MW marker of 2.3 kDa by bioautography of the gel. The bioautogram detects the presence of antifungal material on the gel by allowing the antifungal material to diffuse from the gel and into an agar slab that has been seeded with *L. maculans*. If there is antifungal material present it will inhibit the fungus, and a ZOI is observed.

II.3.1.1.1 Effect of dilution of antifungal material on ZOI determined by well bioassay

The activity of the antifungal material does not conform to a linear dilution response as observed by the diameters of the zones of inhibition in a well bioassay.

Many factors can effect the diameter of the ZOI on solid media, some examples due to the solid medium used in the bioassay include; concentration, depth, uniformity and degree of moisture. Environmental factors that affect ZOI can include, incubation temperature and light intensity. The ZOI in well bioassay can also be affected by the presence of contaminants within the test sample that physically hinder or react with the antibiotic and cause loss of activity, or by the solubility of the antibiotic in the medium.

Sep-pak purified antifungal material, in a constant volume of 75 μl, was diluted with methanol to provide different amounts. The external factors that could affect the ZOI were minimized. The resulting ZOI from each duplicated test sample were similar to each other. The ZOI values were averaged and graphed against the corresponding amount of antifungal material, in a log format (Figure II.3.1.2). The relationship of the line was determined to be an R^2 value of 0.9151.

This lack of a linear response may be due to the solubility of the antifungal material within the water-based agar. The antifungal antibiotic is only slightly soluble in water. The solubility of the antifungal material may also be affected by a contaminant in the antifungal material sample. If a contaminant affected the solubility of the antifungal material then the effect of the contaminant on the solubility of the antifungal material could change as the antifungal material was diluted.

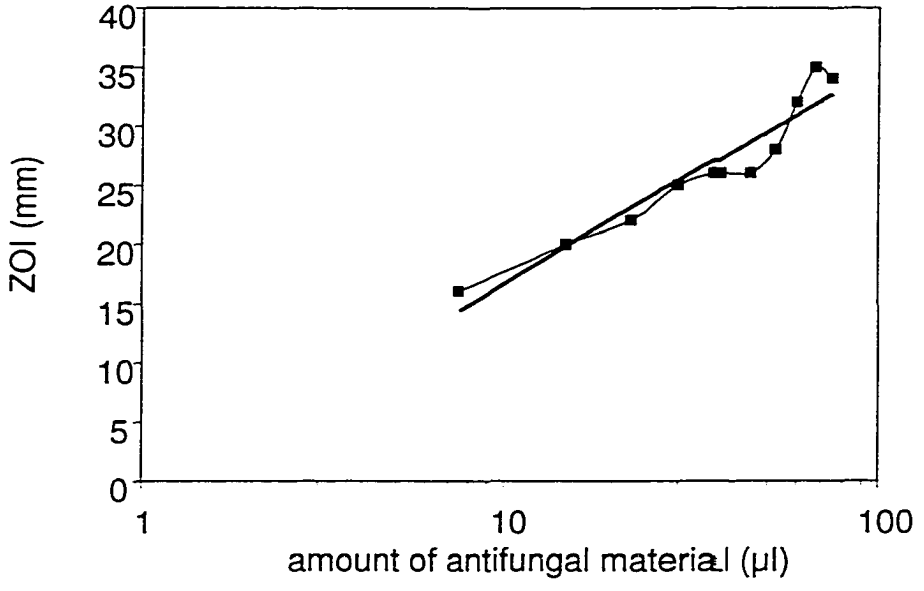

Figure II.3.1.2. The relationship between the amount of Sep-pak purified antifungal material used in a well bioassay and the resulting ZOI measured from the well bioassay. The square points represent the averaged ZOI from a series of dilution assays. The line represents the best fit line of the points on the graph, with an R-squared value of 0.9151. The equation of the line is $y = 7.9784 \ln(x) - 1.7265$.

Due to the lack of a linear relationship between ZOI from different antifungal antibiotic samples, the amount of antifungal antibiotic as determined by well bioassay will be discussed in relative terms only.

II.3.1.2 Methanol extraction of antifungal material

Antifungal antibiotic was extracted with methanol from either freshly harvested *P. polymyxa* cells or from lyophilized *P. polymyxa* cell/spore stocks. Since antifungal antibiotic is only slightly soluble in water, the cell/spore mass from either freshly grown cultures or from lyophilized material was washed with water twice to eliminate or reduce water soluble contaminants. The washed biomass was extracted twice with methanol though most of the antifungal material was found in the first extraction liquid. As the crude methanol extract was concentrated, a large amount of polysaccharide-like material would precipitate out of solution as a gummy yellow substance. This contaminant was separated from the antifungal material that was dissolved in the methanol by centrifugation. The crude methanol extract was reduced to a volume suitable for loading onto the Sephadex LH-20 column.

II.3.1.3 Extraction of antifungal material by a method adapted from Nakajima *et al.* (1975).

The extraction method devised from Nakajima *et al* (1975) for extraction of gatavalin was also tested on *P. polymyxa* cultures. In this method the antifungal material was extracted from the cells by either acidifying and heating the culture thereby releasing the antifungal material from the cells/spores of the culture into the broth, or by removing the cells/spores from the culture broth, resuspending them in acidified water and heating to release the antifungal antibiotic. In either initial procedure the biomass was removed, leaving acidified aqueous extract containing the antifungal material. The antifungal material was extracted from the crude acidic extract with n-butanol. The n-butanol phase was reduced in volume and ethyl acetate was used to precipitate the antifungal material. These two steps were repeated until no more antifungal material precipitated from the n-butanol. The precipitated material was bead-like and an off-white colour. The precipitate was dissolved in 50% methanol in water and passed through a

Sep-pak column before further purification and analysis by C_4 and C_{18} RP-HPLC columns.

The amount of antifungal material extracted from the cells/spores by this acidic extraction method appeared to be greater than the amount of antifungal material extracted from the the same amount of cells/spores by the methanol extraction method. The ZOI obtained from a 75 µl sample of crude extract in a well bioassay was 42 mm for antifungal material precipitated from the crude acidic extract after n-butanol extraction and 34 mm from the crude methanol extract. In each case the total volume of the crude extract was 100 ml derived from an initial culture volume of 400 ml.

This extraction method adapted from Nakajima *et al.* (1975) was tested near to the end of this thesis and so was not used to extract antifungal material *from P. polymyxa* PKB1 for the majority of the characterization studies on the antifungal material.

II.3.1.4 Sephadex LH-20 and C_{18} RP-Sep-pak purification

The crude methanol extract was passed through a Sephadex LH-20 column to separate components of the sample by size. Fractions were monitored at the time of elution by measuring the A_{280} of the eluant, this wavelength can detect aromatic amino acids and other aromatic compounds. The A_{214} of the fractions was also measured to detect other peptidic material, the measured values for these two absorbances were compared in a graph versus time (Figure II.3.1.4). The absorbance profile indicated that the fractions with antifungal activity corresponded to part of the A_{214} peak, and not the A_{280} absorbance. The antifungal material, detected by well bioassay and 16.5% acrylamide tricine SDS-PAGE with bioautography, eluted between 140 and 200 minutes. This suggested that the fraction with antifungal material contained peptide bonds but no aromatic amino acids, but that it was still contaminated by components that were detectable at A_{280}.

The antifungal material that eluted from the Sephadex LH-20 column was passed through a Sep-Pak column as a sample cleaning step prior to RP-HPLC treatment. A Sep-pak column is a small scale C_{18} reverse phase column that separates components on the basis of polarity; from most to least polar (Snyder and Kirkland, 1979). The antifungal material was loaded on the column 50% methanol in water and eluted from the column in methanol and dried.

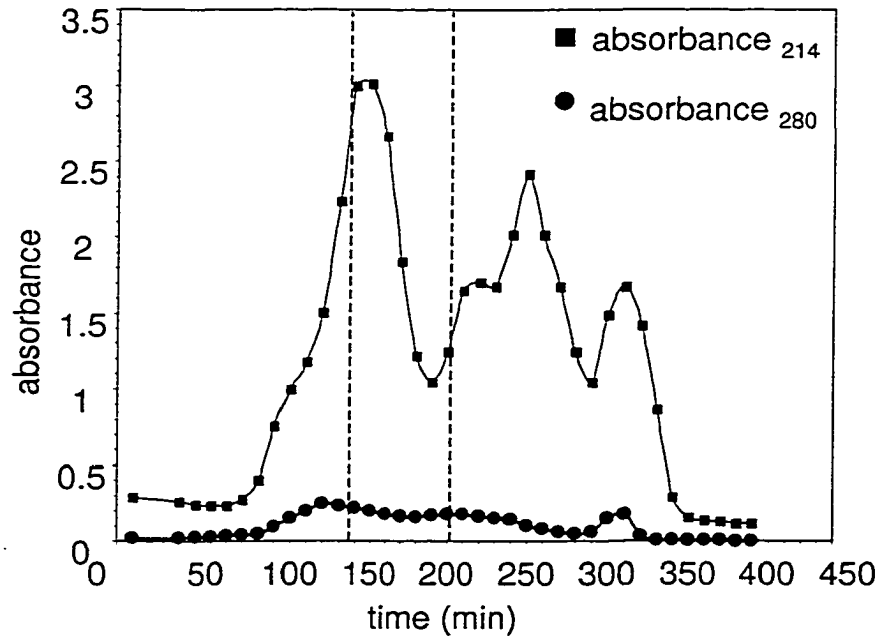

Figure II.3.1.4. Purification of antifungal material from *P. polymyxa* PKB1 by Sephadex LH-20 chromatography. The A214 and A280 measurements of the fractions were taken. Fractions containing activity were from 140 to 200 min as indicated by the dashed lines; fractions numbered 14 to 21, the ZOI of the fractions were 23, 34, 31, 32, 35, 31, 26, and 20 mm, respectively.

II.3.1.5 C_4 and C_{18} RP-HPLC purification

C_4 and C_{18} RP-HPLC were used to purify the antifungal material further and to attempt to separate the peptides comprising the antifungal material. The MS spectrum of the RP-HPLC purified antifungal material (Section II.3.3.6) indicated that it was a mixture of two major peptides and possibly three minor peptides. A variety of chromatographic conditions and mobile phases which affect the retention time of the antifungal material were tested such as; TFA as an ion-pairing reagent, orthophosphate as an ion-pairing reagent, gradient polarity, gradient solvent selectivity, gradient steepness, flow rate and type of stationary phase.

The condition that appeared to affect the retention of the antifungal material the greatest was the addition of an ion-pairing reagent to the mobile phase, in particular the use of orthophosphate.

When the antifungal material was passed through the C_{18} RP-HPLC without the presence of an ion-pairing reagent in the solvent, the activity was found in a very broad peak which gave smaller ZOI compared to fractions from RP-HPLC with an ion-pair reagent in the solvent (Figure II.3.1.5.A).

The use of TFA as an ion-pairing reagent sharpened the elution of the antifungal material in comparison to the same solvent system without an added ion-pairing reagent.

The antifungal material was not very well retained with the use of ms# 3, and the antifungal activity was associated with a peak that had many smaller shoulder peaks coming off of it. Subsequent MS spectrum information (to be described later) suggested that the antifungal material was still contaminated with a co-eluting substance or that the active material was a mixture of closely related peptides that possess very similar retention capabilites. Changes were made to the polarity of the solvent system in an attempt to increase the resolution of the peaks. Increasing the solvent polarity of the isocratic mobile phase (ms # 4) resulted in the antifungal material being associated with two broad, poorly resolved peaks with the same retention time as in ms # 3.

The ion-pairing reagent was changed to orthophosphate to measure the effect of a different ion-pairing reagent on antifungal material retention and resolution. As well, the solvent polarity was increased as compared to ms # 4, a gradient was introduced and the flow rate was decreased (ms # 5 to 8). The gradient of the solvent systems in these mobile phases differed; a sharper increase to the gradient at the beginning of the

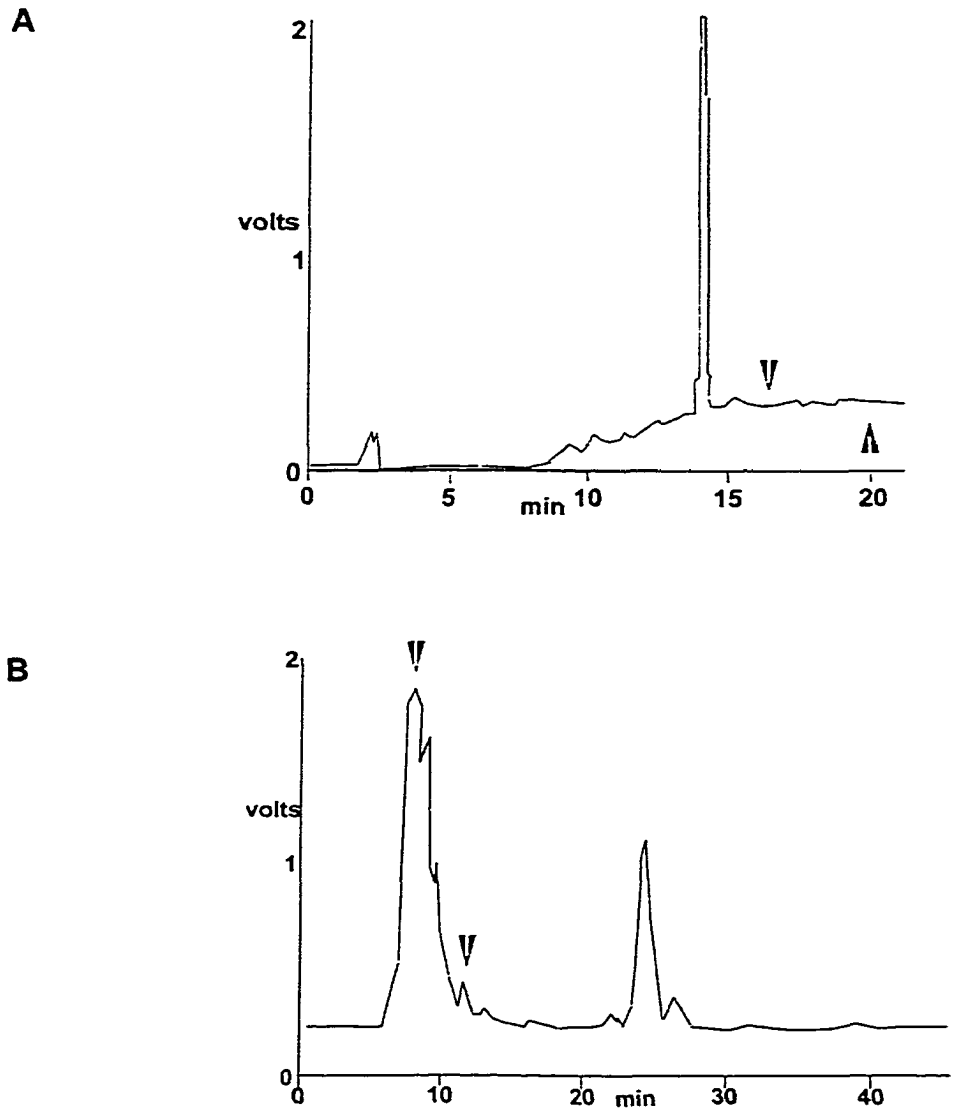

Figure II.3.1.5.A. C_{18} RP-HPLC analysis of Sep-pak purified antifungal material from *P. polymyxa* PKB1 using two different mobile solvent systems. A. Isocratic methanol solvent system without an ion-pairing reagent (ms#1). Antifungal activity was found at 17 to 20 min with ZOI of 16, 17, 17, 14 mm. B. Isocratic 2:1 acetonitrile:water with 0.1% TFA (ms#3). Antifungal activity found at 9 to 11 min with ZOI of 21, 23, 20 mm. Arrows indicate the fractions active against *L. maculans*

run was designed into ms# 6 and a less polar mix of solvent was designed near the end of the run in ms# 7 and ms# 8. The RP-HPLC traces of antifungal material using either ms # 5, 6, 7 or 8 were very similar. The retention of the peptide was increased using these two systems in comparison to the isocratic system of ms# 3 with TFA (Figures II.3.1.5.A versus II.3.1.5.B). The peaks resolved into approximately four extremely close peaks and the antifungal activity was approximately associated with the last peak of the cluster (Figure II.3.1.5.B; uses orthophosphate as the ion pairing reagent).

The organic solvent in ms# 6 was changed to methanol from acetonitrile because more antifungal material could be recovered from the column with methanol in the mobile solvent phase (ms# 9). The purity of the sample appeared to have the same effect on the retention of the peaks associated with the antifungal antibiotic analysed by RP-HPLC using ms# 9 as was observed using ms# 6. The peaks were also smaller and broader than the peaks at the same retention time using ms# 6, suggesting a loss in resolution with methanol in the mobile phase as compared to acetonitrile.

Improvement of the resolution of the peaks associated with antifungal material was attempted by the use of a C_4 RP-HPLC column and varying gradients and flow rates. The ms# 15 provided the best resolution out of the six different gradients tested with the C_4 column, however, this resolution was not an improvement upon any gradients and conditions used with the C_{18} column.

II.3.1.6 Purification table for the antifungal antibiotic from *P. polymyxa* PKB1

An example of a typical purification of antifungal antibiotic extracted from the cells/spores by the methanol extraction method is outlined in Table II.3.1.6.

One unit of activity was defined as the amount of antifungal material needed to give a ZOI of 25 mm, as determined by the well bioassay. This amount was determined from the equation of the line of the graph in Figure II.3.1.2 to be 28.5 µl. The total activity (U) of the antifungal material sample at each purification step listed in the table was derived from the ZOI resulting from the sample by the well bioassay. The amount of antifungal material from the test sample was calculated from the equation of the line from the graph in Figure II.3.1.2, the unit value was calculated by dividing the amount of antifungal material of the test sample by the amount of antifungal material from 25 mm (28.5 µl). The unit value was corrected for the total volume of the test sample and divided by the volume used for the ZOI (75 µl) measurement.

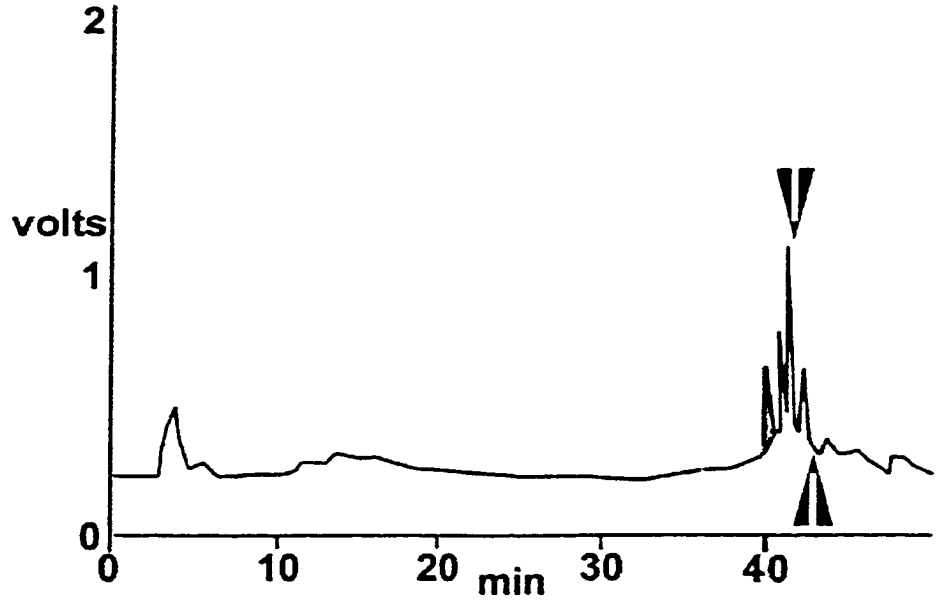

Figure II.3.1.5.B. C_{18} RP-HPLC analysis of HPLC purified antifungal material from *P. polymyxa* PKB1 using solvent system ms#6, with orthophosphate as the ion-pairing reagent. Antifungal activity was found at 43 and 44 min, as indicated by the arrows, with ZOI of 21 and 20 mm.

Table II.3.1.6. Purification table of the antifungal material from *P. polymyxa* PKB1 using the methanol extraction method.

antifungal material	total activity[a] (U)	total protein and peptide[b] (mg)	specific activity (U/mg)	yield (% total activity[c])	purification factor[d]
crude methanol extract	7186	90	80	100	1.0
pooled Sephadex LH-20 fractions	749	7.5	100	10.4	1.25
pooled Sep-pak fractions	540	3.2	169	7.5	2.1
pooled RP-HPLC fractions	58	0.057	1017	0.81	12.7

[a] one unit of activity was defined as the amount of antifungal material needed to give a ZOI of 25 mm as determined by the well bioassay (II.2.4.1), the amount of antifungal material that gave a ZOI of 25 mm was derived from the slope of the graph in Figure II.3.1.2. to be 28.5 μl. The total activity of a sample was calculated by: determining the amount of antifungal material in the sample from the graph in Figure II.3.1.2 from the ZOI of the sample, dividing this by the amount of antifungal material that produces a ZOI of 25 mm (28.5 μl). The volume of the sample was taken into account by multiplying
the unit value by the total volume of the sample and dividing by the volume used in the well bioassay (75 μl).

[b] total protein and peptidic material was determined by the BCA assay.

[c] the % total activity was calculated as the total activity of that step divided by the total activity of the crude methanol extract, multiplied by 100.

[d] the purification factor is the increase in specific activity from the crude methanol extract to that purification step, calculated as the specific activity of that step divided by the specific activity of the crude methanol extract.

The total amount of protein in each sample was determined by the bicinchoninic acid (BCA) assay against a standard curve of bovine serum albumin (BSA). The BCA assay uses the reagent bicinchoninic acid as a colourimetric detector of the biuret reaction. The biuret reaction is the reduction of Cu^{2+} to Cu^{+} by proteins or peptides under alkaline conditions. The amount of colour formation is directly related to the amount of protein present in the sample. The structure of the protein, the number of peptide bonds and the presence of cysteine, cystine, tryptophan and tyrosine all contribute to colour formation and hence the determination of the amount of protein and peptidic material present (Chanda, 1998; Pierce Instruction Booklet). Since the amino acid composition analysis indicates that there are no cysteine, cystine, tryptophan or tyrosine residues in the antifungal material the determination of an amount of total peptide by the BCA assay is considered an estimate of the peptide bonds present in the antifungal material samples.

An example of the antifungal material present at each purification step is seen in Figure II.3.1.6., which shows the Coomassie blue staining pattern of the purified antifungal material in a 16.5% acrylamide tricine SDS-PAGE and the resulting bioautogram against *L. maculans*.

II.3.2 Inhibition of *L. maculans* by *P. polymyxa* subsp. *colistinus koyama*

The amino acid compositional analysis (to be discussed later) suggested that the antifungal material was similar to a previously partially purified and partially characterized peptide antibiotic called gatavalin (Nakajima *et al.*, 1975). Gatavalin was reported to be produced by a Japanese soil isolate, *P. polymyxa* subsp. *colistinus koyama*. This bacterium was obtained and tested for activity against *L. maculans* (Section I.3.7).

P. polymyxa subsp. *colistinus koyama*, was able to inhibit *L. maculans*. The nature of the antifungal material was investigated to determine if it was similar to the antifungal material of *P. polymyxa* PKB1. *P. polymyxa* subsp. *colistinus koyama* culture was grown and the cells/spores pelleted and extracted with methanol as described in Section II.2.5.2. The crude methanol extract (100 ml total; ZOI from a 75 µl sample before concentration was 28 mm) was passed through Sephadex LH-20, Sep-pak and C_{18} RP-HPLC with mobile solvent system no. 3 (Sections II.2.5.4 to 6). Antifungal activity was detected from the fractions eluted from the three columns in the same numbered

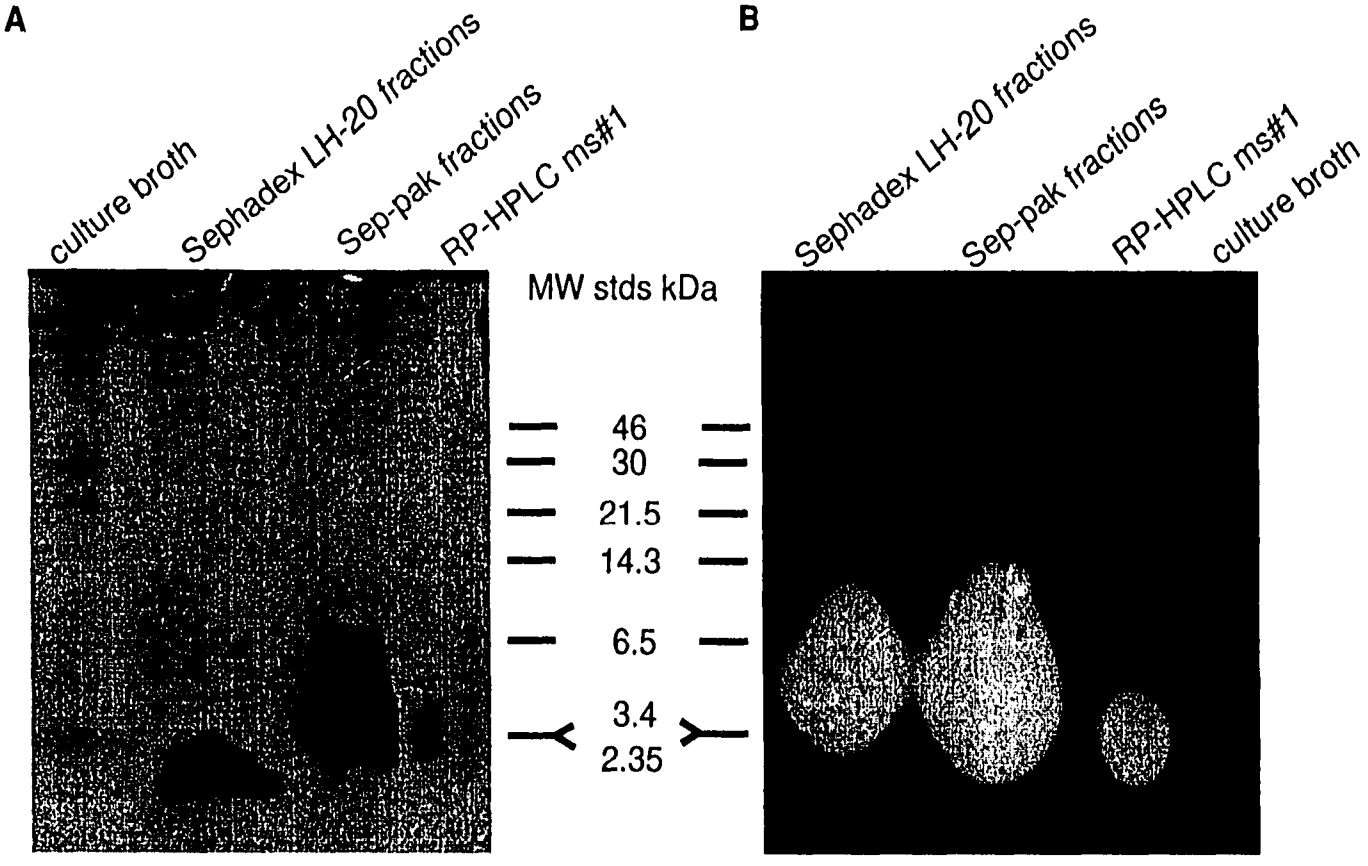

Figure II.3.1.6. SDS-PAGE analysis of antifungal material at various purification steps. A. 16.5% acrylamide tricine SDS-PAGE, stained with Coomassie blue and B. the bioautogram of the 16.5% acrylamide tricine SDS-PAGE against *L. maculans*.

fractions as it is when extracted from *P. polymyxa* PKB1. For example, the range of fractions with antifungal material eluted from Sephadex LH-20 (fractions 14 to 17, corresponding to 140 to 170 min elution) and the diameters of the zones of inhibition obtained from the fractions containing antifungal material (ZOI from each fraction were 26, 32, 33, 28 mm respectively) from *P. polymyxa* subsp. *colistinus koyama* were similar to *P. polymyxa* PKB1 (Section II.3.1.2). Figure II.3.2 shows a 16.5% acrylamide tricine SDS-PAG with samples from the different purification stages and a C_{18} RP-HPLC trace of the antifungal material. The SDS-PAGE shows the same diffuse band at approximately 2.3 kDa as observed for the antifungal material extracted from *P. polymyxa* PKB1 and electrophoresed on a 16.5% acrylamide tricine SDS-PAG as well (compared to Figure II.3.1.6). Antifungal antibiotic was eluted from the HPLC column as determined by well bioassay of the fractions at the same retention time as the antifungal material from *P. polymyxa* PKB1, using the same mobile solvent system; no. 3 (comparison to Figure II.3.1.5.A).

P. polymyxa subsp. *colistinus koyama* is known to produce the peptide antibiotics gatavalin, jolipeptin and colistins A and B (polymyxins E1 and E2; Sections I.1.4 and II.1.4). The colistins and jolipeptin differ from the antifungal material isolated from *P. polymyxa* PKB1 by their inhibition spectrum; these antibiotics are not antifungal and they mainly inhibit Gram negative bacteria. As well, the colistins have different solubilities than the antifungal material and jolipeptin is ninhydrin positive. Gatavalin does have similar characteristics to the antifungal material from *P. polymyxa* PKB1. The inhibition spectrum of gatavalin is of Gram positive bacteria and fungi, it has the same solubility characteristics of the antifungal material and it is ninhydrin negative.

P. polymyxa subsp. *colistinus koyama* appears to be a producer of the same or very similar antifungal antibiotic as is produced by *P. polymyxa* PKB1.

II.3.3 Characterization of antifungal material produced by *P. polymyxa* PKB1.

II.3.3.1 Solubility of antifungal material

HPLC purified antifungal material was soluble in methanol, n-butanol, ethanol, acetonitrile and isopropanol, partially soluble in acetic acid, slightly soluble in water and insoluble in n-hexane, ethyl acetate and methylene chloride. Figure II.3.3.4.1 in Section II.3.3.4, shows the ability of a fixed amount of antifungal material to inhibit *L. maculans*

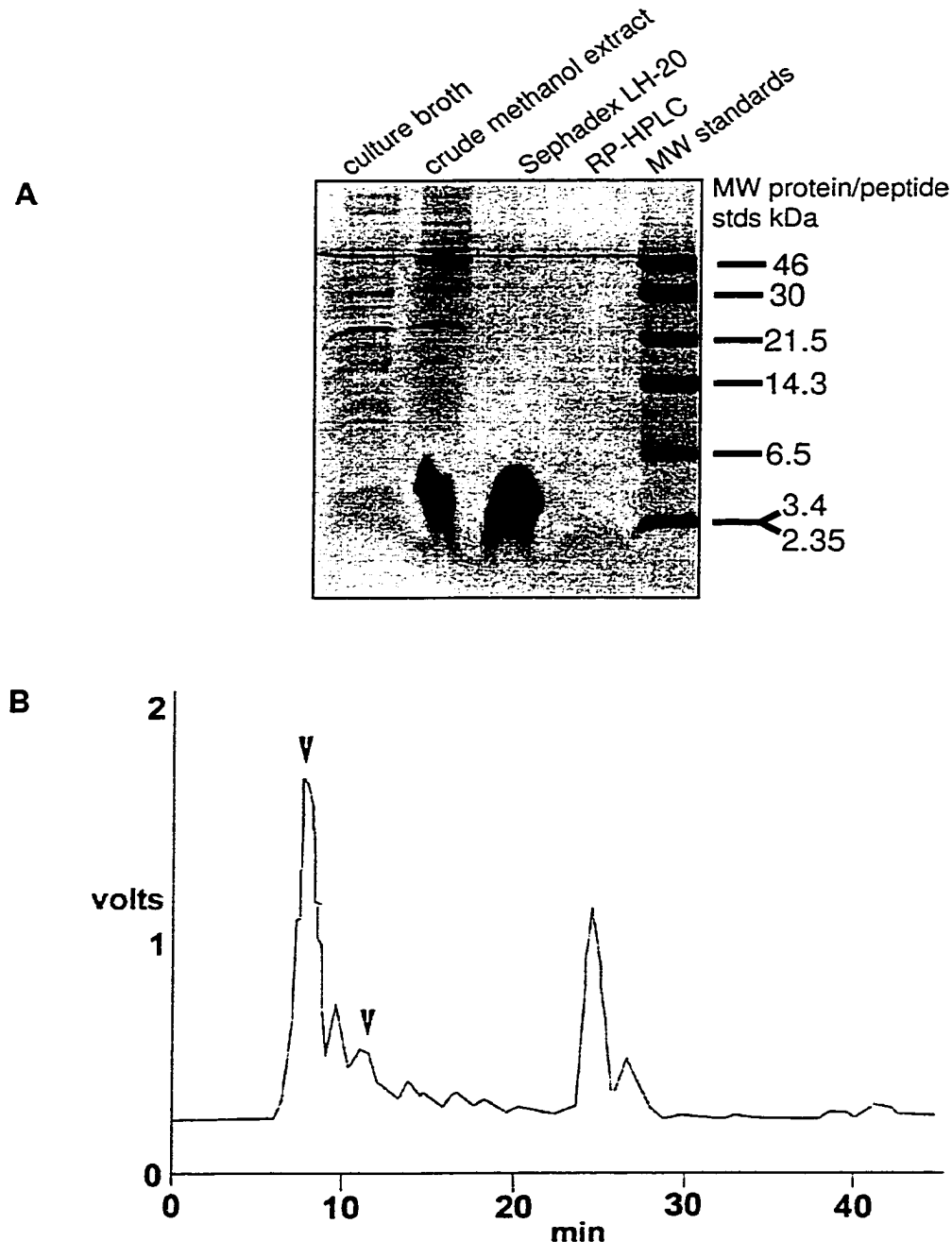

Figure II.3.2 Purification of antifungal material from *P. polymyxa* subsp. *colistinus koyama*, a Japanese soil isolate. A. 16.5% acrylamide tricine SDS-PAGE, showing the antifungal material present at each purification step. B. C_{18} RP-HPLC analysis of antifungal material using ms#3, arrows indicate fractions active against *L. maculans*.

when it was 'dissolved' in methanol, ethyl acetate or water and developed on a TLC. The antifungal material dissolved in methanol and so was present on the TLC to inhibit the fungus. The antifungal material could not dissolve in ethyl acetate, as seen by the lack of a ZOI on the bioautogram, while it was slightly soluble in water, as seen by the small ZOI on the bioautogram that represented a small portion of the material was developed on the TLC.

Comparison of the selectivities of each solvent for the four polarity parameters shows that the solvents that were able to dissolve the antifungal material were different from the insoluble solvents in their H-bonding and dielectric interaction selectivites. The solvents that were able to dissolve the antifungal material had polarity parameters of between 5 and 6; in comparison to the polarity parameters of water and n-hexane: 10.2 and 0.1, respectively (Snyder and Kirkland, 1979). They also show the ability to form H-bonds and in particular to act as H-bond acceptors (i.e. methanol). The range of dielectric constants among these solvents was approximately 25 to 37; in comparison to the dielectric constants of water and n-hexane: 80 and 1.88, respectively (Snyder and Kirkland, 1979). The solubility profile suggests that the antifungal material is neither strongly polar or nonpolar and that it contains amino acids that can act as proton donor groups.

II.3.3.2 Stability of antifungal material

Sep-pak purified antifungal material in methanol was heated to 90°C and flash frozen to -70°C both for 65 min. The methanol in the sample that was heated had evaporated by the end of the test and an equal amount of methanol was added to analyze the stability of the antifungal material by well bioassay. Both samples retained antifungal inhibition (for 90°C, ave. ZOI=31 mm; for –70°C, ave. ZOI=31.5) as compared to antifungal material that was not treated (ave. ZOI=32.5 mm).

The antifungal material retains its activity indefinitely at 4°C and up to 4 wk at 25°C.

II.3.3.3 Reactivity and detection of the antifungal material with ultraviolet radiation, stains, dyes and chemicals

RP-HPLC purified antifungal material, developed on a TLC, showed no fluorescence under either short or long ultraviolet (UV) radiation. Scans of RP-HPLC purified antifungal antibiotic dissolved in methanol were taken from A_{200} to A_{600} and only showed end absorption from A_{210} to A_{215}.

Non-hydrolyzed antifungal material, after development on a TLC, did not react with ninhydrin, suggesting that if the antifungal material is a peptide it must either be cyclic or N-terminally blocked.

Iodine vapours were also used to detect unsaturated fatty acids and –HC=CH- groups in general in the antifungal material. Samples at all purification steps were developed on TLC plates, treated to iodine vapours and examined for brown spots that denote a reaction. The antifungal material samples formed some brown spots after exposure of the TLC plate to iodine vapours, however when the plate was bioautographed against *L. maculans* the point of antifungal activity was not associated with the spots. The R_f value of the spots were from 0.4 to 0.6 while the activity on the bioautogram represented an R_f value of 0.94. The antifungal material does not appear to be derivatized with unsaturated fatty acids or to contain –HC=CH- groups.

Antifungal material was electrophoresed in a 16.5% acrylamide tricine SDS-PAG and treated with silver stain to detect fatty acids, carbohydrates or peptides carrying amino acids with sulhydryl or carboxylic acid side chains. Crude methanol extracts were stained brown-black by silver stain. However, Sephadex LH-20 and RP-HPLC purified antifungal material was not silver stained; though Coomassie blue dye could detect a diffuse band that contained activity as observed by bioautography. This suggests that either the silver stain was detecting a contaminant in the crude methanol extract that was removed by Sephadex LH-20 or that the amount of antifungal material remaining after purification of the crude methanol extract by Sephadex LH-20 was low. The activity values generated for the antifungal material at each purification step listed in Table II.3.1.6 suggests that much antifungal material is lost from the crude methanol extract after purification with Sephadex LH-20. The silver stain procedure involved washing the electrophoresed gel in 50% methanol for a minimum of 2 h and a maximum of 24 h, to reduce background staining of the gel by the silver stain. This step may have allowed antifungal material to diffuse from the gel into the wash due to its solubility in methanol. It is possible that the amount of antifungal material remaining after Sephadex LH-20 purification was low to begin with, then the washing of the gel containing this low amount of antifungal material, further reduced the amount on the gel so that there was too little

for silver stain detection. SDS-PAG that were concomitantly stained with Coomassie blue or used in bioautography against *L. maculans* were able to detect antifungal material because both these gels were washed in 50% methanol for 30 min only.

RP-HPLC purified antifungal material was electrophoresed in a 16.5% acrylamide tricine SDS-PAG and treated with a stain to detect carbohydrate derivitized polypeptides. The stain detects carbohydrates and colours them a pink-red colour. Although some high MW bands were detected using this stain, it was not observed with the band that represented the antifungal material from any step in the purification.

II.3.3.4 Susceptibility of antifungal material to enzymatic and chemical cleavage

II.3.3.4.1 Pronase activity

The effect of pronase on crude methanol extracts of antifungal antibiotic was determined by well bioassay and TLC. Pronase is a mixture of unspecified exo- and endo-peptidases, including carboxy- and aminopeptidases, isolated from *S. griseus*, that can digest proteins to their constituent amino acids.

No reduction of activity from the antifungal material was seen after pronase treatment in comparison to untreated antifungal material on a well bioassay against *L. maculans*. A bioautogram of the TLC plate used to analyze the antifungal material before and after treatment with pronase also indicated that the antifungal material was still active after treatment (Figure II.3.3.4.1). The lack of effect of pronase on the antifungal material suggests that the peptide antibiotic is cyclic or N- and C-terminally blocked; which would protect the antifungal material from cleavage by carboxy- and aminopeptidases, and that it does not contain the amino acid sequences that the exo- and endoproteinases present in the mixture use as cleavage sites. The lack of reactivity could also be an indication of a cleavage site modification by the presence of an amino acid in the D configuration instead of the L-configuration or of non-amide bonds linking amino acids in the structure. The cyclic nature of the peptide could also inhibit cleavage by proteases because the cyclic backbone restricts the secondary structure of the peptide so that it is relatively inflexible (Eckart, 1994). This may make a cleavage site in the peptide unaccessible to the proteases due to steric hindrance.

II.3.3.4.2 Trypsin and carboxypeptidase reactivity

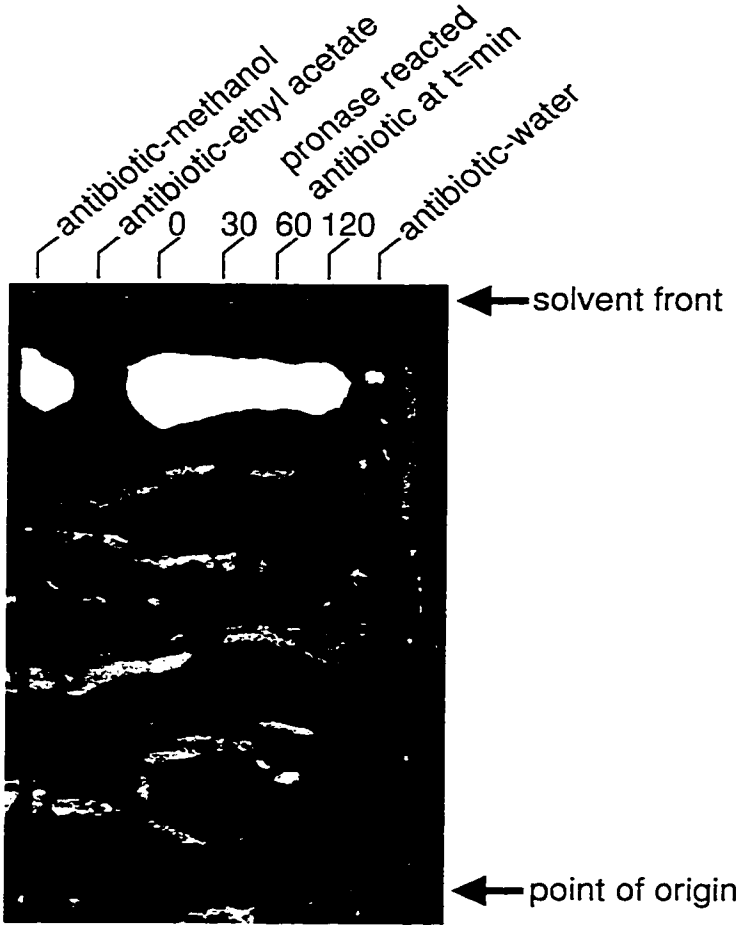

Figure II.3.3.4.1. Solubility and pronase sensitivity of the antifungal material from *P. polymyxa* PKB1. Bioautogram of the TLC plate with antifungal material, using *L. maculans* as the indicator organism.

Trypsin, an endoproteinase enzyme, cleaves amide or ester bonds on the carboxyl side of arginine or lysine residues, forming a free carboxyl group on the arginine or lysine and a free amino group, or hydroxyl group if an ester bond was cleaved, on the other amino acid (Stryer, 1988).

Carboxypeptidase, an exoproteinase, cleaves the peptide bond that links the C-terminal amino acid to the rest of the peptide. It will continue cleavage of the carboxy terminal amino acid until there is no more substrate available (Stryer, 1988).

The antifungal antibiotic, when incubated with either trypsin or carboxypeptidase, retained its antifungal activity. The test samples were analysed by RP-HPLC, then activity against *L. maculans* was measured by a well bioassay of the resulting RP-HPLC fractions.

If there was a free C-terminal on the antifungal antibiotic then the antifungal material should have been degraded and antifungal activity of the sample would have been reduced or lost. However the antifungal material remained active. This result suggested that there was no free C-terminal amino acid available to the enzyme, perhaps due to cyclization of the antifungal antibiotic or derivitization of the C-terminal amino acid.

If there were an arginine or lysine residue in the peptide that could serve as a site for cleavage of the peptide by trypsin then the peptide would have been linearized and potentially lost its antifungal activity. The antifungal material did not lose its antifungal activity upon treatment with trypsin, therefore it appears that the peptide does not contain either of these two residues. The amino acid compositional analysis (Section II.3.3.5) showed that the antifungal material contained an un-identified residue that had a retention time that was most similar to the retention time of the arginine standard. That trypsin could not eliminate the activity of the antifungal material suggests that this un-identified residue is not arginine.

II.3.3.4.3 Endoproteinase Asp-N reactivity

The enzyme endoproteinase Asp-N can cleave amide bonds at either one of two amino acids, depending on the reaction conditions. The enzyme cleaves rapidly on the carboxyl side of aspartic acid residues and cleaves slowly on the carboxyl side of glutamic acid residues.

Treatment of RP-HPLC purified antifungal material with endoproteinase Asp-N under conditions that either supported cleavage of asp only, or asp and glu, did not result in a loss of inhibition of *L. maculans* by the antifungal material as measured in a well bioassay. As well, the antifungal material treated with endoproteinase Asp-N under both conditions still could not be sequenced by the Edman degradation method of N-terminal peptide sequencing. The residues aspartic acid and glutamic acid do not appear to be present in the antifungal material. Amino acid composition analysis of the antifungal material does find asx and glx (Section II.3.3.5), therefore it is possible that these residues are asparagine and glutamine, however, this lack of cleavage may also be due to inability of the enzyme to recognize an existing cleavage site because of modification or steric hindrance to the site from the cyclic nature of the antifungal material.

II.3.3.4.4 Esterase reactivity and Ester hydrolysis of antifungal material

Ester bonds (-C(=O)-O-C-), can be found in antibiotics produced by *P. polymyxa* such as cyclic peptide antibiotics, called depsipeptides. Some examples of cyclic peptides from *P. polymyxa* strains that contain ester bonds are; polypeptin A and B and fusaricidin A, B, C and D, that all use an ester bond to close the ring structure.

Sep-pak purified and RP-HPLC purified antifungal material were tested for esterase sensitivity and ester hydrolysis by ammonia, respectively. The esterase enzyme used was a carboxylic-ester hydrolase that, upon cleavage of the ester, forms a carboxylic acid anion. The esterase was added to the Sep-pak purified antifungal material and samples from the reaction were taken periodically, up to 90 min. The reactions were stopped and the reaction samples were passed through a Sep-pak column and a RP-HPLC column. Fractions from these columns were tested for inhibition of *L. maculans*.

Ester hydrolysis of the antifungal material by ammonia was allowed to occur, on ice, for 2.4 h; fractions were taken periodically and the reaction was stopped at that time point. The treated antifungal material was passed through a RP-HPLC column and analyzing the resulting fractions were tested for inhibition of *L. maculans*.

In both ester hydrolysis treatments the antifungal material was observed to lose antifungal activity as the length of treatment time increased. The ZOI from untreated antifungal material sample was 30 mm. The ZOI from the five fractions sampled from the

ammonia ester hydrolysis reaction at 30, 55, 64, 130, and 144 min after RP-HPLC were: 28; 26; 26, 23 and 21 mm, respectively. The ZOI obtained from the fractions from esterase treatment taken at 30, 60 and 90 min and passed through the Sep-pak column were: 30, 25 and 17 mm, respectively. When these Sep-pak derived fractions were then passed through the RP-HPLC column, the resulting ZOI of the fractions (originally taken at 30, 60 and 90 min) were: 27, 24 and no inhibition, respectively (Figure II.3.3.4.4).

The inhibition of *L. maculans* by the antifungal material was reduced after both ammonia treatment and reaction with an esterase. This indicates the presence of one or more ester bonds in the structure that, when disrupted results in a slow loss of activity.

II.3.3.5 Amino acid composition and N-terminal sequence analysis

The tests on the antifungal material indicated that the activity appeared to be caused by a peptidic structure. This is not unusual as there are many peptide antibiotics produced by bacterial species, especially by many strains of *P. polymyxa*.

RP-HPLC purified antifungal material was analysed by S. Kielland, Victoria Protein Microchemistry Centre, University of Victoria, to determine a peptide sequence by Edman degradation and an amino acid composition by total acid hydrolysis.

The Edman degradation method was not able to determine a sequence, suggesting that the N-terminal is either blocked by a chemical modification or is bound to a component of the peptide, forming a cyclized structure. Both these features are commonly found in *Paenibacillus* peptide antibiotics. However when the antifungal material was partially acid hydrolysed, for 10 to 15 min at 100°C, instead of the 70 min at 150°C required for total acid hydrolysis in the method used by S. Kielland, a sequence was obtained by Edman degradation. The sequence was N-terminal-thr-val-val-X-glu-ala-C-terminal. This is similar to the sequence of fusaricidin B (Figure I.1.4), which is a peptide structure of 897 Da. Fusaricidin B contains a D-allo-threonine in the position where the unknown residue is found in the antifungal material of this study. The amino acid composition and the Edman sequencing of the antifungal material in this study suggests that this unknown amino acid may not D-allo-threonine. However, a known sample of D-allo-threonine will be tested against the unknown amino acid of the peptide mixture comprising the antifungal material. The identity of this unknown amino acid residue will be discussed further with the amino acid composition results of this Section, and the MS spectral information in Section II.3.3.6. Fusaricidin B was determined to

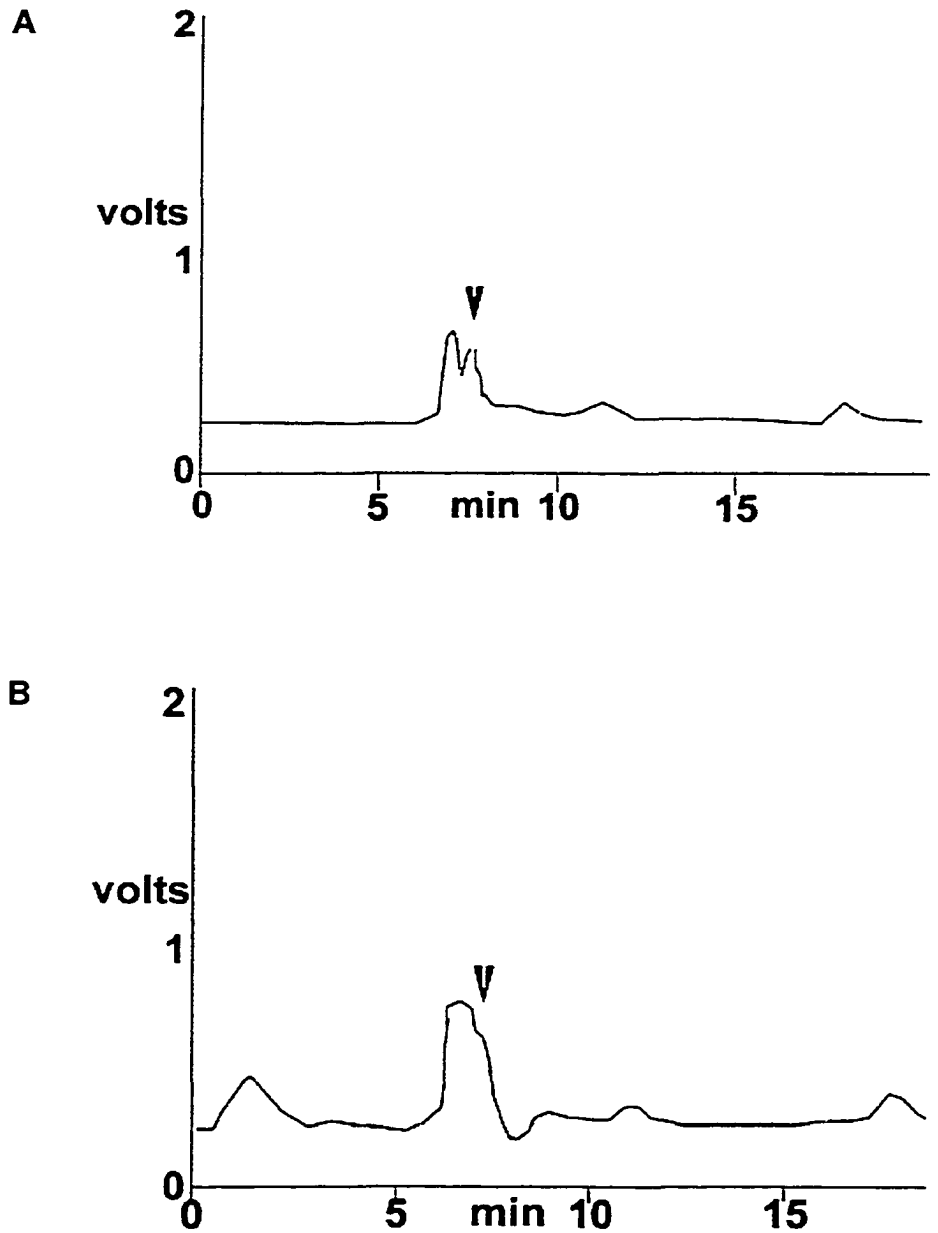

Figure II.3.3.4.4. RP-HPLC traces of esterase treatment of antifungal antibiotic in comparison to no treatment. A. Non-treated antifungal material (time = 0). B. Treated antifungal material (time = 90 min), arrow indicates loss of antifungal activity from this fraction. Mobile phase #3 was used to elute the samples.

contain gln, however the sequencing of this antifungal material demonstrates the presence of glu instead. These two amino acids have been found in other peptides to be readily exchanged for one another by the module of the PS enzyme responsible for activation of the residue and incorporation into the growing peptide chain. It is also possible that the partial hydrolysis conditions deamidated the putative gln residue, forming a glu residue. The fact that a sequence could be obtained from partially hydrolysed antifungal material suggests that this treatment enabled the previously blocked N-terminal amino group of the peptide to be freed. The structure of the fusaricidin peptides show that the N-terminal amino group of thr is blocked because of a guanidino containing fatty acid moiety being bound to it. The researchers who characterized the fusaricidin cyclic depsipeptides had noted that hydrolysis of the fusaricidins resulted in the cleavage of the bond between the thr and the fatty acid moiety, leaving a free amino group on the thr residue. The sequence information on the antifungal material in this study appears to agree with this characteristic.

The amino acid composition was determined by acid hydrolyzing the antifungal material and analyzing the free amino acids by RP-HPLC for comparison of the retention time of the amino acids in the sample to amino acid standards. The amino acid composition was determined to be asx, glx, thr, X, ala, and val. The same antifungal material used to determine amino acid composition and sequence of the partially hydrolysed antifungal material was used in a preliminary analysis of the mass by FAB MS (Section II.3.3.6). The FAB MS spectrum showed the presence of two parent ions with masses of 883.5 and 897.5, suggesting that the material used to obtain the amino acid composition was a mixture of, at least, two components differing in MW by 14 mu. The amino acid composition, the sequence and the FAB-MS spectral anaylsis of the antifungal material, are very similar to the known sequences and structures of fusaricidin A and B. Fusaricidin A and B have the same sequence and structure except that A has an asn and B has a gln residue and their masses are 883 and 897 Da, respectively. The antifungal material in this study contains two molecular ions at 883 and 897 Da each, therefore the amino acid composition would reflect that there is a mixture of two peptides. This is suggested by the amino acid composition of the antifungal material in this study which found both asx and glx present along with the other amino acids that are also found to be in fusaricidin A and B. When the ratios of the amino acids in the composition are calculated based on the presence of one ala residue, as it is in fusaricidin A and B, the amino acid residues are in these ratios: asx (0.5), glx (0.6), thr

(0.8), X (1.0), ala (1.0) and val (1.6). These values correlate with the presence of two peptides in the antifungal material, one with an asx residue and another with a glx residue, as well, it agrees with the sequence information that was obtained on the partially hydrolysed antifungal material that suggests that instead of two thr residues in the material, as is the case for fusaricidin A and B, there is one thr and one unknown residue. The ratio of 1.6 val in this antifungal material is also comparable to fusaricidin A and B which contain two val. The amount of val detected by acid hydrolysis of a peptide can be underestimated because the val-val amide bond can be difficult to cleave. The unknown amino acid residue labelled as X showed a retention time on RP-HPLC that was most similar to the amino acid standard for arginine suggesting the presence of an arginine-like amino acid residue or component in the structure. The retention time of the X residue was 8.43 min and arginine has a retention time of 8.59 min, in the HPLC system used by S. Kielland. Citrulline is an amino acid residue that elutes just prior to arginine in RP-HPLC, as the X residue from the antifungal antibiotic did. However, a citrulline standard that was analysed by RP-HPLC concomittantly with the hydrolysed antifungal material showed that it had a different retention time than the unknown component of the material. The possible identity of the unkown residue will be discussed further in Section II.3.3.6.

II.3.3.6 Mass spectrometry of the antifungal material from *P. polymyxa* PKB1

The mass spectrum of the antifungal material has been derived by a number of different soft ionization procedures. All of the spectra show the two molecular ions with masses of 883.5 and 897.5. The ESI spectra that were performed at the University of Alberta Mass spectrometry Facility, show two other molecular ions at masses 870 and 912 (Figure II.3.3.6.A). The FAB spectra also shows the 870 Da molecular ion but not the other fragment ion of 912 Da. The ESI spectra that were performed at the Harvard Microchemistry Facility, show the four molecular ions listed above (869.5, 883.5, 897.5, 912) as one cluster and the molecular ions 947 and 961 as another cluster. The ions in the clusters are 14 mass units (mu) in difference. This pattern suggests a group of peptides with very similar compositions. Some peptidic characteristics that are observed by a difference of 14 mu in the molecular ion include a val to ile or an asx to glx switch or methylation of functional groups. The PSD spectra also shows $[M+Na]^{+1}$ and $[M+Na+H]^{+2}$ ions for the molecular ions of 897 and 912. Peptide antibiotics of these MW

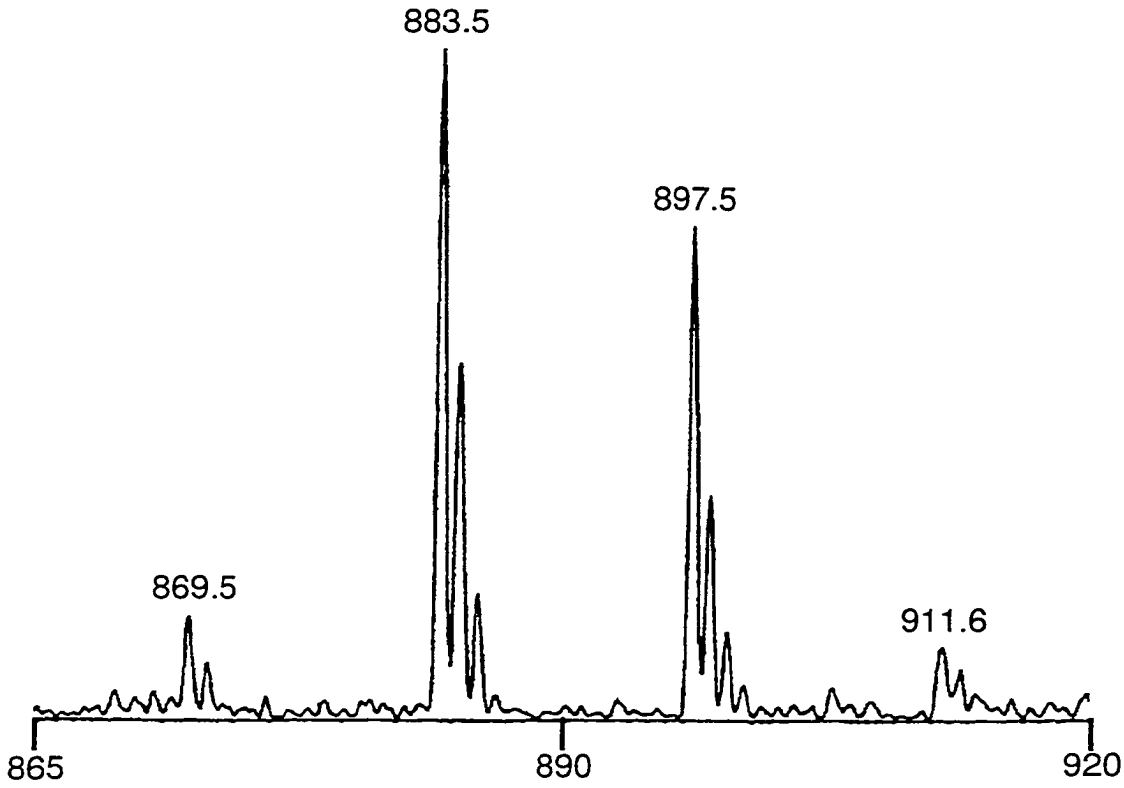

Figure II.3.3.6.A. An ESI MS spectrum of the molecular ions of the RP-HPLC purified antifungal material from *P. polymyxa* PKB1.

from *P. polymyxa* strains have been reported in the literature before (Table I.1.4). The HPLC separated peptidic material called LI-F04 contained molecular ions of 883 and 897 Da and LI-F03 contained molecular ions of 947 and 961 Da. Fusaricidin A has a MW of 883 Da and fusaricidin B has a MW of 897 Da, as discussed in Section II.3.3.5.

The amino acid compositon results and the sequence of the antifungal material generated by partial acid hydrolysis and Edman degradation suggested that the antifungal material of this study was similar to fusaricidin A and B. The structure of these peptides is of a cyclic peptide ring closed with an ester bond between the β-hydroxy group of thr and the C-terminal carboxyl group of ala and a guanidino containing fatty acid side chain bound to the N-terminal amino group of thr. This side chain was identical for all four of the cyclic peptides of the fusaricidin group; 15-guanidino-3-hydroxypentadecanoic acid (GHPD; Figure I.1.4). Although MS spectral analysis of these cyclic peptides was not reported by the researchers working with the fusaricidins, the authors did report that fusaricidin A and B were acid hydrolysed, separately, for amino acid composition analysis. The authors found a component of each of the fusaricidins that had fragmented from the rest of the structure during acid hydrolysis that did not extract from the hydrolysis sample in the aqueous layer as the amino acid residues had, instead it was found in the organic solvent layer. The researchers analysed this component by FAB-MS and found a $[M+H]^+$ molecular ion of 298 Da. Further analysis of this ion by a variety of NMR procedures determined it as the GHPD structure. The authors of the study on fusaricidin structure suggested that GHPD underwent dehydration during acid hydrolysis of the peptides to form the αβ-unsaturated fatty acid containing the guanidino group that had separated from the N-terminal amino group of the threonine in the peptide ring.

In the present study of the antifungal peptide from *P. polymyxa* PKB1, an ion of 256 Da was abundant in every MS spectrum generated of the antifungal material. The PSD MS of the $[M+H]^{+1}$ molecular ion 883 had the 256 Da peak, as well as a peak at 628 Da which is the difference of the molecular ion and 256 Da (Figure II.3.3.6.B). The 256 ion peak may represent a side chain from the cyclic peptide structure that was easily fragmented from the ring structure, leaving this ring intact. Since the antifungal material had previously been determined to be ninhydrin negative and the N-terminal sequence of non-hydrolysed antifungal material could not be derived by Edman degradation, it was assumed that either the N-terminal amino acid on the side chain was modified so that it was blocked, if the side chain comprised amino acid residues, or that the side chain was

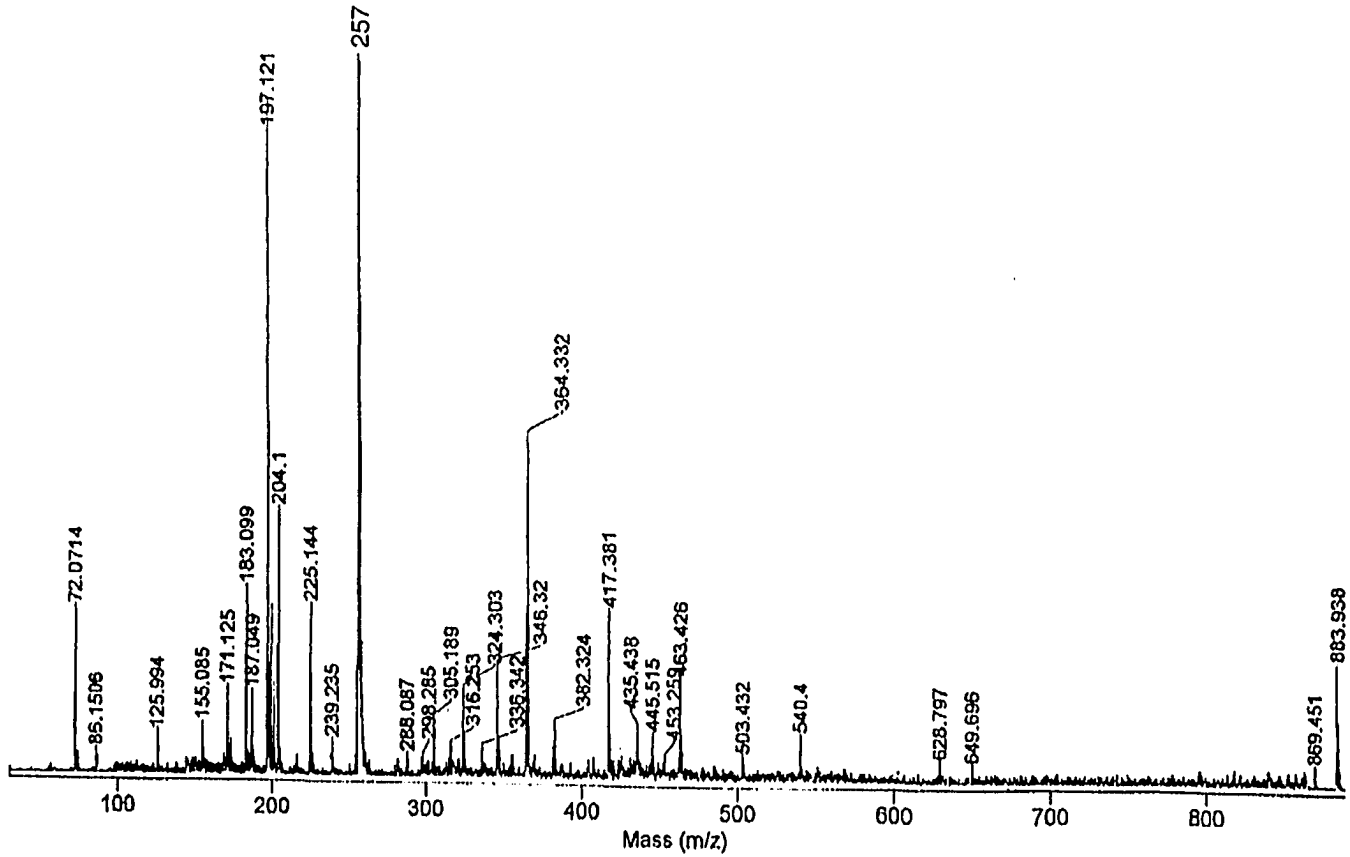

Figure II.3.3.6.B. MALDI-PSD MS spectrum of the $[M+H]^+$ molecular ion fragment of 883 Da from the antifungal material produced by *P. polymyxa* PKB1.

another type of component. If the 256 Da ion is considered as a side chain, then the 628 ion peak could be the cyclic ring structure of the peptide. In this way the structure would not contain a free N-terminal or C-terminal amino group, as had been indicated in previous studies with the antifungal material. Tandem MS was performed on the 256 Da ion seen in the MS spectra of this study, and the fragmentation pattern suggests that the ion is part of a GHPD structure. Ionization by a variety of 'soft' methods and resulting fragmentation of the ring structure and GHPD side chain appears to have allowed proton migration to the hydroxyl group of C_3, then cleavage of the GHPD structure at the bond between C_2 and C_3 (Figure II.3.3.6.C and D; Table II.3.3.6). This would result in the $CH-(OH_2)-(CH_2)_{12}$ with the attached guanidino group coming off of the ring structure to form the 256 Da ion. This would leave a 42 Da acetyl group that was part of the GHPD structure attached to the threonine on the ring (Figure II.3.3.6.D). Those two structures make up the entire GHPD structure of 298 Da that is also present in fusaricidin A, B, C and D. An ion at 239 mu is seen in the tandem MS of 256 which correlates with the loss of the protonated hydroxyl group (water) of the 256 mu ion. The protonated guanidino group from the GHPD structure is also seen as an ion peak in the tandem MS of 256, at 60 Da.

The fragmentation pattern of the ring structure from the MW 883 ion is depicted in Figure II.3.3.6.D. The fragmentation of the peptide ring, suggests a peptide structure similar to fusaricidin A and correlates with the sequence information of the antifungal material from partial hydrolysis and Edman degradation. The ring structure appears as an ion of 628 Da after it appears to have broken at the ester bond between the side chain (β) hydroxy group of threonine and the amino group of alanine, with the formation of a carboxyl group at the C-terminal alanine residue and threonine as the N-terminal residue, with the acetyl group that was part of GHPD bound to it. The linear peptide then fragmented at amide bonds to form acylium mono-, di-, tri- and quattro-peptide ions of acetyl-thr (125.9), acetyl-thr-val (225 mu), acetyl-thr-val-val (324mu), acetyl-thr-val-val-X-asn (539 mu), respectively. The MS fragmentation of the ring structure suggests that the mass of the unknown residue (X) is 101. It would appear to be thr according to the MS spectra and the structure of the fusaricidins that the antifungal material closely ressembles. However the sequence information of the partially hydrolysed antifungal material and the amino acid compostion ratios suggest otherwise. Since the unknown residue appears to have the same mass as a thr residue, according to MS analysis, one possible candidate is 2, 4-diaminobutyric acid (DAB), which has a residue mass of 101

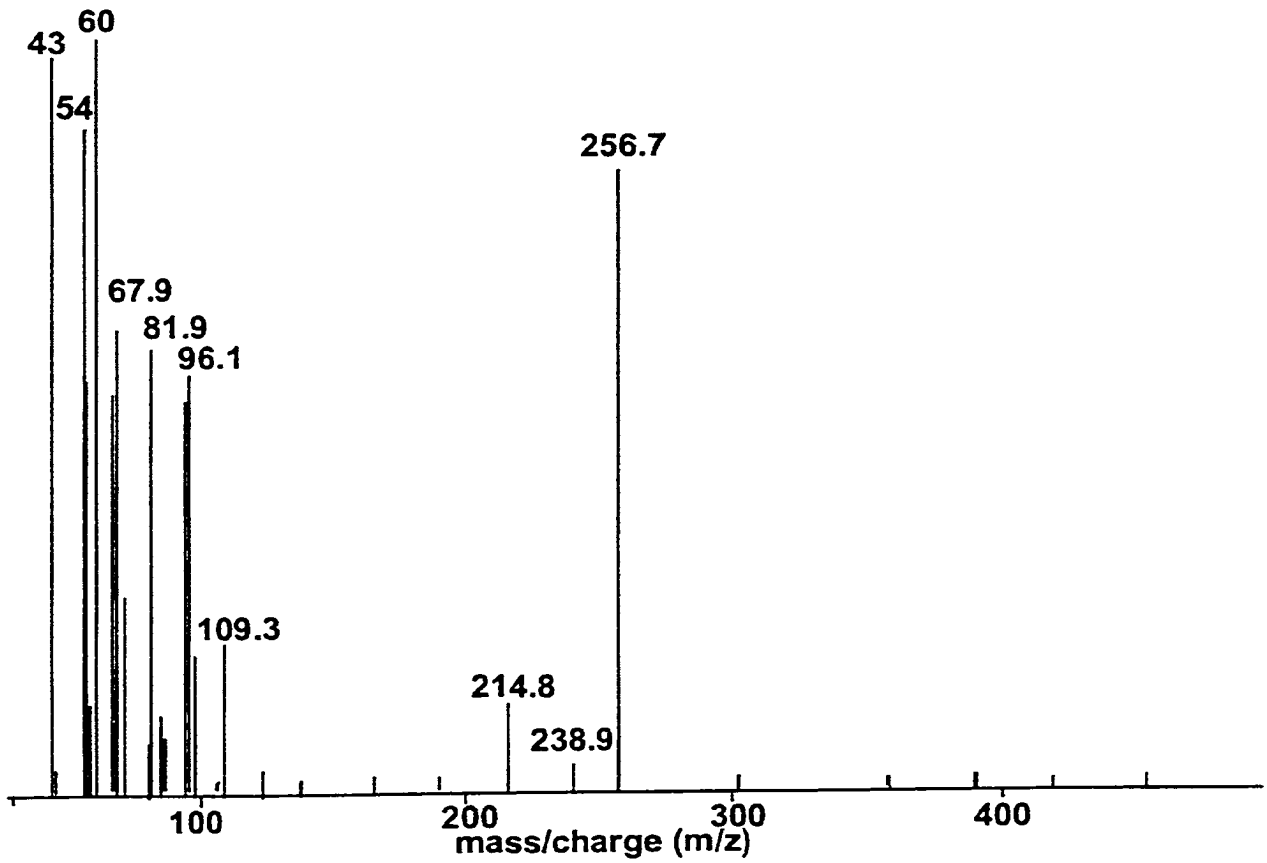

Figure II.3.3.6.C. Tandem MS spectrum of the 256 Da fragment ion generated from ESI-MS of the antifungal material produced by *P. polymyxa* PKB1.

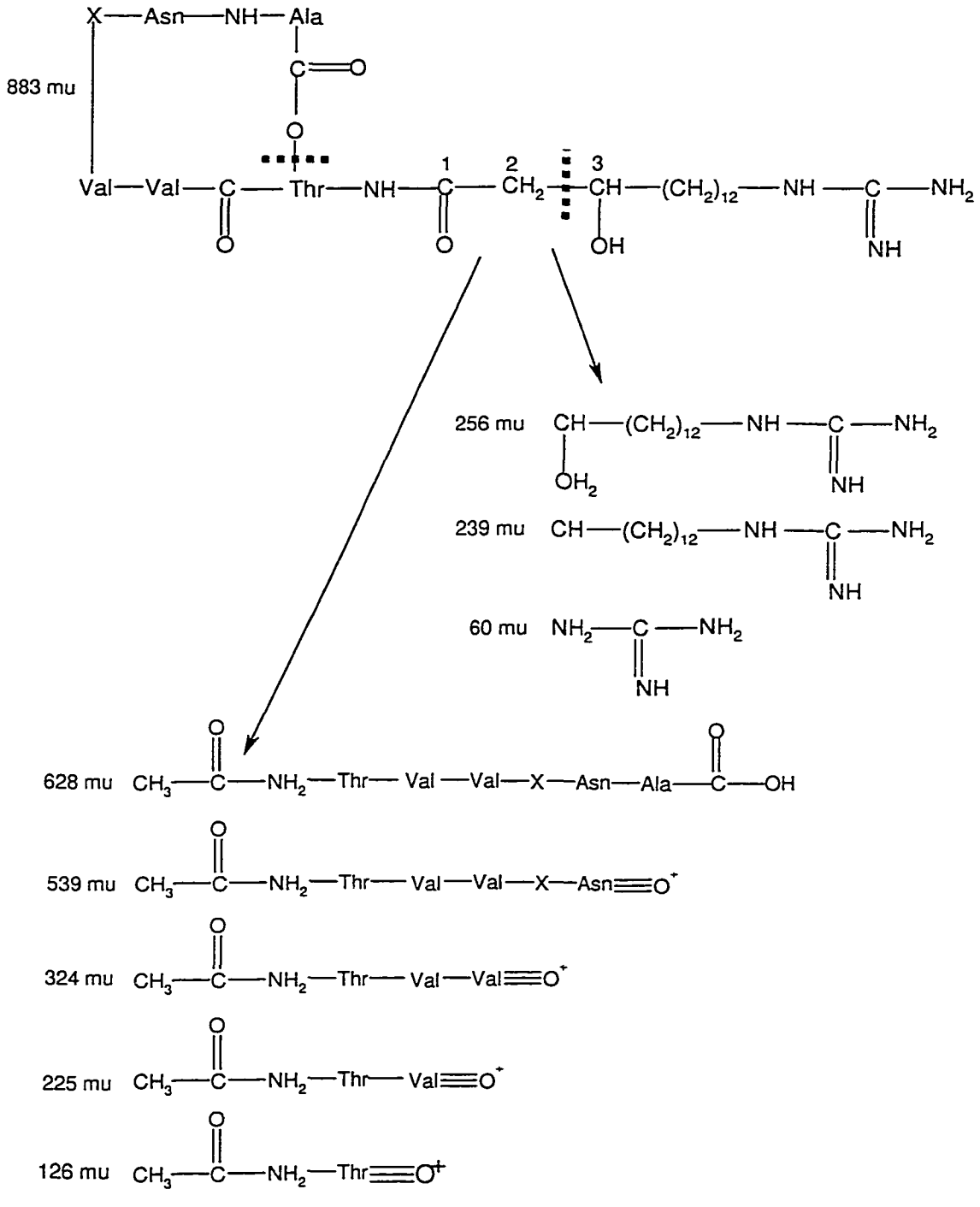

Figure II.3.3.6.D. The proposed structure and fragmentation of the molecular ion 883 Da from the antifungal material produced by *P. polymyxa* PKB1. The dashed lines indicate possible fragmentation sites in the structure. The fragment ions are listed in Table II.3.3.6.

Table II.3.3.6. Interpretation of MS[a] ion masses derived from the 883 m/z molecular ion and the 256 ion fragment in the antifungal material purified from *P. polymyxa* PKB1.

mass/charge (m/z)	MS type	possible sequence
883	PSD, ESI, FAB	$[M+H]^{1+}$; major molecular ion
870	PSD, ESI, FAB	$[M+H]^{1+}$; minor molecular ion
fragmentation of major molecular ion 883		
628	PSD	complete ring structure plus acetyl group of GHPD (acetylthr [-side chain O]-val-val-X-asn-ala-COOH
539	PSD	acetylthr (-side chain O)-val-val-X-asn
445	PSD, ESI	$[M+H+Na]^{2+}$ of 870 $[M+H]^{1+}$
453	PSD, ESI	$[M+H+Na]^{2+}$ of 884 $[M+H]^{1+}$
324	PSD	acetylthr (-side chain O)-val-val
225	PSD	acetylthr (-side chain O)-val
125.9	PSD	acetylthr (-side chain O)
72	PSD, ESI	val immonium ion
fragmentation of 256 ion		
256	PSD, ESI, FAB	part-GHPD
239	PSD, ESI	loss water from part-GHPD
60	ESI	guanidino group of part-GHPD
43	ESI	acetyl group from GHPD

[a] the different types of MS ionization and or instrumentation used to generate the ion fragments are: PSD=matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) post source decay (performed by H. von Dohren, Technische Universitat Berlin), FAB=fast atom bombardment ionization with quadrupole mass filter (performed by M. McGillivry, University of Victoria Chemistry Department), ESI=electrospray ionization with in source collisionally induced dissociation (CID) and tandem MS analysis of ions (performed by Harvard Microchemistry Department and University of Alberta Mass Spectroscopy Laboratory)

and is found in many peptide antibiotics produced by *P. polymyxa*. As can be seen by the proposed sequences for the fragment ions 539 mu and 324 mu, these two ions differ by the dipeptide X-asn. However, comparison of the hydrolysed unknown amino acid residue of the antifungal material with known DAB illustrated that the unknown residue did not show the same retention on RP-HPLC as DAB.

The PSD and ESI spectra showed ions at 445 mu and 453 mu that corresponded to doubly charged $[M+H+Na]^{+2}$ molecular ions 870 and 883, respectively.

The whole bacterium was also used as a sample in a MALDI-TOF experiment (Figure II.3.3.6.E). *P. polymyxa* PKB1 was mixed with the ionization matrix that included organic solvents that extracted the secondary metabolites from the bacteria. The spectrum showed the two clusters of $[M+H]^{+1}$ molecular ions of 870, 883, 897, and 912 mu plus 947 and 961 that were observed in the spectra of the antifungal material and three other higher MW ions at 1203, 1225 and 1241. These molecular ions represent three other secondary metabolites that are produced by *P. polymyxa* PKB1.

Based on amino acid composition, sequence and MS fragmentation analysis of the antifungal material, it is proposed to be a mixture of cyclic depsipeptides that are either closely related to fusaricidin A, B, C and D or are these antibiotics.

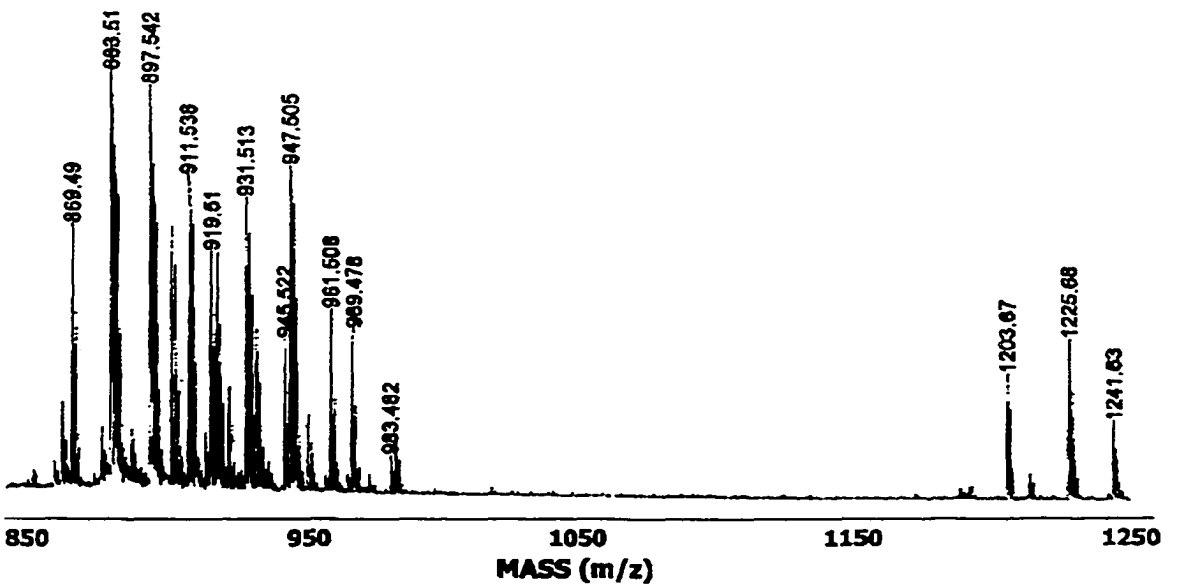

Figure II.3.3.6.E. MALDI-TOF spectrum of the secondary metabolites from *P. polymyxa* PKB1, using the whole bacterium in the ionization matrix

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III.1 Introduction: Peptide Synthetase Genes of *P. polymyxa* PKB1

An environmental bacterium, isolated from canola stubble, was found to be able to inhibit the fungus *L. maculans.* The bacterium was determined to be *P. polymyxa* and was given the strain designation PKB1. Studies on the inhibitory nature of *P. polymyxa* PKB1 against *L. maculans* suggested that inhibition was due, at least in part, to the presence of a low molecular weight, amino acid containing material. Strains of *P. polymyxa*, as with most spore-forming bacteria, are known to produce peptide antibiotics that are active against Gram negative and Gram positive bacteria, or fungi and Gram positive bacteria. These peptide antibiotics are also generally known to be produced non-ribosomally, by peptide synthetase (PS) enzyme complexes. The PS enzymes are highly structured, containing modules with many conserved sequences. There are a number of modules within the PS enzyme responsible for activating and incorporating each amino acid of the peptide that the PS enzyme constructs (Section A.7). The production of the antifungal material by *P. polymyxa* PKB1 was investigated based on the assumption that it would involve participation of one or more PS enzymes.

III.1.1 Peptide synthetase genes

A number of PS genes have been partially or fully characterized to date, including the genes for the production of: surfactin, gramicidin S, tyrocidine, bacitracin, bialaphos, fengycin, lichenysin, syringomycin and saframycin in bacteria, and of: cyclosporin, HC-Toxin, cephalosporin C and destruxin in fungi (Ishihara *et al.*, 1989; Coque *et al.*, 1991; Raibaud *et al.*, 1991; Turgay *et al.*, 1992; Cosmina *et al.*, 1993; Weber *et al.*, 1994; Nikolskaya *et al.*, 1995; Pospeich *et al.*, 1995; Bailey *et al.*, 1996; Mootz and Marahiel, 1997; Lin *et al.*, 1998; Guenzi *et al.*, 1998; Konz *et al.*, 1999). The genes encoding the PS enzymes appear to form clusters within the genome of the producing organism. From this sequence information on PS genes gathered from both bacteria and fungi, it appears that bacteria encode the PS enzyme components on more than one gene, generally from three to five genes. In contrast, fungi tend to encode the PS enzyme within one gene, which can be as large as 45.8 kb, as is the gene encoding the cyclosporin PS enzyme (Weber *et al.*, 1994; Marahiel, 1997). The ACV synthetase, an enzyme that constructs δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine and is found in both

bacteria and fungi, is an exception to this since it appears to be encoded by one gene (Coque *et al.*, 1991).

One module of a PS enzyme; containing an amino acid adenylation domain, a pan motif and an elongation domain, is encoded on approximately 3 kb of DNA. Additional genetic material can encode domains responsible for modification of particular amino acids of the peptide under construction, or cleavage of the peptide from the PS enzyme complex. In bacteria, the DNA encoding the last module of a PS enzyme can include a thioesterase-like domain encoded on 1 kb of DNA. As well, PS enzymes producing peptides that have an attached fatty acid component will carry an extra elongation domain in the module that activates the first, or N-terminal, amino acid of the peptide, which is encoded by approximately 1.5 kb of DNA (Konz *et al.*, 1999). Some PS enzymes of fungi have also been found to be modified with an N-methylation domain that is found between the amino acid adenylation domain and the pan motif of a module that is encoded on 1.2 kb of DNA (Stein and Vater, 1996). In both fungi and bacterial PS enzymes, an epimerizing domain can also be found within the module that activates any amino acid that will be epimerized. The epimerizing domain is encoded on approximately 1.5 kb of DNA. Due to the conserved nature of PS enzymes, the size of the gene(s) encoding the peptide synthetase can be predicted if the number of amino acids present in the peptide structure that the PS enzyme produces is known providing any modifications to the amino acids of the peptide are also known.

III.1.2 The genetic similarity of amino acid activating modules of PS enzymes

Amino acid activating modules of PS enzymes that activate the same amino acid have been found to be genetically similar (Konz *et al.*, 1999). Although PS enzyme modules contain regular conserved sequences, referred to as core sequences, that are found in all PS enzymes, they also contain a variable segment between cores two and three. This region is considered to be the amino acid recognition site for the module, and is encoded on approximately 0.57 kb of DNA (Marahiel, 1997; Konz *et al.*, 1999). Konz *et al.* (1999) aligned the amino acid sequence of this variable region of amino acid adenylation domains from many modules of different PS enzymes from various *Bacillus* spp. and displayed the result in a phylogenetic tree diagram. Modules known to activate the same amino acid were found to be grouped together on the tree diagram, indicating

the amino acid recognition sites of modules activating the same amino acids were similar in amino acid sequence.

A group of researchers (Tosato *et al.*, 1997) sequenced the operon containing genes encoding fengycin synthetase enzymes from *B. subtilus*. The researchers used this similarity of the variable region to other variable regions to identify the amino acids that the modules in the PS enzymes were responsible for activating and incorporating into fengycin. In this way the amino acid sequence of the fengycin, produced by the PS enzymes, was determined.

III.1.3 Genetic characteristics of *P. polymyxa*

P. polymyxa appear to contain a circular genome of approximately 4.3 Mb with a G+C% mol of 45%.

The only report of a plasmid isolated from a *P. polymyxa* strain is of a cryptic linear plasmid (pFS1) in *P. polymyxa* SCE2 (Rosado and Seldin, 1993). Many species of bacteriophage have been isolated from *P. polymyxa* strains; mainly of the *Siphoviridae* family (phage with long, noncontractile tails; Ackermann *et al.*, 1994).

III.1.4 Research design

This chapter will focus on the investigation of PS encoding genes of *P. polymyxa* PKB1. DNA fragments from genes encoding PS enzymes were amplified from the *P. polymyxa* PKB1 genome by PCR using primers based on conserved sequences found in PS enzymes (called PS-PCR products). These DNA fragments were used as probes to identify recombinant phage carrying PS encoding gene inserts from a LambdaGEM®-11 *P. polymyxa* PKB1 genomic library. A number of phage were found that could hybridize to the PS-PCR product probes. These recombinant phage were analysed for PS genes by PCR with primers based on conserved sequences of PS enzymes. They were also analysed by digestion of the *P. polymyxa* PKB1 genomic inserts of the phage with a series of restriction enzymes and Southern analysis using the PS-PCR products as probes.

One of the digestion fragments of one of the recombinant phage that was found to contain an insert of DNA encoding PS enzymes, was used to test for the feasibility of using an integrating vector in gene disruption studies. For this to occur, a method to

introduce DNA into *P. polymyxa* PKB1 was developed. The success of the insertion of the integrating vector into the *P. polymyxa* PKB1 genome was assessed by Southern hybridization of the digested genomes of putative transformants with radiolabelled vector as a probe.

III. 2 Materials and Methods

III.2.1 Strains of bacteria, storage conditions and reagents

Escherichia coli DH5α was obtained from Bethesda Research Laboratories, Inc., (Gaithersburg, Maryland). *E. coli* XL1-Blue was obtained from Stratagene (Cambridge, United Kingdom). *E. coli* ER1447 was obtained from T. Arrowsmith, SmithKline Beecham Pharmaceuticals, (Bristol, England). A permissive *E. coli* lambda bacteriophage host LE392 was purchased from Promega (Madison, Wisconsin). All *E. coli* strains were stored at -70°C as 20% glycerol stocks.

Antibiotics (ampicillin, chloramphenicol, tetracycline) were obtained from Sigma. Restriction endonucleases, ribonuclease A and DNA modifying enzymes were from Boehringer Mannheim (Indianapolis, Indiana), New England Biolabs (Beverly, Massachusetts), Sigma (St. Louis, Missouri) and Promega (Madison, Wisconsin). The [γ-^{32}P]dCTP was obtained from Amersham (Arlington Heights, Illinois).

III.2.2. *E. coli* and *Bacillus* plasmids and LambdaGEM®-11 bacteriophage

The cloning vectors used (Table III.2.2) were obtained from the following sources: pUC118 was obtained from J. Vieira (Waksman Institute of Microbiology, Rutgers University, Piscataway, New Jersey). The plasmids pJH101, pBGSC-6 and pDG364 were provided by W. Nicholson from University of Arizona (Tuscon, Arizona). The plasmid, pC194 was provided by D. Zeigler (*Bacillus* Genetic Stock Centre, Columbus, Ohio).

The genetically altered lambda bacteriophage LambdaGEM®-11 was purchased from Promega for use as a vector to prepare a genomic library of *P. polymyxa* PKB1 DNA. The LambdaGEM®-11 *P. polymyxa* genomic library (producing recombinant lambda bacteriophage) was made by R. Mosher and T. Palmer-Stone, Department of Biological Sciences, University of Alberta. The titre of the library at the time of use in this study was 3.0 X 10^4 pfu/ml.

III.2.3. Preparation of plasmids and LambdaGEM®-11 bacteriophage

Table III.2.2. Plasmid vectors used in this study.

plasmid name	plasmid description	insert	resistance marker	reference
pUC118	*E. coli* cloning vector	no insert, vector 3.2 Kb	amp^r	Vieira and Messing, 1987
pC194	*Bacillus* cloning vector	no insert, vector 2.9 Kb	CAT	*Bacillus* genetic stock centre
pJH101	*E. coli* to *Bacillus* shuttle vector, *Bacillus* integrating vector	no insert, vector 3.2 Kb	amp^r in *E.coli*, CAT in *Bacillus*	W. Nicholson, University of Arizona
pDP4	pUC118 with PS[a] fragment No.4	404 bp PCR[b] fragment	amp^r	this thesis
pDP16	pUC118 with PS[a] fragment No.16	382 bp PCR[b] fragment	amp^r	this thesis
pDP17	pUC118 with PS[a] fragment No.17	406 bp PCR[b] fragment	amp^r	this thesis
pDP26	pUC118 with PS[a] fragment No.26	337 bp PCR[b] fragment	amp^r	this thesis
pDP31	pUC118 with PS[a] fragment No.31	359 bp PCR[b] fragment	amp^r	this thesis
pPB4	pJH101 with PS[a] fragment No.4	same insert as for pDP4	amp^r / CAT	this thesis
pPB16	pJH101 with PS[a] fragment No.16	same insert as for pDP16	amp^r / CAT	this thesis
pPB17	pJH101 with PS[a] fragment No.17	same insert as for pDP17	amp^r / CAT	this thesis
pPB26	pJH101 with PS[a] fragment No.26	same insert as for pDP26	amp^r / CAT	this thesis
pPB31	pJH101 with PS[a] fragment No.31	same insert as for pDP31	amp^r / CAT	this thesis

Table III.2.2. continued.

plasmid name	plasmid description	insert	resistance marker	reference
pSac2	pUC118 with DNA from rp19[c]	2.0 kb *Sac* I fragment of PS	amp^r	this thesis
pSac6A	pUC118 with DNA from rp19[c]	1.2 kb *Sac* I fragment of PS	amp^r	this thesis
pSac8A	pUC118 with DNA from rp19[c]	2.0 kb *Sac* I fragment of PS	amp^r	this thesis
pSac14	pUC118 with DNA from rp19[c]	600 bp *Sac* I fragment of PS	amp^r	this thesis
pSac20	pUC118 with DNA from rp19[c]	500 bp *Sac* I fragment of PS	amp^r	this thesis
pSac21	pUC118 with DNA from rp19[c]	700 bp *Sac* I fragment of PS	amp^r	this thesis
pSac32	pUC118 with DNA from rp19[c]	1.6 kb *Sac* I fragment of PS	amp^r	this thesis
pBsa49	pUC118 with DNA from rp19[d]	2 kb *Bsa* I fragment of PS	amp^r	this thesis
pBsa52	pUC118 with DNA from rp19[d]	500 bp *Bsa* I fragment of PS	amp^r	this thesis
pBsa65	pUC118 with DNA from rp19[d]	500 bp *Bsa* I fragment of PS	amp^r	this thesis
pJT1	pJH101 with DNA from rp19[e]	2.0 kb Sac I fragment insert of pSac8A	CAT	J. Taylor, University of Alberta

[a] PS, peptide synthetase gene.
[b] PCR product prepared using degenerate primers; FDP4 and RDP6
[c] recombinant phage (rp) 19 DNA was digested with *Sac* I and subcloned into pUC118.
[d] rp19 DNA was digested with *Bsa* I and subcloned into pUC118.
[e] rp19 DNA was digested with *Sac* I and ligated into pJH101

For plasmid preparation from *E. coli*, overnight cultures were grown at 37°C in Luria-Bertani Broth (LB) medium containing 100 μg/ml ampicillin, agitated on a tube roller. LB contained 1% tryptone, 0.5% yeast extract and 1% NaCl; the pH was adjusted to 7.5.

Plasmids replicated in *E. coli* were isolated and purified according to the method of Birnboim and Doly (1979) as described in Sambrook *et al.* (1989).

P. polymyxa plasmids were prepared using overnight roller tube cultures grown at 37°C in glucose-beef (GB) broth (1% glucose, 1% Difco peptone, 0.2% Difco meat extract, 0.1% Difco yeast extract, 0.5% NaCl; pH 7.0; Seldin *et al.* 1983) containing 5 μg/ml chloramphenicol. Plasmids replicated in *P. polymyxa* were isolated and purified according to the method reported by Belliveau and Trevors (1989).

Recombinant lambda bacteriophage were grown in *E. coli* LE392, purified and harvested by the liquid phage lysate method adapted from the Promega Protocols and Applications Guide (Titus, 1991) and protocols outlined by Silhavy *et al.* (1984). A starter culture of *E. coli* LE392 was grown in 5 ml TB (1% tryptone, 0.5% NaCl; after autoclaving 10 ml 1 M $MgSO_4$ [sterile] was added to 1 L broth) with 0.2% maltose at 37°C on a tube roller as an overnight culture. The cells were pelleted at maximum speed in a clinical centrifuge for 10 min and resuspended into 2.5 ml 10 mM $MgSO_4$. A 500 μl aliquot of cell suspension was added to 20 μl of phage stock in lambda-dilution buffer (0.1 M Tris-HCl, 0.05 M $MgSO_4 \cdot 7H_2O$, 0.1% gelatin [Difco], pH 7.4, titre was approximately 10^{10} to 10^{11} pfu/ml) or phage lysate broth and incubated at room temperature for 5 min. This mixture was transferred to 100 ml of LB broth, prewarmed to 37°C, with 0.2% maltose and 10 mM $MgSO_4$ in a 250 ml flask and incubated at 37°C, shaking at 200 rpm until lysis occurred, after approximately 5 to 7 h. Five hundred microlitres of chloroform was added to the lysate. Cell debris was pelleted by centrifugation at 15 300 X g for 10 min and the phage lysate was reserved for recombinant lambda DNA extraction. The recombinant lambda DNA was extracted from the phage lysate preparation following the phage 'mini-prep' procedure in the Promega Protocols and Applications Guide (Titus, 1991) with the these two modifications that were adapted from Sambrook *et al.* (1989). The first modification was; after 100 μl of 10% SDS and 100μl of 0.5 M EDTA were added to the 10 ml bacteriophage particle containing phage buffer and incubated at 68°C for 15 min, 10 ml chloroform was added to the mixture, vortexed and separated from the aqueous mixture by centrifuging in a

clinical centrifuge at 890 X g for 5 min. This was repeated with the aqueous mixture two more times. The second modification was; the recombinant lambda DNA was spooled onto a glass rod from ice-cold 95% ethanol instead of being pelleted by centrifugation.

III.2.4 Gel purification of DNA fragments

DNA fragments were purified by electrophoresis on a 0.8% agarose gel (ICN, Aurora, Ohio) made with 1 X TAE (0.04 M Tris-acetate, 0.001 M EDTA), in a 1 X TAE running buffer. Gels were run at 90 V (5 V/cm). Bands containing the DNA fragments of interest were excised and the DNA was recovered using a Geneclean® II kit (Bio 101, Vista, CA), following the manufacturers' instructions with these changes: DNA was eluted from the glassmilk into 15 µl distilled H_2O by heating the mixture to 50°C for 3 min. DNA was eluted from the glassmilk a second time into 5 µl distilled H_2O by incubating at room temperature for 30 s. Eluted DNA was pooled and stored at 4°C if the DNA preparation was to be used within 7 d or at -20°C if the DNA preparation was not to be used within 7 d. If the DNA preparation was for long term storage then it was eluted from the glassmilk into TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) following the same procedure.

DNA fragments were also purified from 4% polyacrylamide gels (10% TBE [0.045 M Tris-borate, 0.001 M EDTA], 10% 29:1 [40%] acrylamide solution (Bio Rad), 4.6% ammonium persulfate, 0.06% TEMED) run in 1X TBE running buffer at 150 V (18 V/cm). DNA bands were excised and the DNA was recovered using the 'crush and soak' method as outlined in Sambrook *et. al* (1989) with one modification; before the crushed gel was incubated at 37°C overnight, it was placed at -20°C for 10 min to aid in the release of DNA from the gel.

III.2.5 Measurement of DNA concentration

DNA was quantified and its purity was determined by measuring its A_{260} and A_{280} using a UV/VIS Philips Model PU 8740 UV/VIS scanning spectrophotometer that was set to scan the sample from A_{320} to A_{230}.

DNA concentrations were estimated by agarose gel electrophoresis and comparison with a DNA standard such as lambda bacteriophage DNA (New England Biolabs, Beveryl, Massachesetts) digested with the restriction enzymes *Pst* I or *Bst* EII.

Fluorometry was done to quantify DNA for sequencing using a Hoefer Scientific Instruments Model TKO 100 fluorometer. Samples of 2 μL were mixed with 2 mL Hoefer Scientific H33258 fluorometry dye solution, used according to the specifications of the manufacturer, and analyzed.

III.2.6 Purification of genomic DNA from *P. polymyxa* PKB1

Genomic DNA was prepared using an overnight culture of *P. polymyxa* PKB1 grown in 25 mL of PDB or LB medium per 250 ml flask at 28°C, shaken at 200 rpm using an adaptation of the method described by Mavingui *et al.* (1992). The culture was divided in half and centrifuged at 12 000 X g for 20 min at 4°C. The cell pellet was resuspended in 0.5 ml of TES buffer (30mM Tris-HCl, 5 mM EDTA and 50 mM NaCl, pH 8.0), and centrifuged at 16 000 X g for 2 min. The TES supernatant was discarded and pellets were resuspended in 600 μL of TES buffer, with 150 μL of a fresh 5 mg/mL lysozyme (Sigma) in TE buffer. Samples were incubated at 37°C for 60 min, then 45 μL of 10% w/v SDS in TES buffer was added, mixed by inversion, and incubated at 37°C for 15 min before the addition of 2.5 μL of 10 mg/mL ribonuclease A (Sigma), and a third incubation at 37°C for 60 min. At this time 50 μL of Proteinase K (Sigma), 25 mg/ml, was added and the samples were incubated for 90 min at 37°C. The genomic DNA preparation was extracted with 1 volume of neutral phenol/chloroform/isoamyl alcohol solution (25:24:1 v/v), then with 1 volume of chloroform/isoamylalcohol solution (24:1 v/v). DNA was precipitated with 2 volumes of 95% ethanol and 50 μL of 3 M sodium acetate for 1 h at -70°C, then rinsed with 70% ethanol. DNA was resuspended in 1 ml TE buffer and stored at 4°C.

III.2.7 Generation of *E. coli* and *P. polymyxa* electrocompetent cells and electroporation

An overnight culture of the desired strain of *E. coli* incubated in 10 ml LB broth at 37°C, rotating on a test tube roller was used as an inoculum. Electrocompetent *E. coli* cells were prepared using a 1% suspension of the inoculum in 100 ml LB broth incubated in a 500 ml flask at 37°C, shaken at 200 rpm. The OD_{600} of the culture was monitored until it reached 0.35 to 0.5 OD units. The cells were centrifuged at 10 000 X g

for 15 min at 4°C to form a pellet. The cell pellet was resuspended in an equal volume of 10% (v/v) glycerol in distilled H_2O. This procedure was done twice. The washed cell pellet was resuspended in 10% (v/v) glycerol in dH_20 at 40 µl per 1 ml culture. The electrocompetent cells were flash frozen (-70°C) in a dry ice and ethanol bath in 40µl aliquots and stored at -70°C until used.

For electroporation of plasmids into *E. coli*, a 40 µl aliquot of an electrocompetent *E. coli* strain was thawed on ice. Plasmid DNA (1 µl volume; 4 ng) from a plasmid preparation or from a ligation mixture (3 µl volume; 4 ng), was added to the electrocompetent cells in a 0.2 cm gapped electrocuvette (BTX, San Diego, California) and mixed by gentle agitation. The cuvette was placed in the Gene Pulser II electroporation instrument (Bio Rad) and pulsed with an electric charge of 2.5 kV (12.5 kV/cm) and 25 µF for 5 ms. The cells were transferred to a 1.5 ml Eppendorf tube containing 960 µl of recovery medium (such as LB broth without antibiotic). The cells were incubated at 37°C for 60 to 120 min, then plated onto LB agar containing 100 µg/ml ampicillin in 200 and 800 µl portions and incubated at 37°C overnight. When plasmids that allowed blue/white selection were used in the electroporation, 100 µl 2% isopropyl-β-D-thiogalactopyranoside (X-gal; Chem Alta Ltd., Edmonton, Alberta) in dimethylsulfoxide (DMSO; Sigma) and 50 µl isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) were spread on each plate prior to inoculation.

Electrocompetent *P. polymyxa* cells were prepared using a method adapted from the protocols used by Belliveau and Trevors (1989) and Rosado and Seldin (1993). *P. polymyxa* PKB1 was added to 100 ml GB broth in a 500 ml Erlenmyer flask as a 1% (v/v) inoculum from a 10% glycerol stock (Section II.2.3.) and grown at 28°C, shaken at 200 rpm for 24 h for use as a starter culure. The starter culture (1 ml) was used to inoculate 250 ml GB broth in a 500 ml Erlenmeyer flask, which was then incubated at 28°C, shaken at 200 rpm, overnight. When the culture reached an OD_{600} between 0.85 and 1.6, with the optimum at 1.26, it was centrifuged at 10 000 X g for 15 min at 4°C. The cells were washed two times with distilled H_2O and then once with electroporation buffer, HEB (272 mM sucrose, 1 mM $MgCl_2$, 7 mM HEPES buffer solution [BDH], pH 7.3) by resuspending in 60 ml and centrifuging the cells as before. The cells were then resuspended in HEB at 1% of the original culture volume. The resuspended cells were aliquotted into sterile 1.5 ml Eppendorf tubes in 400 µl volumes, flash frozen in a dry ice, ethanol bath to -70°C and stored at this temperature.

For electroporation of plasmids into *P. polymyxa*, a method was adapted from those used by Belliveau and Trevors (1989) and Rosado and Seldin (1993). Two 400 µl aliquots of electrocompetent cells were thawed on ice and placed in a 0.4 cm gapped electrocuvette (BTX). Plasmid DNA (about a 30 µl volume; 0.5 to 1 µg of DNA) was added to the cells. The cuvette was gently agitated to mix the two components, then left on ice for 5 min. The cells were pulsed (Gene Pulser II instrument) with an electric charge of 1.5 kV (3750 V/cm) and 25 µF for 3.20 ms. The electroporated cells were transferred to a 15 ml disposable centrifuge tube containing 1 ml of recovery medium such as GB broth, and kept on ice for 15 min, then 7 ml GB broth was added to the cells and they were incubated at 28°C on a tube roller or shaken at 100 rpm for 2 h. The cells were pelleted in a clinical centrifuge at 890 X g for 7 minutes, then resuspended in 500 µl of GB broth and plated in 100 µl aliquots onto GB agar with 4 or 5 µg/ml chloramphenicol. *P. polymyxa* PKB1 colonies that were chloramphenicol resistant were visible on GB agar after 24 h incubation at 37°C. The transformants were screened for antifungal activity by patching them (with a sterile toothpick) on PDA with 5 µg/ml chloramphenicol (in a square 245 mm petri plate) that had been inoculated with 800 µl of *L. maculans* spore stock. The plate was incubated at room temperature, in the light, for 48 h.

III.2.8. Generation of calcium chloride competent *E. coli* and transformation

The ligation reactions for subcloning DNA fragments generated from the restriction enzyme digestion of recombinant lambda bacteriophage No. 19 into pUC118 contained polyethylene glycol (PEG; BDH). This chemical interferes with electrotransformation so these ligation reactions were transformed into *E. coli* DH5α using freshly made competent *E. coli* prepared using the calcium chloride procedure as described in Sambrook *et al.* (1989), with the following modifications. An overnight culture of *E. coli* XL1Blue was grown in 5 ml LB broth at 37°C, shaken at 100 rpm. Fresh LB (5 ml) was inoculated with 100 µl of the overnight culture and incubated at 37°C for 2 h, shaken at 100 rpm. This culture was centrifuged at 7740 X g for 5 min at 4°C. The cell pellet was resuspended in 3 ml of ice cold 0.1 M $CaCl_2$ and incubated on ice for 20 min. The cells were pelleted as before and resuspended in 1 ml of ice cold 0.1 M $CaCl_2$ for use as competent cells in the transformation procedure. The competent cells (200 µl)

and the ligation mixture or plasmid to be transformed were placed in an Eppendorf tube and incubated on ice for 45 min with gentle resuspension every 15 min. The tranformation mixture was heat shocked at 43°C for 2 min and plated on LB agar with 100 μg/ml ampicillin in 100 μl aliquots.

III.2.9. Southern blotting

The method for transferring DNA from an agarose gel to a nylon membrane was adapted from the method designed by Southern (1975). Restriction enzyme digested DNA was separated by electrophoresis in a 0.8% agarose gel with TAE running buffer, stained for 10 min in an ethidium bromide bath and photographed with a photoilluminator under UV light. The gel was bathed for 10 min in depurination solution (0.125 M HCl) with gentle agitation, then submerged for 25 min in denaturation buffer (1 M NaCl, 0.5 M NaOH) with gentle agitation, then soaked for 25 min in neutralization buffer (1 M NaCl, 0.5 M Tris-acetate, pH 7.5) with gentle agitation. A Southern blot apparatus was prepared: a 30 x 15 cm pyrex dish filled with transfer buffer (10X SSC; 0.3 M tri-sodium citrate, 3 M NaCl) supporting a glass plate covered with three 90 x 16 cm sheets of blotting paper with the ends of the papers folded under the glass sheet to act as a wick for the 10 X SSC (Ahlstrom Paper Group; Mt. Holly Springs, Philadelphia). The blotting paper was pre-moistened with 10 X SSC. The gel was placed on the blotting papers of the Southern blot apparatus, then a Hybond-N nylon membrane (Amershan Life Sciences, Little Chalfont, England) was placed on the gel, followed by three sheets of blotting paper pre-moistened with 10 X SSC. The membrane and blotting papers that were placed on the gel were cut to the same size as the gel. A three inch stack of paper towels and a weight was placed on top of the blotting papers and the apparatus was left overnight. After the transfer occurred, the lanes on the gel were marked on the corresponding membrane and the DNA was fixed to the membrane by heating the blot at 80°C for 2 h in an oven. The fixed membranes were either hybridized to a radiolabelled probe immediately, or stored wrapped in plastic and kept in a dark, dry place until used.

III.2.10. Hybridization of Southern blots

The nylon membrane to be hybridized (Section III.2.9) was placed in a hybridization bottle with 20 ml hybridization solution (5X SSPE [0.9 M NaCl, 0.05 M

dibasic sodium phosphate, 5 mM EDTA, pH 7.7], 5 X Denhardt's solution [0.1% (w/v) BSA fraction V (Boehringer Mannheim), 0.1% (w/v) Ficoll 400 (BDH), 0.1% (w/v) polyvinylpyrroleidone (Sigma)], 0.5% (w/v) SDS and150 μg/ml denatured salmon sperm DNA [Sigma]) and heated to 65°C. The membrane was incubated in a roller hybridization oven at 65°C for 1.5 h, then radiolabelled DNA probe was added to the bottle and it was incubated overnight at 65°C in the roller oven. The membrane was washed with prewarmed (50°C) 2X SSPE and 0.1% (w/v) SDS for 10 min at room temperature twice, then once with prewarmed (65°C) 1X SSPE and 0.1% SDS for 15 min at 65°C and once with prewarmed (65°C) 0.1X SSPE and 0.1% SDS for 10 min at 65°C. The membranes were wrapped in plastic, placed in a film cassette with two sheets of X-ray film (Scientific Imaging Systems, Eastman Kodak Company, New Haven, Conneticut). The film was exposed at -70°C. Exposure times varied from 5 h to 7 d ,depending on the strength of the radiolabelled probe. Exposed X-ray film was developed using a Fuji RGII X-ray film processor (Fuji Photo Film Company, Tokyo, Japan), according to manufacturers' instructions.

III.2.11. Membrane stripping

Nylon membrane blots hybridized with radiolabelled DNA probes were stripped of the hybridized probe using two methods.

One method involved soaking the membrane in boiling 0.1% (w/v) SDS. The SDS bath was allowed to cool to room temperature and the wash was repeated, then the membrane was rinsed with 2 X SSC.

The other method involved bathing the membrane in 42°C 0.2 M NaOH for 10 min with gentle agitation. This was repeated, then the membrane was washed with 2 X SSC for 15 min.

The extent of stripping was analysed after either method by exposing an X-ray film to the blot overnight. The membranes were kept moist to prevent the radiolabelled probe from permanently fixing to them.

III.2.12. Nick translation radiolabelling of DNA probes

Gel purified DNA fragments (Section III.2.4) or linearized plasmid were labelled with α^{32}P-dCTP using a nick translation method adapted from Hopwood *et al.* (1985). The nick translation reaction contained: from 0.1 to 0.5 μg DNA, 1 μl each of 1 mM dATP, dTTP and dGTP (Boehringer Mannheim), 1 μl (10μCi) of α^{32}P-dCTP, 3 μl of 10 X nick translation buffer (0.5 M Tris-HCl pH 7.5, 0.1 M $MgSO_4$, 1 mM dithiothreitol (DTT; Sigma), 2.5 μl of a 10^{-4} dilution of DNAse I (Boehringer Mannheim), 0.5 μl of DNA polymerase I (Boehringer Mannheim) and distilled H_2O to bring the reaction volume to 30 μl. This reaction mixture was incubated at 15°C for 1.5 h. The reaction was stopped by adding 15 μl of 0.5 M EDTA. The radiolabelled DNA was separated from unincorporated nucleotides using a G-50 Sephadex (Amersham Pharmacia) gel filtration mini column after 15 μl of bromthymol blue (Sigma) was added to the mixture to visualize the passage of the probe through the column. The probe DNA was added to the hybridization solution (Section III.2.10) after denaturing at 90°C for 5 min, then cooling on ice for 2 min. A 1 μl sample of the radiolabelled probe was taken prior to addition to the hybridization buffer to measure the extent of incorporation of radioactivity using the Beckman LS 3801 scintillation counter (Beckman Instruments; Fullerton, California).

III.2.13 Restriction enzyme digestion of DNA

The restriction enzymes were used in reactions according to manufacturers' instruction and Sambrook *et al* (1989).

III.2.14. Peptide synthetase gene fragments generated by PCR

Peptide synthetase (PS) gene fragments were generated from *P. polymyxa* PKB1 using an adaptation of a method outlined by Turgay and Marahiel (1994). These are referred to as PS-PCR products in this thesis.

III.2.14.1 Primer design

Primers were synthesized by P. Murray and the primer stocks were treated as stated in Section I.2.5.1.

The forward and reverse PCR primers used to generated the PS gene fragments were designed based on the codons for the amino acid sequences of the conserved regions of cores 4 (degenerate primer 4; FDP4) and 6 (degenerate primer 6; RDP6) that are found in all PS enzyme domains (Section A.7). The codons used in the primers were (mostly) based on the Universal codon list (Sambrook *et. al.*, 1989), however *Bacillus sp.* codon bias (Ogasawara, 1985) was also taken into account. The primers were designed to be degenerate, with the incorporation of deoxyinosine triphosphates (dITP) and mixes of two or three different nucleotides (Innis *et al.*, 1990). If a particular amino acid in the conserved motif was encoded by multiple codons with each of the four nucleotides represented in the third position, inosine was substitiuted in the third position in the primer. If there were two or three different codons for a certain amino acid that varied at the third position of the codon, those nucleotides were incorporated into the primer, in equal proportions at that position. Three restriction enzyme sites, *Eco* RI, *Xba* I and *Sac* I, were introduced at the 5' end of the primer (Section I.2.5.1). Other primers were designed to hybridize to DNA encoding the conserved amino acid sequences of cores 1 and 2, and for the reverse of 4, or with different restriction enzyme sites incorporated into the 5' end of the primer. Table III.2.14.1 lists the primers designed to hybridize to conserved sequences of PS domains and describes the restriction enzyme sites present on each oligonucleotide.

III.2.14.2. PCR protocol for generation of PS fragments

The PCR reactions were carried out in an MJ Research Minicycler as stated in Section I.2.5.2.

The PS fragments were amplified from the *P. polymyxa* PKB1 genome using primers FDP4 and RDP6. A 'touchdown' PCR protocol was developed to compensate for the degeneracy of the primers. Since each primer was actually a mixture of many slightly different primers, the melting temperature of the primer mix was unknown; to compensate, the annealing temperature was 71°C in the first cycle, then the temperature was decreased in each following cycle until the annealing temperature of 64°C was reached, then the PCR program cycled 20 more times (Table III.2.14.2).

The PCR reaction mixture contained; 0.7 µl of 38 ng/µl *P. polymyxa* PKB1 genomic DNA, 1.1 µl of 19 pmol/µl primer FDP4, 0.61 µl of 33 pmol/µl primer RDP6, 0.4

Table III.2.14.1. PCR primers designed to anneal to regions of PS enzyme encoding genes.

primer[a]	RE sites	primer sequence 5' to 3'[b]
FDP1	*Sac*1 *Xba*1 *Eco*R1	TGAGAGCTCTAGAATTCGTXCTXAA(AG)GCXGGXGGXGC
F2DP1	*Spo*1 *Not*1	TCGCGAGCGGCCGCTXAA(AG)GCXGGXGGXGC
FDP2	*Sac*1 *Xba*1 *Eco*R1	TGAGAGCTCTAGAATTC(AGCT)GG(AGCT)AC(TA)AC(CG)GG(AGCT)
FP3	*Apa*L1 *Dra*III	GTGCACATGGTGCGAACAGGCGAA
FDP4	*Sac*1 *Xba*1 *Eco*R1	TGAGAGCTCTAGAATTCGXGGXCA(TC)CGXAT(TCA)GA(AG)CT
RDP4	*Sac*1 *Xba*1 *Eco*R1	TGAGAGCTCTAGAATTCAG(CT)TC(TGA)ATXCG(AG)TGXCCXCG
RP4	*Spo*1 *Not*1	TCGCGAGCGGCCGCTTGTGCCTCGACCTCACC
RP6	*Eag*1 Fse1	CGCGGCCGGCCGCTATCTCCTCCTAG
RDP6	*Eco*R1 *Xba*1 *Sac*1	ATGAGAATTCTAGAGCTCXGA(AG)TGXCCXCC(AC)AG
R2DP6	none	AGATCTGC(TC)TTXAGXGA(AG)TCXCCXCCA

[a] the primer name is coded; F: forward primer, R: reverse primer, DP: degenerate primer, the last number refers to the PS enzyme conserved sequence number that the primer is designed from.

[b] the nucleotides in brackets indicate an equal mixture of these were available for incorporation into the oligonucleotide at that position and X refers to the addition of dITP at that position.

[c] restriction enzyme (RE) sites added to primers at the 5' end are listed.

Table III.2.14.2. Touchdown PCR protocol used to generate PS fragments from *P. polymyxa* PKB1 with degenerate primers.

step	temperature (°C)	time (s)	protocol
1	94	90	denaturation of template and primers
2	94	60	denaturation of template and primers
3	71	30	annealing of template and primers
4	72	30	extension of PCR product
5	94	60	denaturation of template and primers
6	70	30	annealing of template and primers
7	72	30	extension of PCR product
8	94	60	denaturation of template and primers
9	69	30	annealing of template and primers
10	72	30	extension of PCR product
11	94	60	denaturation of template and primers
12	68	30	annealing of template and primers
13	72	30	extension of PCR product
14	94	60	denaturation of template and primers
15	67	30	annealing of template and primers
16	72	30	extension of PCR product
17	94	60	denaturation of template and primers
18	66	30	annealing of template and primers
19	72	30	extension of PCR product
20	94	60	denaturation of template and primers
21	65	30	annealing of template and primers
22	72	30	extension of PCR product
23	94	60	denaturation of template and primers
24	64	30	annealing of template and primers
25	72	30	extension of PCR product
26	--	--	go to step 23 20 more times
27	72	5 min	extension of PCR product
28	4	--	cool down
29	4	--	end

μl of 25 mM dNTP mix, 5 μl of 10 X PCR buffer, 0.75 μL of 0.1 M $MgCl_2$, 28.25 μL of distilled H_2O and 1 μl of 5 units/μl Taq DNA polymerase.

PCR reactions using λ DNA from recombinant phage and a primer pair mix of one degenerate primer and one non-degenerate primer were perfomed using a thermocycler program of 30 cycles with the annealing temperature set to 72°C and an extension time of 50 sec. The rest of the conditions were the same as for the touchdown thermocycler program.

The PCR reaction mixture contained, 0.5 μl or 1 μl of 40 ng/μl λ DNA, 1.67 μl of 30 pmol/μl each of FDP1 or FDP2 and RP4, 1 μl of 25 mM dNTP mix, 5 μl of 10 X PCR buffer, 0.5 μl of 100 mM $MgCl_2$, 37 μl or 37.5 μl of distilled H_2O and 1 μl of 5 units/μl Taq DNA polymerase.

III.2.14.3. Blunt-end ligation of PS-PCR products into pUC118

Blunt-end ligation of DNA into a plasmid was used to clone PCR products amplified from the *P. polymyxa* PKB1 genome and DNA fragments that were digested using a blunt cutting restriction enzyme. The PCR products were made blunt using the Geneclean II PCR products cloning protocol outlined in the manufacturers' instruction booklet.

The blunt ended DNA was ligated into *Sma* I digested pUC118. The ligation reaction contained 5% polyethelyne glycol 8000 (Sigma), 1 X ligation buffer (Boehringer Mannheim), 1 unit T4 DNA ligase (Boehringer Mannheim) and DNA at either 1:3 or 1:6 vector:insert ratio. The ligation reaction was incubated at room temperature, overnight, then was heat inactivated at 65°C for 10 min.

III.2.14.4. Sequencing of PS-PCR DNA products

The concentration of plasmids carrying PS-PCR products was adjusted for sequencing as described in Section I.2.5.3.

III.2.14.5 Computer analysis of DNA and amino acid sequences

III.2.14.5.1 DNA Strider program

The DNA Strider program was used to analyse DNA sequences for restriction enzyme digestion sites, and to determine which DNA sequence frame would translate the DNA into the correct amino acid sequence. One way involved the translation of DNA into the 6 possible amino acid sequences and analysis of the resulting amino acid sequence for the presence of known conserved amino acid sequences (Section A.7; Stein and Vater, 1996). The other way involved analysing the nucleotide sequences for putative open reading frames (ORF; no in frame stop codons). The nucleotide sequence in the frame that appeared to be a part of an ORF was translated into the corresponding amino acid sequence.

III.2.14.5.2 Basic local alignment search tool (BLAST)

BLASTn and BLASTp were used as described in Section I.2.5.4.1. to search for similar sequences to the target sequence from the databases available with this search tool.

III.2.14.5.3 Genetics Computer Group (GCG) programs

DNA and amino acid sequences were analyzed for similarity to each other using the software package from the Genetics Computer Group (GCG, Wisconsin Package version 9.1, Madison, Wisconsin); in particular using gap analysis, pileup and pretty programs. The programs were used according to the GCG instruction manual.

III.2.14.6 Cohesive-end ligation of PS-PCR DNA products

Cohesive-end ligation was used to clone DNA fragments that were digested with restriction enzymes that leave overhangs at the cleavage site according to Sambrook *et al.* (1989). The cohesive-end ligation reaction contained 1 X ligation buffer (Boehringer Mannheim), 1 unit T4 DNA ligase (Boehringer Mannheim) and DNA at either 1:3 or 1:10 vector:insert ratio. The vector and insert DNA were combined and heated at 55°C for 3 min then put on ice before the rest of the reaction mixture was added. The ligation reaction was incubated at room temperature, overnight, then was heat inactivated at 65°C for 10 min.

III.2.15. Probing of LambdaGEM®-11 *P. polymyxa* PKB1 genomic library

Three of the PS DNA fragments, No.s 4, 16 and 17, were used as probes to analyze both a non-amplified and an amplified library of recombinant lambda bacteriophage carrying *P. polymyxa* PKB1 genomic inserts. Two different protocols were used to generate agar plates of *E. coli* LE392 plaques for plaque lifts and subsequent hybridization with the radiolabelled probes (Sections III.2.9 and III.2.11). The plaque growth protocol that was used to discover the positive recombinant lambda bacteriophage No. 19 was as described in the Promega Protocols and Applications Guide (Titus, 1991).

The protocol that was used to find the positive recombinant lambda bacteriophage No. 44 and No. 67 was derived from Silhavy *et al.* (1984). A culture of *E. coli* LE392 was grown overnight at 30 °C in 5 ml TB medium with 0.2% (w/v) maltose in a tube roller. The cells were pelleted by centrifugation in a clinical centrifuge at 890 X g for 10 min, then resuspended in 2.5 ml 10 mM $MgSO_4$ and stored at 4°C until used (for up to 7 d). A 50 µl aliquot of these cells was incubated at room temperature for 5 min with an aliquot of either a non-amplified or an amplified phage library preparation that had been serially diluted to 1 X 10^{-6}. After 5 min, 4 ml molten 0.7% (w/v) water agarose was added to the cell-phage mixture and poured onto LB agar that were prepared 2 to 3 d previously so that they would be dried out. The plates were incubated overnight at 37°C, then cooled to 4°C for 1 h. A nylon membrane (to lift the plaques from the agar) was placed on the agar and left for 30 s, orientation marks were applied to the membrane and the outside of the plate and the membrane was lifted off and placed plaque side up on 3 3MM Whatman filter papers saturated with denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 3 min. The membrane was then placed onto 3 3MM Whatman filter papers saturated with neutralization buffer (1 M Tris-HCl, 1.5 M NaCl, pH 7.5) twice, for 3 min each time. The membranes were vigorously shaken in a 2 X SSC bath for 3 min, air dried, and baked at 80°C for 2 h. Up to three plaque lifts were taken from each plate. The membranes were hybridized separately with the radiolabelled DNA probes, PCR PS fragments No.'s 4, 16 and 17, washed stringently (Section III.2.10), and used to expose X-ray film in a film cassette overnight, then developed. The plaques that hybridized to the DNA probes were picked using sterile toothpicks, and stabbed into a fresh TB plate that had previously been seeded with *E. coli* LE392 (50 µl of the 10 mM $MgSO_4$ suspension) in a soft agar overlay (either 0.7% (w/v) TB agarose or 0.7% (w/v)

water agarose). The same type of plates were also streaked with the putative positive plaque using a sterile toothpick and incubated at 37°C, overnight. A plaque lift of the stab plate plaques was probed a second time with the same DNA probes. The plaques of interest were then prepared as phage stocks by placing the agar plug containing the plaque into 1 ml of lambda-dilution buffer with a drop of chloroform and storing them at 4°C.

III. 3 Results and Discussion: Peptide synthetase genes of *P. polymyxa* PKB1

The method outlined by Turgay and Marahiel (1994) for identification of PS genes from the genome of an organism was adapted for use with *P. polymyxa* PKB1. This study was undertaken to determine the optimal experimental methods and conditions to find PS genes from this environmental isolate as well as to attempt to obtain a portion of the PS gene(s) encoding the PS enzymes responsible for production of the antifungal material.

There are strains of *P. polymyxa* known to produce different peptide antibiotics made by PS enzymes (Section I.1.4). Some of these are known to produce two or three structurally unrelated peptide antibiotics such as *P. polymyxa* subsp. *colistinus koyama* that produces jolipeptins, colistins and gatavalin; where probably three different PS enzyme complexes and subsequently three different PS genetic clusters would be in the bacterial genome. It is possible that *P. polymyxa* PKB1 also produces more than one type of peptide antibiotic, with the result that there would be more than one PS genetic operon within the genome of this organism. According to a MALDI-TOF MS spectral anaylsis of the whole *P. polymyxa* PKB1 bacterium (Section II.3.3.6), there are apparently three other secondary metabolites in addition to the components of the antifungal material. This suggests that there may be at least three other PS genomic operons in addition to the putative PS operon encoding the PS enzymes responsible for the production of the antifungal material.

PS gene fragments were amplified from the *P. polymyxa* PKB1 genomic DNA to be used as probes in the screening of a LambdaGEM®-11 *P. polymyxa* PKB1 genomic library for recombinant bacteriophage carrying PS genes. Due to the possibility of multiple PS operons within the *P. polymyxa* genome, the recombinant phage library was screened with three of the PS-PCR products as probes in an effort to isolate recombinant phage that may contain PS genes from different putative PS operons.

The bacteriophage library was made by T. Palmer-Stone and R. Mosher (Department of Biological Sciences, University of Alberta). Two versions of the library were tested; the original non-amplified and the subsequent amplified. The library contained fragments of *P. polymyxa* genome partially digested with *Sau* 3AI with an insert size range of 9 to 23 kb. A unique restriction enzyme site, *Sac* I, flanking the insert can be used to excise the insert DNA from the recombinant phage.

III.3.1 Amplification of fragments of PS gene(s) from *P. polymyxa* PKB1

III.3.1.1 Primer design to amplify conserved regions of PS gene(s)

Oligonucleotides were designed and synthesized for use as primers for amplification of regions of the amino acid adenylation domains and 4'-phosphopantetheine (pan) motifs of PS gene(s) by the polymerase chain reaction (PCR). The conserved amino acid sequences of cores one, two, three, four and six (Figure A.7.A and Table A.7.B) were used as the basis of the nucleotide sequences for the design of a series of forward and reverse primers as listed in Table III.2.14.1.

The conserved core amino acid sequences of PS modules from a large range of organisms; both bacteria and fungi were compared to design the sequences for the cores that were used to synthesize oligonucleotides for PCR. The PS core sequences are not completely conserved among species and so the nucleotide primers designed to anneal and amplify PS gene fragments from *P. polymyxa* PKB1 had to be flexible to accommodate possible alterations in the core sequences for this organism. The primers were designed to be degenerate to incorporated flexibility of annealing to the template genomic DNA. Three restriction enzyme digestion sites were also included in some of the primer nucleotide sequences so that the amplified PCR products could be manipulated more easily.

Primers based on cores four and six (FDP4 and RDP6) were used to amplify an approximately 400 bp fragment from the PS genes of *P. polymyxa* PKB1 (Figure III.3.1.1). Cores four and six were chosen as the basis of the amplification because they were the best candidates to reduce amplification of non-PS gene fragments. Core four is a lengthy conserved sequence which allowed the core four primer to be more stringent in annealing to the *P. polymyxa* PKB1 genomic template. The stringency of core four was important in the reduction of amplification of non-PS segments because amino acid adenylation domains are common to other enzymes besides PS, such as fatty acid synthetases. Core six is the conserved amino acid sequence of the pan carrier motif. This core sequence was chosen as a primer site because it is not found within fatty acid synthetase enzymes. By using this sequence for one of the amplification primers, the amplification of non-PS genes was reduced further.

Non-degenerate primers, FP3 and RP6, based on the conserved sequences for cores three and six were initially designed and used in an amplification reaction with *P.*

forward degenerate PCR primer based on core four of PS amino acid adenylation domains: FDP4

```
aa                                  I   R   G   H   R   I   E   L
nt  5' TGAGAGCTCTAGAATTCGIGGICATCGIATTGAACT 3'
       Sac 1 Xba 1 Eco R1            C       C   G
                                             A
```

reverse degenerate PCR primer based on core six of PS 4' phosphopantetheine motif: RDP6

```
aa                                  S   H   G   G   L
nt  5' ATGAGAATTCTAGA GCT C IGAATGICCICCAAG 3'
       Eco R1 Xba 1 Sac 1           G       C
```

Figure III.3.1.1. The forward and reverse PCR amplification primers designed to anneal to cores four and six of PS amino acid activating modules.The amino acids of the conserved core are listed above the primer sequence. Annealing of these primers to DNA representing cores four and six in PS encoding genes would result in amplification of an approximately 400 to 450 bp sized DNA fragment.

polymyxa PKB1 genomic DNA as a template. A PCR product of approximately 800 nucleotides was expected. The PCR products that were obtained were 1 kb, 0.8 kb and 0.5 kb in size, with the 0.8 kb band as the lesser of the three bands. This indicated that use of non-degenerate primers led to amplification of non-specific PCR products that, in this case, were amplified more than the product of interest.

III.3.1.2 Amplification of PS gene(s) fragments by touchdown PCR

The touchdown PCR protocol (Table III.2.14.2) was implemented for use as the temperature control protocol in amplification reactions with degenerate primers. It was designed, based on the protocol outlined by Don *et al.* (1991), to optimize the temperature and timing of each reaction for the improvement of amplification of the DNA of interest from the *P. polymyxa* PKB1 genomic DNA with the degenerate primers. This protocol allowed for a stringent annealing temperature to be used first to decrease amplification products due to spurious annealing of the primers to the genomic DNA. With the use of degenerate primers, which represent a pool of primers with slight modifications in the nucleotide sequence, the touchdown protocol was necessary to use since the melting temperatures of the primers were unknown.

Initial PCR experiments with amplification of the PS gene segment with FDP4 and RDP6 produced many PCR products, including a band of approximately 400 to 450 bp in size which was the expected size of PCR product. The other PCR products ranged from 2 kb to 0.8 kb. The PCR mixture and PCR conditions other than the touchdown annealing temperature protocol were optimized according to the guidelines specified by Innis *et al.* (1990) to reduce non-specific products so that the 400 to 450 bp band was the major PCR product. The concentrations of gDNA and MgCl were changed in the PCR mix to determine the concentrations of reagents that would allow for less high molecular weight nonspecific products (Figure III.3.1.2). A 'hot start' technique was also used to decrease nonspecific band production; the Taq polymerase was added to the reaction mixtures after they were incubated at 94°C for I min. The final optimized concentrations of the components in the PCR mix are listed in Section III.2.14.2.

III.3.2 Sequencing of PS-PCR products from *P. polymyxa* PKB1

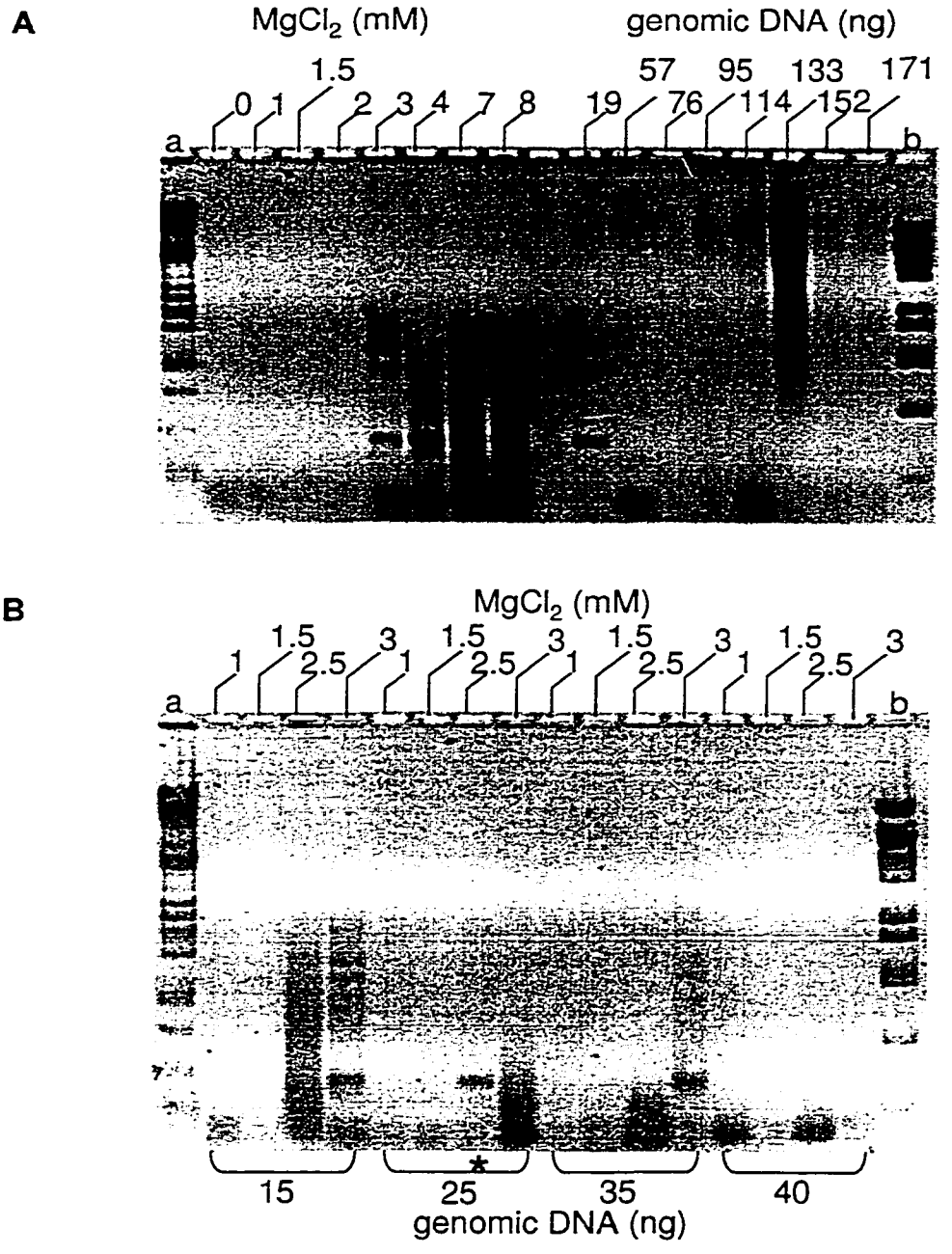


Figure III.3.1.2. Optimization of the expected 400 to 450 bp PCR product amplified from the *P. polymyxa* PKB1 genome with primers FDP4 and RDP6, based on cores four and six, respectively. The concentrations of $MgCl_2$ and genomic DNA were varied to obtain the combination that would allow for the least nonspecific product formation. The combination of $MgCl_2$ and genomic DNA concentrations used routinely to obtain the 400-450 bp products were 25 ng of genomic DNA in a 50 µl total reaction volume and 2.5 mM $MgCl_2$ as seen in the starred lane of gel B. Lane a: *Pst* 1 digested lambda marker, lane b: *Bst* EII digested lambda marker. The rest of the components of the PCR mixtures are listed in Section III.2.14.2.

III.3.2.1 Blunt-end ligation of PS-PCR products into pUC118 for sequencing

The DNA in the band corresponding to an approximately 400 to 450 bp size that was amplified from *P. polymyxa* genomic DNA with primers FDP4 and RDP6 was ligated into the *E. coli* vector pUC118. This was done using the blunt-ended *Sma* I site on the plasmid after the amplified DNA in the 400 to 450 bp PCR product was modified to remove single stranded DNA tails that are added to PCR products during extension. The ligated plasmids were used to transform *E. coli* XL1B by electroporation. The plasmid pUC118 allows for selection of transformant colonies carrying recombinant plasmid based on colour of the colony; blue indicates a plasmid without insert DNA and white indicates a plasmid with insert DNA. There were a total of 78 white colonies after two electroporation reactions of 3 μl of ligation mixture each. The sizes of the DNA inserts in the plasmids of the transformants were analysed by digestion with *Sac* I which would excise the insert. Only 13 of the 78 colonies appeared to contain an insert; one was about 600 bp, three were about 300 bp and nine were approximately 450 bp in size as judged by electrophoresis on a 0.8% agarose gel.

III.3.2.2 Analysis of PS-PCR product sequences

The nine approximately 450 bp sized PCR-product inserts in pUC118 were sequenced using the forward and reverse universal primers for pUC118 as described in Section I.2.5.3.

Analysis of the sequences of the nine PCR-product inserts by the DNA Star program indicated that none were identical nucleotide sequences. Homology between PS encoding genes is not always evident at the DNA level therefore to determine if any of these nine PCR products were PS gene fragments the six frames of each nucleotide sequence were translated into the resulting amino acid sequences. The six possible amino acid sequences for each of the nine PCR products were analysed for the conserved sequence of core five which should be present if the amino acid sequence was generated from a PS gene fragment. Five of the nine PCR-products (labelled as numbers 4, 16, 17, 26, 31) contained the core five sequence NGK. BLASTp was used to search for amino acid sequences in the protein databases that were similar to these five PCR products as described in Section I.2.5.4.1. The five PCR-products were all similar to known PS enzyme amino acid sequences such as gramicidin S synthetase I and II

and a tyrocidine synthetase (Figure III.3.2.2.A). Protein sequences similar to the amino acid sequences of the other four PCR-products that did not contain the core five sequence were also found in a BLASTp search of the protein. All four PCR-product sequences were most similar to cystathionine gamma synthase, an enzyme involved with the biosynthesis of L-methionine (Singleton and Sainsbury, 1993) but not to any PS sequences.

The DNA sequences of the five PCR products that were similar to known PS enzymes (called PS-PCR products 4, 16, 17, 26, and 31) were analysed by the gap analysis program from the Genetics Computer Group (GCG). This program aligns two sequences together, by maximizing the number of matches and minimizing the number of gaps, and determines the percent similarity and identity between the matched sequences. The five PS-PCR products ranged from 46% to 94% identical in DNA sequence paired alignment (Table III.3.2.2). The five PS-PCR product DNA sequences were also aligned together using the pileup program from the Genetics Computer Group (GCG). This program can align all the sequences using the same parameters as the gap analysis program. The alignment showed that PS-PCR product 4 contained a 6 nt section of DNA that was not present in the other PS-PCR products (Figure III.3.2.2.B). This was reflected in the paired matches of PS-PCR product 4 to the other PS-PCR products that were the lowest percent identies of all the paired matches.

III.3.3 The first screening of LambdaGEM®-11 *P. polymyxa* genomic library with the PS-PCR products

The recombinant λ *P. polymyxa* PKB1 genomic library was screened for phage carrying PS genes using the PS-PCR products as probes in a Southern hybridization experiment. Phage aliquots, that represented both the non-amplified and the amplified *P. polymyxa* PKB1 genomic library were used to infect the *E. coli* phage host strain and allowed to form plaques in a lawn of the host strain on agar plates. Multiple plaque lifts onto nylon sheets were taken to be screened in a Southern experiment. The PS-PCR products, 4, 16, 17 and 26 were used as radiolabelled probes of these plaque lifts that represented the first *P. polymyxa* PKB1 genomic library aliquot tested. Due to PS-PCR product 31 having such a high identity percentage with PS-PCR 16, it was not used as a probe. Table III.3.3 shows that the PS-PCR product 17 was able to hybridize with the most phage in the second round of hybridization, followed by PS-PCR products 16, 4

Sequences producing High-scoring Segment Pairs:		Reading Frame	High Score	Smallest Sum Probability P(N)
PS-PCR product 4				
gi|2623773	(AF004835) tyrocidine synthetase ...	-3	228	9.8e-28
gi|39369	(X15577) pid:g39369 [Bacillus bre...	-3	246	4.8e-27
sp|P14687|GRSA_BACBR	GRAMICIDIN S SYNTHETASE I (GRAMIC...	-3	246	4.8e-27
PS-PCR product 16				
gi|2623773	(AF004835) tyrocidine synthetase ...	+2	358	1.2e-40
pir||JX0340	gramicidin S synthase 2 - Bacillu...	+2	355	3.2e-40
gi|2623772	(AF004835) tyrocidine synthetase ...	+2	352	8.2e-40
PS-PCR product 17				
pir||JX0340	gramicidin S synthase 2 - Bacillu...	-2	383	1.0e-43
gi|2623773	(AF004835) tyrocidine synthetase ...	-2	354	9.2e-40
gi|2623772	(AF004835) tyrocidine synthetase ...	-2	341	5.5e-38
PS-PCR product 26				
gi|2623772	(AF004835) tyrocidine synthetase ...	-3	360	3.6e-41
gi|2623773	(AF004835) tyrocidine synthetase ...	-3	318	2.2e-35
pir||JX0340	gramicidin S synthase 2 - Bacillu...	-3	312	1.5e-34
PCR product 28				
gnl|PID|e1183208	(Z99110) similar to cystathionine...	-2	367	1.5e-50
sp|P56069|METB_HELPY	CYSTATHIONINE GAMMA-SYNTHASE (CGS...	-2	334	5.7e-44
gi|1934606	(U93874) cystathionine gamma-lyas...	-2	314	3.4e-42
PS-PCR product 31				
gi|39369	(X15577) pid:g39369 [Bacillus bre...	-3	220	6.8e-29
sp|P14687|GRSA_BACBR	GRAMICIDIN S SYNTHETASE I (GRAMIC...	-3	220	6.8e-29
sp|P14688|GRSB_BACBR	GRAMICIDIN S SYNTHETASE II (GRAMI...	-3	211	1.7e-28
PCR product 57				
gnl|PID|e1183208	(Z99110) similar to cystathionine beta-lyase [.		192	1e-48
sp|P56069|METB_HELPY	CYSTATHIONINE GAMMA-SYNTHASE (CGS) (O-SUCC.		174	3e-43
gi|1934606	(U93874) cystathionine gamma-lyase [Bacillus subtili.		174	3e-43

Figure III.3.2.2.A. The first three BLASTx search results for the PCR products from *P. polymyxa* PKB1 as a template and FDP4 and RDP6 as primers. The nucleotide sequences of the PCR products were translated into the most probable amino acid sequence to use in a search for similar amino acid sequences from the protein databases. The sequences determined as PS-PCR products were 4, 16, 17, 26, and 31.

Table III.3.2.2. The percent identity[a] of the PS-PCR products from *P. polymyxa* PKB1.

PS-PCR product No.	length of PS-PCR product (bp)	4	16	17	26
4	404				
16	382	47			
17	406	48	59		
26	337	51	66	54	
31	359	46	94	56	63

[a] the analysis was done using gap analysis from GCG (Madison, WI) that aligns two sequences to maximize the number of matches and minimize the number of gaps.

```
        1                                                          50
16rcv   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~GCTGGG TGAGGTCGAG GCACAAATTT
31rcv   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~TTTT
26rcv   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~GGG CGAAGTTGAG GCGGCTATGC
17rcv   GGTACCCCGG GGGCATCGGA TCGAACTGGG CGAGATCGAA GCTCAGCTTC
 4rcv   ~~~~~~~~GG GGGCATCGGA TCGAGGCGGG TGAAATTGAA GGAGCTTTAT

        51                                                        100
16rcv   TGAAAGTGGA GGACGTACAG GAGGTCATCG TACTGGCCCA GGCAGACGAG
31rcv   GAAAGTGGGA GGACGTACGA GAGGTCATCG TACTAGCCCA GGCAGACGAG
26rcv   TGAAGGCAGA TCGCATGCAA GAGGTCATCG TACTGGCACA TGCAAACAGC
17rcv   AGAAAGTGGA GGGAATTCGG AAAGCAACGG TATTCGCGAG GGAAGACGTC
 4rcv   CCTCCTATCC TGACATCCAG CATGCTGTCG TAAACGTTGT GGAAACAGAC

        101                                                       150
16rcv   CAGGGGCAAA ACCAAC.... ..TGGTGGCG TACTATGTCG CTGAACGAGA
31rcv   CAGGAGCAAG ACCAAC.... ..TGGTGGCG TACTATGTCG CTGAACGAGA
26rcv   CAAAACCAAT ACGAAC.... ..TGGTTGCC TACTACGTGG CGGAACGCGA
17rcv   TCCGGCGAGA AGCAGC.... ..TTTGCGCG TATTATGAAG CGGACCGCGA
 4rcv   GATGCGAATA GATATCCCGT TTTGTGTGCC TATTATGTAT CTGATCGTGC

        151                                                       200
16rcv   TGTAAGCGCT GGTGAGCTAC GCAGTTTACT GGGTGAGGAG TTTGCAAACT
31rcv   TGTAAGCGCT GATGAGCTAC GCAGTTTAGT GGGTGAGGAG TTGCCAAAC.
26rcv   AGTGACGGGG AGCGAACTTC GCAAGCAGCT GAGTGAAGAA CTCCCGAATT
17rcv   GCTTCCGGCG GCCGAGCTGA AGAGCGTGCT GTCCCAGGAA CTGCCGGCCT
 4rcv   TATCTCTCCC AAGCTTCTCA AAGATTATTT ACATGCTTGT CTTCCCCATT

        201                                                       250
16rcv   ACATGGTGCC TTCGTATTTC ATCCAGTTGG AGCAGATGCC GCTGACACCC
31rcv   ACATAGTGCC TTCGTATTTC ATCCAGCTGG AGCAGATGCC GCCGACACAC
26rcv   ATATGGTTCC CTCGTACTTT GTTCAGTTGG AGCAAATGCC GCTCACACCC
17rcv   ATATGATTCC GGCGTACCTG ATCCAGTTGG AGCGGCTCCC GCTGACGACG
 4rcv   ATATGGTACC AAGCCACATA CTCCAGCTGG AAATCATCCC GCTTACATCC

        251                                                       300
16rcv   AATGGTAAAA TCGACCGCAA GGCCCTGCCG GCACCAGAGG GCAGCCTGCA
31rcv   AATGGTAAAA TCGACCGCAA GGCCCTGCCG GCACCAGAGG GCAGCCTGCA
26rcv   AACGGCAAAA TCGACCGTAA GGCATTGCCG GCACCGGAGC AGATTTCCCG
17rcv   AACGGCAAGG TCGACCGCCG ATCACTCCCG GCGCCGGAGG CGAGCTTGCA
 4rcv   AATGGCAAGG TGGACAAGCA AAAGCTTCCT GTACCAGACG TGTCCTCTGG

        301                                                       350
16rcv   AAGCGGAGCG GACTATGTAG GGCCGCGCAC AGCGCTGGAG CAGACCATGG
31rcv   AAGCGGAGCG GACTATGTAG GGCCGCGCAC AGCGCTGGAG CAGACCTTGG
26rcv   TTCCACGCAG GAATATAGGG CTCCGCAGAC AGCAGAGGAG CAGGCGCTGG
17rcv   GCCGGGCGAA GAACGTACTC CGCCTCGGAC TCCGCTGGAA GCCAGCTTGG
 4rcv   ATTAACTGAC ATATATGAGG AAGCCCATAA TTCGGTGGAA AGCATTCTCG

        351                                                       400
16rcv   TTGCAGTTTG GCAGTCCGTA TTAGGCGCCA AGAGAGTCGG GATTTTGGAT
31rcv   TTGCAGTTTG GCAGTCCGTA TTAGGCGCCA AGAGAGTCGG GATTTTGAAT
26rcv   CCCATGTGTG GGAGGCTGTG ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
17rcv   CGGGAATTTG GAAAAGCGTG CTCGGACTGG AGCACATCGG GGTTCATGAC
 4rcv   TGCAGGTTTG GGAAGAGATG TTCCATGAGT CTATCATAGG TATCCACGAT

        401       412
16rcv   AATTTCTTTG AT
31rcv   AATTTCTTTG AT
26rcv   ~~~~~~~~~~ ~~
17rcv   AACTTGTTCG AC
 4rcv   AATTTCTTTG CA
```

Figure III.3.2.2.B. Alignment of the nucleotide sequences of the PS-PCR products 4, 16, 17, 26 and 31 using the pileup program from GCG. The underlined sequences are the three codons of the amino acid sequence for core five: NGK.

and 26. The first round of hybridizations were done using identical plaque lifts for each of the Southern hybridization experiments with the radiolabelled PS-PCR product probe. Each of the four probes hybridized to different plaques in the first round of hybridization, however, after separating the recombinant phage from other phage in the second round of hybridizations, they were able to cross-hybridize with the other PS-PCR product probes. The λ DNA from these recombinant phage carrying putative PS genes were purified and the *P. polymyxa* genomic insert was excised from the linear λ DNA using the restriction enzyme *Sac* I. The inserts from the recombinant phage ranged in size from approximately 11 to 14 kb, as judged by electrophoresis on a 0.8% agarose gel.

III.3.3.1 PCR with the positive recombinant phage and primers based on cores one and four of PS enzymes

To confirm that the recombinant λ phage that hybridized with the PS-PCR probes in the secondary hybridization carry PS genes, the DNA from these positive recombinant phage were used in a PCR reaction as template DNA with primers based on the conserved amino acid sequences of cores one and four (FDP1 and RDP4 or RP4; Table III.2.14.1) from the amino acid adenylation domain of PS enzymes. Degenerate primers, FDP1 and RDP4, were designed as well as the non-degenerate primer, RP4. This non-degenerate primer was based on the known DNA sequence of core four from PS-PCR product 16. This primer was made in an attempt to increase the amplification of the expected PCR-product of approximately 950 bp in size. The nine recombinant phage that originally hybridized to PS-PCR product 17 all showed the expected ~ 950 bp PCR product, however, the recombinant phage that originally hybridized to either PS-PCR products 4, 16 or 26 did not show the expected PCR product after amplification with either the degenerate primers for cores one and four or the non-degenerate primer core four coupled with FDP1 (Table III.3.3). The PCR reaction using these primers produced many other products ranging in size from 2.5 kb to 500 bp and the expected 950 bp product was not the major product in the PCR.

III.3.3.2 PCR with the positive recombinant phage and primers based on cores two and four of PS enzymes

Table III.3.3. The numbers of λ phage with *P. polymyxa* PKB1 genomic inserts that hybridized to the PS-PCR probes and produced PCR products from amino acid adenylation core sequence primers in the first screening of the genomic library.

probe	first round of hybrid.[a]		second round of hybrid.[b]				
	non-amp.	amp.	total positive phage	PCR[c] ~950 bp	PCR[d] ~700 bp	*E. coli* clone[e] ~700 bp	hybrid. of digested DNA[f]
4	2	4	2	all -	all +	0	----
16	1	6	3	all -	2 +	0	----
17	1	18	13	8[g] +	8[h] +	3	rp19, yes
26	0	1	1	-	-	-	-

[a] first round of hybridization; initial probing of the non-amplifed and amplified recombinant λ phage libraries.
[b] second round of hybridization; re-probing of phage from the first round of hybridization.
[c] the recombinant λ DNA from hybridizing phage was used as template in PCR with primers FDP1 and RP4. Yes or no indicates if the expected ~950 bp band was produced or not.
[d] the recombinant λ DNA from hybridizing phage was used as template in PCR with primers FDP2 and RP4. Yes or no indicates if the expected ~700 bp band was produced or not.
[e] the number of clones carrying an ~700 bp insert amplified from the recombinant phage (labelled rp7, rp15, rp25).
[f] the recombinant phage that showed digestion products with the restriction enzymes tested are listed and if the digested DNA hybridized with at least one or all, of the PS-PCR product probes 4, 16 and 17, a yes is indicated.
[g] eight of the thirteen phage that hybridized with PS-PCR 17 were tested for production of the ~950 bp PCR product, they all showed the expected band (labelled rp7, 10, 14, 15, 16, 24, 25, 26).
[h] eleven of the thirteen phage that hybridized with PS-PCR 17 were tested for production of the ~700 bp PCR product, eight of these showed the expected band (the eight listed above plus rp31).

It was possible that the recombinant phage that could hybridize with the four PS-PCR products containing DNA encoding cores four to six, but were not able to produce PCR products with primers based on cores one and four, contained a segment of a PS gene that did not contain core one. Cores one and two are separated by approximately 250 bp. Those phage were re-analysed in another PCR reaction using the conserved amino acid sequences of cores two and four as the basis of the nucleotide sequences of the primers (FDP2 and P4; Table III.3.3), and the expected size of the product was approximately 700 bp. Both of the recombinant phage that originally hybridized with PS-PCR product 4 showed an approximately 700 bp sized DNA band using the cores two and four primers. Of the three recombinant phage that hybridized with PS-PCR product 16, two had a ~ 700 bp PCR product. Of the nine recombinant phage that hybridized with PS-PCR product 17, eight had a ~ 700 bp product, and the single recombinant phage that hybridized to PS-PCR product 26 did not produce a ~ 700 bp band. As was seen in the PCR using the recombinant phage as template with the primers based on cores one and four, this PCR with primers based on cores two and four generated PCR products other than the expected 700 bp sized DNA.

The PCR products generated from amplification of the various recombinant phage with the primers FDP2 and RP4, were ligated into pUC118 and transformed into *E. coli* XL1B. Transformants with vectors carrying a 700 bp sized insert were searched for among the total number of transformants. None of the transformants that hybridized with PS-PCR products 4 or 16 had a ~ 700 bp insert, but the inserts from three out of eight recombinant phage that hybridized to PS-PCR product 17 appeared to contain a ~ 700 bp product; these three phage were labelled rp7, rp15 and rp25.

III.3.4 The second screening of LambdaGEM®-11 *P. polymyxa* genomic library with the PS-PCR products

Due to the low numbers of recombinant phage that hybridized with PS-PCR products 4 and 16 in the first screening of the library, a second screening of the library was performed, using these two PS-PCR products as probes. The PS-PCR product 26 was not used because the DNA appeared to have re-arranged in pUC118 so that it did not give a reproducible restricition enzyme digestion pattern. Both the non-amplified and amplified libraries were screened again, and the numbers of hybridized plaques from this second screening are listed in Table III.3.4. The second round of hybridization of the

Table III.3.4. The numbers of λ phage with *P. polymyxa* PKB1 genomic inserts that hybridized to the PS-PCR probes and produced PCR products from amino acid adenylation core sequence primers in the second screening of the library

probe	first round of hybridization[a]		second round of hybrid.[b]	third round of hybrid[c]	
	nonamplified	amplified	total pos. phage	total pos. phage	hybridization of digested DNA[d]
4	13	2	5	1	rp44, yes
16	16	10	4	2	rp67, rp79 yes

[a] first round of hybridization; initial probing of the non-amplifed and amplified recombinant λ phage libraries.
[b] second round of hybridization; re-probing of phage from the first round of hybridization.
[c] third round of hybridization; re-probing of the positive phage from the second round of hybridization as a determinant of pure phage stock.
[d] the recombinant phage that showed digestion products with the restriction enzymes tested are listed and if the digested DNA hybridized with at least one or all, of the PS-PCR product probes 4, 16 and 17, a yes is indicated.

recombinant phage that hybridized with either PS-PCR product 4 or 16 in the first round in this screening, showed five recombinant phage that hybridized to PS-PCR product 4 and four recombinant phage that hybridized to PS-PCR product 16. These recombinant phage were hybridized a third time (third round) with the respective PS-PCR probes to ensure that the phage preparation was not a mixture of phage. Each of the recombinant phage stocks were used to re-infect the *E. coli* host strain and spread onto agar plates. Plaque lifts were taken from the agar plates and hybridized to the PS-PCR probes. The Southerns indicated that one recombinant phage (rp44) out of the five that originally hybridized to PS-PCR product 4 was pure since approximately 95% of the plaques generated from this one phage stock re-hybridized to PS-PCR product 4 and two recombinant phage (rp67 and rp79) out of the four that originally hybridized to PS-PCR product 16 were pure since approximately 95% of the plaques generated from these two phage stocks re-hybridized to PS-PCR 16. The other four recombinant phage that had originally hybridized to PS-PCR product 4 and the other two recombinant phage that had originally hybridized to PS-PCR product 16 showed approximately 50 to 75% of the plaques not hybridizing to the respective probes indicating that the phage stocks were still a mixture of different recombinant phage.

III.3.4.1 Analysis of the positive recombinant phage by restriction enzyme digestion and Southern hybridization with the PS-PCR products

The pure recombinant phage that hybridized to PS-PCR product 4 and PS-PCR 16 from the second phage library aliquot (rp44, rp67 and rp79), and a pure recombinant phage that hybridized to PS-PCR product 17 (rp19) were analysed further for PS genes. The recombinant DNA from each of these phage were purified and digested with a series of restriction enzymes. The digests were electrophoresed on two agarose gels and transferred to two nylon membranes for Southern analysis. The two membranes were each hybridized with PS-PCR products 4, 16 and 17, respectively. The two membranes were completely stripped of the previous radiolabel before the next hybridization experiment.

The recombinant DNA from these phage were digested with *Bgl* II, *Bsa* BI, *Bsa* I, *Bst* EII and *Sac* I. The DNA bands seen in the agarose gels from the recombinant λ DNA digestions of all, except digestion of rp19 with *Sac* I, were blurry and difficult to resolve. The λ DNA preparation from the phage involves the addition of a large amount of

polyethylene glycol which, if not fully removed from the DNA preparation, can interfere with the migration of the DNA in the agarose gel (Roy, 1999).

The rp44 DNA was digested, at least in part, with *Bgl* II, but did not appear to be digested with the other restriction enzymes. This may be due to an inhibitory agent in the recombinant λ DNA preparation or it may be that the insert DNA did not contain sites for the other restriction enzymes. Lack of digestion of the insert DNA by *Sac* I was observed for many of the recombinant phage initially tested, even though the enzyme was able to excise the insert from the λ DNA. The rp67 appeared to have been digested by *Bsa* I, *Bsa* BI and *Sac* I; rp79 appeared to have been digested with *Bsa* BI, *Bst* EII and *Sac* I, and rp 19 appeared to have been digested with *Bsa* I, *Bst* EII and *Sac* I. The rp67 and 79 did not show clear digestion patterns but it was discernable that the rp19 digestion pattern was different from both rp67 or rp79 suggesting that this recombinant phage insert was different from the other two phage.

Cross-hybridization of each of these recombinant phage digests with the radiolabelled probes; PS-PCR products 4, 16 and 17, showed that all of the recombinant phage contained DNA that was able to hybridize to PS genetic fragments (Figure III.3.4.1). However, it also showed that the recombinant phage did not carry the same PS genetic information because, although cross-hybridization took place, the level of hybridization differed.

Film exposed for 24 h to the Southern membrane probed with PS-PCR product 4 showed that the probe, which originally had identified rp44 from the library, had hybridized with rp44 and with the PS-PCR product 16, but not with rp67 or 79 (originally identified from the library with PS-PCR product 16), or with PS-PCR product 17 or rp19 (originally identified from the library with PS-PCR product 17).

Film exposed for 24 h to the Southern membrane probed with PS-PCR product 16 showed that the probe had hybridized with rp67 and 79 and with PS-PCR product 4 but not with rp44. The probe was also able to hybridize with PS-PCR product 17 and rp19.

Film exposed for 24 h to the Southern membrane probed with PS-PCR product 17 showed that the probe had hybridized with rp19 and with PS-PCR product 16, rp67 and 79, but with less intensity. The probe was able to hybridize with PS-PCR product 4 slightly, but not with rp44.

The hybridization patterns of the three PS-PCR products with the recombinant phage indicated that PS-PCR product 4 is not represented in the *P. polymyxa* genomic

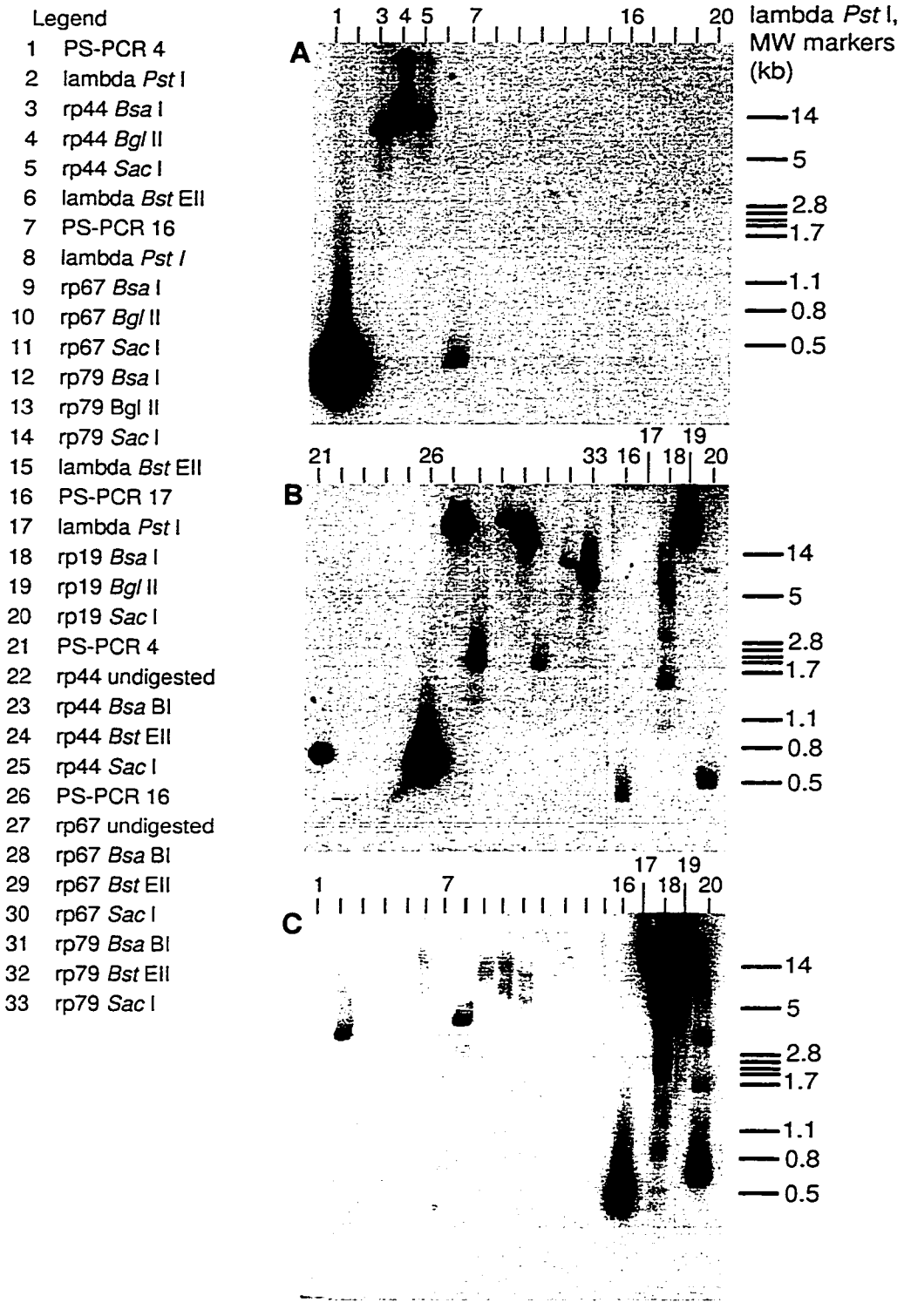


Figure III.3.4.1. Southern hybridization of the *Bsa* I, *Bsa* BI, *Bgl* II, *Bst* EII and *Sac* I digests of rp44, rp67, rp79 and rp19 with a radiolabelled probe of either A. PS-PCR product 4, B. PS-PCR product 16 or C. PS-PCR product 17.

inserts of the phage (rp67, rp79 or rp19) that were originally identified from the library with PS-PCR products 16 and 17. However when PS-PCR product 16 was used as the radiolabelled probe of the phage digests, it was able to hybridize with digested bands from rp67, as expected, and with some of the digestion bands from rp19. In particular the radiolabelled PS-PCR product 16 hybridized strongly with a rp19 *Sac* I fragment of approximately 450 to 500 bp. In contrast, the rp67 DNA was only weakly hybridized with PS-PCR product 17 when it was used as a radiolabelled probe. It appears that *the P. polymyxa* genomic insert in rp67 may contain a sequence that is also part of the *P. polymyxa* PKB1 insert in rp19, and so these two phage may be carrying two sections of one PS encoding gene cluster. This is discussed further in Section III.3.6.

III.3.5 Analysis of the insert DNA from recombinant phage 19

The recombinant phage 19 was chosen for further analysis because it was identified from the *P. polymyxa* PKB1 genomic library with PS-PCR product 17, which had the highest BLASTp scores with PS enzymes (Figure III.3.2.2.A) and because the preparation of the recombinant DNA from rp19 allowed for the cleanest looking digestion pattern.

Partial sequence information of the *P. polymyxa* PKB1 genomic insert from rp19 was obtained in order to construct a partial map of the insert and to confirm that the insert contained portions of a PS enzyme encoding gene. If the nucleotide sequence was shown to be part of a PS gene then it would demonstrate the usefulness of PCR derived PS gene fragments for the identification of PS genetic information from a genomic library. If the sequenced portions of the insert contained PS genetic information then it might also be possible to determine if it is part of the PS genes responsible for the production of the antifungal material.

III.3.5.1 Subcloning of the DNA fragments from the recombinant phage 19 after digestion with *Sac* I and *Bsa* I

The *P. polymyxa* PKB1 genomic insert from rp19 was digested separately with *Sac* I and *Bsa* I restriction enzymes, and resulting DNA fragments were subcloned into pUC118. The only *Sac* I sites in the λ DNA were the two flanking the insert that were designed into the LambdaGEM®-11 vector as a method of excision of the insert. There

were two *Bsa* I sites in the λ DNA; one at 11 kb and the other at 42.7 kb. The LambdaGEM®-11 vector is truncated at the 3' end so that the λ DNA is 43 kb in length instead of the 48 kb of the unaltered λ DNA. Knowing this, it can be predicted that digestion of rp19 by *Bsa* I would produce two λ DNA fragments of 11 kb and 0.3 kb, and the rest of the DNA fragments would potentially contain either λ DNA and *P. polymyxa* genomic insert or insert DNA only.

The *Sac* I enzyme digested the rp19 insert into DNA fragments coresponding to seven DNA bands of approximate sizes 3.5, 2.2, 1.7, 1.5, 1.2, 0.7 and 0.5 kb. for a total of approximately 11.3 kb of DNA (Figure III.3.5.1). These fragments all appeared to hybridize to PS-PCR product 17 in Southern analyses, although some hybridized strongly and other weakly, which will be discussed further in Section III.3.6.2. The *Bsa* I digests of the rp19 insert were not as well resolved as the *Sac* I digests, however, the enzyme appeared to digest the recombinant λ DNA into about six DNA fragments ranging in size from approximately 11 kb to 0.6 kb. The sizes of the *Bsa* I digested bands that hybridized to PS-PCR product 17 in the Southern were approximately 2.2, 1.4, 0.9, 0.8, 0.6, and 0.5 kb.

The digestion products from each restriction enzyme were ligated into either *Sac* I or *Sma* I linearized pUC118. Each of the *Sac* I digested DNA bands that had hybridized to the radiolabelled PS-PCR product 17 probe was gel purified and ligated into pUC118 separately. Due to the less resolved *Bsa* I digestion of rp19, the total *Bsa* I digestion mixture was used in the ligation reaction into pUC118 with the intent that at least a few transformants would contain plasmids bearing *Bsa* I digested DNA fragments with PS genetic information. The recombinant plasmids were used to transform *E. coli* DH5α. The inserts from plasmids purifed from the *E. coli* transformants were digested with either *Sac* I or with a combination of *Xba* I and *Eco* RI to excise the insert DNA from the plasmid, depending on if it was a *Sac* I or a *Bsa* I fragment, respectively. The digested plasmids were electrophoresed on an agarose gel to determine approximate sizes of the inserts and to transfer the DNA from the gels onto nylon membrane for Southern analysis. The rp19 DNA fragments that were excised from the plasmids were re-screened for the presence of PS gene fragments by a Southern with PS-PCR product 17 as the radiolabelled probe. The inserts that hybridized with the probe were considered as candidates for sequencing.

The Southern hybridizations indicated that all the plasmids with *Sac* I fragment inserts hybridized with the PS-PCR product 17, although some of these hybridized

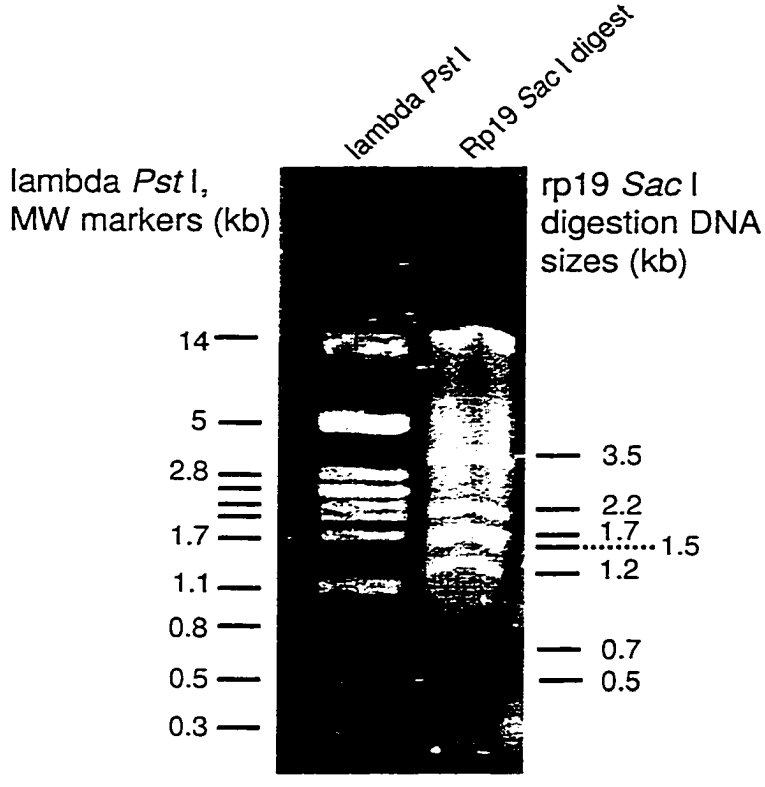


Figure III.3.5.1. The *Sac* I digest of the *P. polymyxa* PKB1 genomic insert of rp19

weakly, and 5 of the 15 plasmids with *Bsa* I fragment inserts hybridized with the PS-PCR product 17 probe. The numbers of *Sac* I digested plasmid inserts were; four of 2 kb, one of 1.6 kb, one of 1.2 kb, seven of 700 to 650 bp and one of 500 bp. The only rp19 *Sac* I fragment that appeared to hybridize to the PS-PCR product 17 that was not represented by these clones was the 3.5 kb fragment. The *Bsa* I fragment inserts that were successfully sucloned included; three of approximately 2 kb, and two of 500 bp. Three of the *Bsa* I digested bands that hybridized with PS-PCR product 17 were not represented in the subclones, they were 0.6, 0.8, 0.9 and 1.3 kb in size.

III.3.5.2 Sequencing of subcloned recombinant phage 19 DNA fragments

The *Sac* I and *Bsa* I fragment subclones that hybridized to PS-PCR product 17 were sequenced in pUC118 using the reverse and forward universal primers. For the *Sac* I fragment inserts; two of the plasmids, pSac2 and pSac8A, of the four with 2 kb sized inserts, were sequenced and determined to be the same fragment of DNA. The plasmids pSac32 and pSac6A contained a 1.6 and a 1.2 kb insert, respectively, these were both sequenced. Of the seven plasmids with inserts of approximately 600 to 700 bp, four of them hybridized strongly with the radiolabelled probe and three of them weakly, therefore one plasmid from each group was selected for sequencing. The insert in pSac21 was chosen to represent the group of 600 to 700 bp sized *Sac* I fragments that hybridized strongly and the insert from pSac14 was chosen to represent the group of 600 to 700 bp sized *Sac* I fragments that hybridized weakly. For the *Bsa* I fragment inserts; one of the three plasmids, pBsa49, with a 2 kb insert was sequenced, the other two plasmids, pBsa52 and pBsa65 both with a 500 bp insert were also sequenced. These two plasmids were determined to contain the same insert. The plasmids are listed in Table III.2.2.

III.3.6 Partial map of the rp19 *P. polymyxa* PKB1 genomic insert

The highly conserved nature of PS enzymes can aid in the mapping of PS genetic fragments, especially when translated into the amino acid sequence of the PS enzyme. The amino acid sequence and location of conserved sequences in the adjacent valine and ornithine modules of the PS enzyme TY3 that is part of the multienzyme complex that produces tyrocidine (Figure III.3.6.2.B) are typical for PS enzymes. Not

only are the core regions conserved among PS enzymes, but distances between the cores are also conserved.

The amino acid adenylation domain of amino acid activating modules, has a section located between cores two and three of approximately 450 bp that appears to be responsible for recognition of the amino acid that the module activates. This, in turn, can provide information regarding the amino acid sequence of the peptide that the PS enzyme is responsible for producing.

III.3.6.1 DNA Strider and BLAST search analysis of the sequenced rp19 DNA fragments

Open reading frame analysis on the six possible nucleotide sequences from the six different frames was performed to determine which frame would most likely encode the amino acid sequence of the PS enzyme. The presence of multiple stop codons within the nucleotide sequence of a particular frame would suggest that that frame was not the encoding frame.

Proteins with similarity to the amino acid sequences translated from the nucleotide sequence for each DNA fragment, were searched for using BLASTp. All of the sequenced fragments were most similar to PS enzymes, but especially to an unnamed PS from *B. subtilis*, tyrocidine, bacitracin and gramicidin S synthetases (Figure III.3.6.1). In particular, the sequenced portions of fragments that contained the amino acid recognition site, between cores two and three, were most similar to particular amino acid activating modules of these PS enzymes, suggesting the identity of the amino acid that was activated by the module of the PS in the present study.

III.3.6.2 Positioning of the sequenced *Sac* I and *Bsa* I fragments of the rp19 insert in relation to a PS enzyme model

Each of the fragments was analysed for the core conserved sequences. These conserved sequences provided information about the location of the fragment within the rp19 insert in relation to the core sequences of known PS enzymes. For the fragments of 500 to 650 bp that were sequenced, the sequence information was double-stranded. For the larger fragments the two ends of the fragment were sequenced, single-stranded only.

The position of fragments, relative to others, were placed according to these factors: the location of the core sequences of the sequenced portions of the digestion

Sequences producing High-scoring Segment Pairs:	High Score	Smallest Sum Probability P(N)
pSac14 with cores M-N-O-P-Q		
gi|3142696 (AF064551) peptide synthetase [Bacillus subtilis]	268	3e-71
gb|AAC06346| (AF007865) bacitracin synthetase 1; BacA [Baci...	253	8e-67
gi|2623773 (AF004835) tyrocidine synthetase 3 [Brevibacillu...	244	4e-64
pSac20 with cores 5-6		
gi|2623773 (AF004835) tyrocidine synthetase 3 [Brevibacillu...	115	3e-25
emb|CAA74213| (Y13917) peptide synthetase [Bacillus subtilis]	111	3e-24
emb|CAA84364| (Z34883) peptide synthetase ORF5 [Bacillus su...	111	3e-24
pSac21 with cores 6-K-L		
pir||JX0340 gramicidin S synthase 2 - Bacillus brevis >gi|5...	124	2e-28
sp|P14688|GRSB_BACBR GRAMICIDIN S SYNTHETASE II (GRAMICIDIN...	124	2e-28
gi|2623773 (AF004835) tyrocidine synthetase 3 [Brevibacillu...	124	3e-28
pSac32 forward with cores 3-4		
gi|3142696 (AF064551) peptide synthetase [Bacillus subtilis]	152	1e-36
gb|AAC06347| (AF007865) bacitracin synthetase 2; BacB [Baci...	150	6e-36
gi|2623773 (AF004835) tyrocidine synthetase 3 [Brevibacillu...	148	1e-35
pSac8A forward with cores M-N-O-P-Q		
gi|3142696 (AF064551) peptide synthetase [Bacillus subtilis]	225	3e-58
gi|2522214 (AF023465) fengycin synthetase FenE [Bacillus su...	217	8e-56
gi|2522212 (AF023464) fengycin synthetase FenA [Bacillus su...	217	8e-56
pSac8A reverse with cores D-3-4		
gi|2623773 (AF004835) tyrocidine synthetase 3 [Brevibacillu...	200	9e-51
gb|AAC06347| (AF007865) bacitracin synthetase 2; BacB [Baci...	198	3e-50
pir||JX0340 gramicidin S synthase 2 - Bacillus brevis >gi|5...	198	3e-50
pSac32 reverse with cores A-1		
gi|2623773 (AF004835) tyrocidine synthetase 3 [Brevibacillu...	125	2e-28
gb|AAC06346| (AF007865) bacitracin synthetase 1; BacA [Baci...	120	4e-27
gb|AAC06348| (AF007865) bacitracin synthetase 3; BacC [Baci...	120	4e-27
pBsa49 forward with core N		
gi|2522212 (AF023464) fengycin synthetase FenA [Bacillus su...	53	1e-06
gi|2522214 (AF023465) fengycin synthetase FenE [Bacillus su...	52	3e-06
gi|3643187 (AF087452) peptide synthetase [Bacillus subtilis]	51	4e-06
pBsa49 reverse with cores D-E-3		
pir||JX0340 gramicidin S synthase 2 - Bacillus brevis >gi|5...	118	2e-26
gi|2623773 (AF004835) tyrocidine synthetase 3 [Brevibacillu...	116	6e-26
gb|AAC06347| (AF007865) bacitracin synthetase 2; BacB [Baci...	112	2e-24
pSac6A with cores R-A-1-2		
pir||JX0340 gramicidin S synthase 2 - Bacillus brevis >gi|5...	346	1e-94
sp|P14688|GRSB_BACBR GRAMICIDIN S SYNTHETASE II (GRAMICIDIN...	337	9e-92
gi|2623773 (AF004835) tyrocidine synthetase 3 [Brevibacillu...	331	7e-90
pBsa65 with cores 4-5-6		
pir||JX0340 gramicidin S synthase 2 - Bacillus brevis >gi|5...	346	1e-94
sp|P14688|GRSB_BACBR GRAMICIDIN S SYNTHETASE II (GRAMICIDIN...	337	9e-92
gi|2623773 (AF004835) tyrocidine synthetase 3 [Brevibacillu...	331	7e-90

Figure III.3.6.1 BLASTp search results using the amino acid sequence translated from the nucleotide sequences of the *Sac* I and *Bsa* I digestion fragments from rp19. The first three of the most similar proteins to the digestion fragments are listed.

fragments, the apparent size of the digestion fragments measured by electrophoresis compared to the size suggested from alignment with the PS model and the similarity or lack of similarity of sequenced fragments carrying the same core sequences. Three other factors were also considered; *Sac* I was used to excise the insert from rp19 therefore *Sac* I fragments had to define the boundaries of the insert; there had to be a *Sac* I fragment of approximately 3.5 kb containing at least a portion of cores 4 to 6 to match the medium to strongly hybridized 3.5 kb *Sac* I fragment that was not sequenced; and the approximate size of the rp19 insert that was calculated from the *Sac* I digested fragments had to correlate with the possible number of modules in the insert according to the other factors.

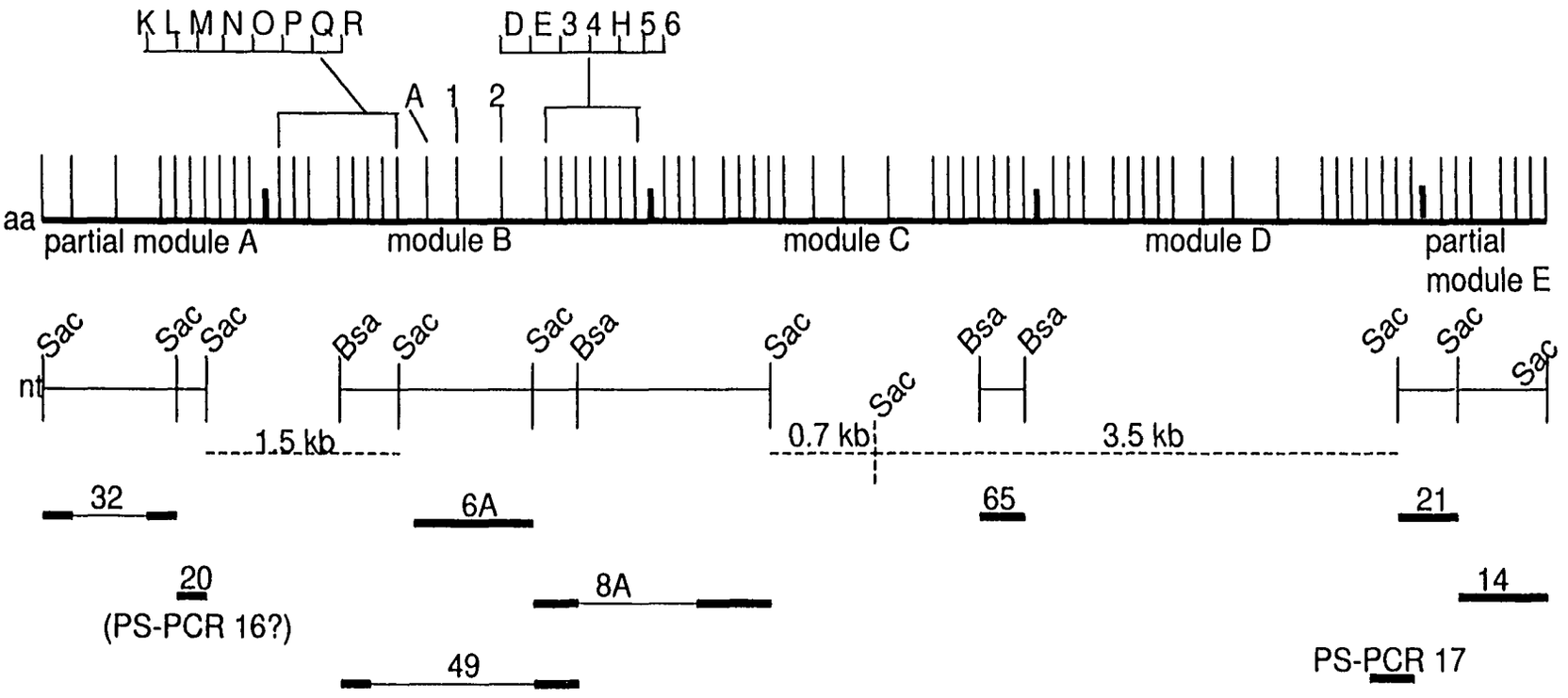
The insert size, from the sum of the *Sac* I fragments, was approximately 11 kb. One PS module is approximately 2.88 kb. The proposed order of the fragments in relation to a PS enzyme model would support the number of modules proposed to be within this insert according to the apparent insert size (Table III.3.6.2; Figure III.3.6.2.A).

The inserts from pSac8A and pBsa49 carry a 200 bp overlapping sequence that includes cores D-three. In many of the PS enzymes studied to date, there is another core sequence in between these two; core E, however both of the pSac8A and pBsa49 inserts have that section of the sequence omitted, as can be seen when the fragments are aligned with the sequence of the module that they are most similar with from the BLASTp search. The DNA sequence that the amino acid sequence was derived from does not appear to be interrupted or difficult to interpret at this section in either fragment. The insert from pSac8A also contains the cores M-N-O-P-Q, on a section of DNA of approximately 500 bp. When these two sequenced ends of 8A are aligned with the PS enzyme model, the suggested size of the fragment is 2 kb which is in agreement with the size of the fragment according to electrophoresis on an agarose gel of 2 kb. The insert from pBsa49 is approximately 2 kb by gel electrophoresis. It contains DNA that includes core N at one end of the fragment and cores D-three at the other end, as previously mentioned. The sequence of the end of the fragment that includes core N is not similar to the core sequence of and around the core N of the pSac8A insert. This suggests that the cores M-N-O-P-Q within pSac8A are a part of the module after module B. In this way, the insert from pSac8A bridges the modules B and C. Since the insert from pBsa49 is linked to pSac8A in the first of the two modules, there should be a *Sac* I fragment that would contain the DNA encoding part of a module prior to the pBsa49 fragment, that may or may not have been sequenced, or the module with the pBsa49 fragment could

Table III.3.6.2. The arrangements[a] of the subcloned *Sac* I and *Bsa* I fragments from rp19 in comparison to known PS enzymes.

plasmid insert	cores present (5' to 3')	identical with	adjacent to	similar PS module[b]	PS model (kb)	agarose gel (kb)	hybrid. intensity
partial module A							
pSac32	A-1----3-4	----	pSac20	~ valine	1.5	1.7	weak
pSac20	5-6	----	pSac32	----	0.46	0.5	weak
module B							
pBsa49	N-----D-3	D-3 of pSac8A	---	ornithine	2.1	2	medium
pSac6A	R-A-1-2		pSac8A	ornithine	1.14	1.2	medium to weak
part of pSac8A[d]	D-3-4--	D-3 of pBsa49	pSac6A	ornithine	2 (total)	2.2 (total)	medium
module C							
part of pSac8A[d]	--M-N-O-P-Q	----	----	----	----	----	----
pBsa65[c]	4-5-6	----	----	----	0.42	0.45	weak
module D							
part of pSac21	-6-	PS-PCR product 17	----	---	0.66 (total)	0.65-0.7 (total)	strong
partial module E							
part of pSac21	-K-L	----	pSac14	---	----	----	----
pSac14	M-N-O-P-Q	----	pSac21	---	0.55	0.6-0.7	weak

[a] the order of the modules is based on the digestion pattern of rp19 and the lack of homology of some core sequences that are found in different fragments.
[b] if the insert encompasses the amino acid recognition sites, between cores two to three, the amino acid that the module that is most similar to the plasmid insert recognizes, is listed.
[c] same as insert of pBsa52
[d] identical insert with pSac2.



approximate nucleotide distances of PS enzyme model in kb: K to 6 (1 module) =2.88, K to L = 0.16, L to M = 0.21, M to N = 0.33, N to O = 0.06, O to P = 0.045, P to Q = 0.06, Q to R = 0.06, R to A = 0.39, A to 1 = 0.12, 1 to 2 = 0.27, 2 to D = 0.42, D to E = 0.04, E to 3 = 0.2, 3 to 4 = 0.06, 4 to H = 0.2, H to 5 = 0.04, 5 to 6 = 0.18, 6_n to K_{n+1} = 0.22

Figure III.3.6.2.A. The possible alignment of *Sac* I and *Bsa* I digested rp19 fragments compared with a model of the core sequences of PS enzymes. Thick lines indicate sequenced sections of the fragments. The dashed lines in the nucleotide sequence of the rp19 inert are predicted *Sac* I fragments.

not represent the first module in the rp19 insert because a *Sac* I site is expected to border the insert DNA. The insert from pSac6A was completely sequenced and found to include cores R-A-one-two. The BLAST search results from both pSac6A and pSac8A showed high similarity to the ornithine module from tyrocidin synthetase 2, bacitracin synthetase 2, and gramicidin S synthetase 2. Aligning the amino acid sequences of pSac6A and pSac8A to the ornithine module of tyrocidin 2 showed a two amino acid gap between these two inserts (Figure III.3.6.2.B). The codons for these two amino acids correspond to the *Sac* I restriction enzyme site. Since the inserts of pSac6A and pSac8A contain the amino acid recognition sites between cores two and three between them, the module that they are in is proposed to be an ornithine activating module.

The insert from pSac21 was found to include sequence prior to and including cores six-K-L and was placed in the putative modules D and E, with the core six portion of module D and the K-L portion of module E. The portion of the insert containing sequence information prior to core six is identical to the corresponding sequence of PS-PCR product 17. The PS-PCR product 17 contains sequence from cores four to six. A segment of the pSac21 insert was identical to the last 47 amino acids of this 134 amino acid sequence. The insert from pSac21 was proposed to be 700 bp according to the PS model and it appeared to be 700 bp according to gel electrophoresis. This insert had strongly hybridized to the PS-PCR product 17 radiolabelled probe in Southern hybridization, as expected since it contains part of the PS-PCR product 17 sequence. There was no other sequenced *Sac* I fragment containing the other 87 amino acids of the probe sequence, however, there was another *Sac* I fragment that hybridized strongly with the radiolabelled probe, at approximately 3.5 kb. It is possible that this 3.5 kb *Sac* I fragment contains the other portion of the PS-PCR product 17 sequence. If this was the case then the 3.5 kb fragment would comprise parts of two modules, one part would contain the core sequences two to six of the putative module C, the module prior to the module that the core six of pSac21 was placed in (module D) and the other part would contain the putative module D sequence up to the pSac21 fragment insert. Since the *Sac* I digest of the recombinant phage 19 was assumed to be complete, this implies that there is no sequenced *Sac* I fragments associated with those sections of modules C and D already. This meant that part of module C and most of module D could contain a 3.5 kb *Sac* I fragment and so there could be no other *Sac* I fragments within this 3.5 kb section of the module. The insert from pSac14 contains the cores M-N-O-P-Q. The sequence of this insert was not similar to the two other fragments, pSac8A and pBsa49,

A partial valine module of TY3 (cores A to six)

3541 dgatalreis llseeerriv tvdfnntfaa yprdltigel feqgaaktpe haavvmdgqm pSac32R
3601 LTYRELMERA NQlahvlrqn gvgkesivgl ladrslemit gimgilKAGG Aylgldpehp
3661 serlaymled ggvkvvlvqk hllplvgegl mpivleeesl rpedcgnpai vngasdlayv
3721 myTSGSTGKP KGvmvehrnv trlvmhtnyv qvresdrmiq tgaigfdamt feifgallhg
3781 aslylvskdv lldaeklgdf lrtnqittmw ltsplfnqls qdnpamfdsl ralivggeal
3841 spkhinrvks alpdleiwnG YGPTenttfs tcylieqhfe eqipigkpia nstayivdgn
3901 ngpgpigvpG ELCVGGDGva rgyvnkpelt aekfvpnpfa pgetmyrTGD larwlpdgti pSac 32F
3961 eylgridqqv KIRGYRIELg eietvlsqqa qvkeavvavi eeanggkalc ayfvpeqavd
4021 aaelreamsk qlpgymvpay yvqmeklpltaNGKvdrral pqpsgerttg safvaaqndt pSac20
4081 eaklqqiwqe vlgipaigih dnffeIGGHS lkamnvitqv hktfqvelpl kalfatptih

B ornithine module of TY3 (cores K to six)

4141 elaahiaesa feqfetiqpv epaaFYPVSF AQKRMYilhq fegsgisynv psvlvlegkl
4201 dydrfaaaiq SLVHRHESLR TSfhsvngep lqrvhpdvel pvrlleated qsesliqeli
4261 qpfdleiapl frvnliklga erhlffmdmh HIISDGVSLA VIVeeiasly agkqlsdlri
4321 qykdfavwqt klaqsdrfqk qedfwtrtfa geipllnlph dyprpsvqsf dgdtvalgtg
4381 hhlleqlrkl aaetgtTLFM VLLAAYHVLl skyagqeeIV VGTPIAGRsh adveriVGMF
4414 VNTLALKNta agslsfrafl EDVRQNALHA FEHQDYPFeh lveklqvrrd lSRNPLFDTM pSAC6A
4501 fslglaesae qevadlkvsp ypvnghiakf dlsldamekq dgllvqfsyc tklfaketvd
4561 rlaahyvqll qtitadpdie larisvlska etehmlhsfl atktayptdk tfqklfeeqv
4621 ektpneiavl fgneqLTYQE LNAKANQlar vlrrkgvkpe stvgilvdrs lymvigmlav
4681 lKAGGTfvpi dpdyplerqa fmledseakl lltlqkmnsq vafpyetfyl dtetvdqeet
4741 gnlehvaqpe nvayiiyTSG TTGKPKgvvi ehrsyanvaf awkdeyhlds fpvrllqmas
4801 fafdvstgdf aralltgggl vicpngvkmd paslyetirr heitifeatp alimplmhyv
4861 yeneldmsqm kllilgadsc paedfktlla rfgqkmriin sYGVTeacid tsyyeetdvt pSac8AR
4921 airsgtvpig kplpnmtmyv vdahlnlqpv GVVGELCIGG AGVargylnr pelteekfvp
4981 npfapgerly rTGDlakwra dgnveflgrn DHQVKIRGVR IELgeietql rkldgiteav
5041 vvaredrgqe kelcayvvad hkldtaelra nllkelpqam ipayfvtlda lpltaNGKvd
5101 rrslpapdvt mlrtteyvap rsvwearlaq vweqvlnvpq vgalddffaL GGHSLramrv

C partial leucine module of TY3 (cores K to R)

5161 lssmhneyqv diplrilfek ptiqelaafi eetakgnvfs iepvqkqayy pVSSAQKRMY
5221 ildqfeqvgi synmpstmli egklertrve aafqrLIARH ESLRTSFavv ngepvqnihe
5281 dvpfalayse vteqearelv sslvqpfdle vaplirvsll kigedryvlf tdmHHSISDG pSAC8AF
5341 VSSgillaew vqlyqgdvlp elriqykdfa vwqqefsqsa afhkqeaywl qtfaddipvl
5401 nlptdftrps tqsfagdqct igaqkalteg lhqlaqatgt TLYMVLLAAY NVllakyagq
5461 EDITVGTPIT GRSHadlepi VGMFVNTLAM RNkpqrektf seflqeVKQN ALDAYQHQDY
5521 PFeelvekla iardlSRNPL fdtvftfqns teevmtlpec tlapfmtdet gqhakfdltf

Figure III.3.6.2.B. The alignment of the inserts (underlined) of A. pSac32 (R) reverse and (F) forward sequences and pSac20 against the valine module of TY3, the PS enzyme that produces tyrocidine and of B. pSac6A and pSac8A reverse and forward sequences against the ornithine module of TY3. The core sequences are indicated by the grey capital letters.

that also carry some or all of these cores, which eliminated the insert from being associated with either two of the modules that those DNA fragments have been assigned to (modules B or C). As well, if the assumptions that the *Sac* I fragment of approximately 3.5 kb contains a portion of the probe sequence and that the *Sac* I digestion was complete are correct, then pSac14 could not be a part of module C, and so was assigned to the putative partial module E with pSac21 by process of elimination. These two *Sac* I fragments are not incompatible, they may share a common *Sac* I site since they contain adjacent core sequences. Placing the insert from pSac14 in the partial module E also ensures that there would be a *Sac* I site at one of the rp19 DNA insert borders.

The insert from pSac32 was determined to contain the cores A-one at one end and three-four at the other end of the fragment. The size of this fragment according to the PS enzyme model was 1.5 kb which is similar to the apparent size of approximately 1.7 kb measured by gel electrophoresis. Although this fragment contains a core four sequence, it is not similar to the PS-PCR product 17 sequence. It also was seen to hybridize only weakly to the probe in a Southern hybridization. This eliminated it from being in module D as the core six sequence of pSac21 was assigned to. It was also not similar in sequence to either pSac8A or pSac6A, both of which contain some of the cores that are found in pSac32, therefore this DNA fragment could not be assigned to modules B or C. This *Sac* I fragment was assigned to module A by elimination, and also to allow for the other *Sac* I rp19 insert border. One of the sequenced ends of pSac32 contains a section of amino acid sequence prior to, and including core three and therefore part of the amino acid recognition site. The PS enzyme modules that were most similar to pSac32, were ones that recognized and activated valine. This suggests that the module encompassing the sequence of the insert from pSac32 may be a valine activating module, although further sequence information from this DNA fragment would be needed to confirm this hypothesis. The insert from pSac20, containing cores five and six, was placed adjacent to pSac32 because it was not similar in sequence to any of the other fragments containing those cores. As well, this insert corresponds sequentially with the core sequences of pSac32 and when these two sequences are aligned with the sequence of the module that they both are similar to from BLASTp analysis, they are separated by two amino acids. The band corresponding in size to the insert from pSac20 hybridized weakly with PS-PCR product 17 but strongly with PS-PCR product 16 (Section III.3.4.1). This suggests that this insert may contain the sequence of PS PCR

product 16 that encompasses cores five and six, suggesting that the partial module A in the rp19 insert may also be contained within the rp67 insert, that hybridized strongly with PS PCR product 16.

The insert from pBsa65 contained the core sequences four-five-six, which were not similar to the other digestion fragments containing these core sequences, nor was it similar to the sequence of PS-PCR product 17. It was placed in module C. This arrangement agrees with the proposal that the unsequenced *Sac* I fragment of 3.5 kb would contain the section of the PS-PCR product 17 adjacent to pSac21 of module D and hence would contain a large portion of module C as well.

The arrangement of the *Sac* I and *Bsa* I fragments into two partial and three whole modules as pictured in Figure III.3.6.2.A, suggests that there were two *Sac* I fragments that were not accounted for in the hybridization of the *Sac* I fragments. These two fragments are predicted from the PS enzyme model to be approximately 1.5 kb and 0.7 kb in size. There were fragments of those sizes seen in the *Sac* I digest. When the *Sac* I fragments of each size were ligated into pUC118 and cloned into *E. coli*, there was more than one clone that carried 1.5 and 0.7 kb inserts that were able to hybridize to PS-PCR product 17. However, only one of the 1.5 kb inserts and two of the 0.7 kb inserts were sequenced, therefore the predicted 1.5 kb and 0.7 kb *Sac* I fragments may be cloned but not sequenced.

III.3.7 Transformation of *P. polymyxa* PKB1

In order to gain evidence as to whether the PS genes under investigation might be involved in production of the antifungal material, a gene disruption experiment was attempted. As a first step in gene disruption, however, it was necessary to develop a procedure for the introduction of DNA into *P. polymyxa* PKB1 cells.

Transformation of DNA into both *E. coli*, as a model for transformation into Gram negative bacteria, and *B. subtilus*, as a model for transformation into Gram positive spore-forming bacteria has been studied extensively, but much less is known about the corresponding process in *Paenibacillus* species. The genetic background of *P. polymyxa* in general and this environmental *P. polymyxa* isolate in particular has not been fully characterized.

III.3.7.1 Adaptation of electroporation procedure to produce *P. polymyxa* PKB1 transformants

A 2.9 kb plasmid called pC194, originally isolated from *S. aureus*, was found to be able to replicate in *B. subtilus* and transform the cells to chloramphenicol (CAM) resistance with the chloramphenicol acetyl transferase gene (Horinouchi and Weisblum, 1987). This plasmid was used in tests of different transformation procedures with *P. polymyxa* PKB1.

The concentration of CAM in solid media that inhibited growth of wild type *P. polymyxa* PKB1 was determined to be 5 μg/ml, cells plated onto media with 3 μg/ml of CAM were reduced in growth only and cells plated onto 4 μg/ml of CAM would grow after 7 days of incubation.

An electroporation procedure for transformation was developed to utilize the potential for higher transformation frequencies that have been found with *E. coli and B. subtilus* when compared to other methods of incorporating foreign DNA into cells. Although many different variables were investigated, the most important aspects for successful transformations were the age and the number of washes of the cells, the number of cells and the amount of DNA used in the transformation. The most optimal point in the culture growth to harvest the cells for electroporation was late exponential phase. If the cells were harvested prior to this time they were able to take up pC194 but the transformation frequency was much lower. If cells were harvested for electroporation after late exponential growth, there were no transformants. Spore-forming bacteria have been known to become naturally competent just prior to sporulation, which would imply that the late exponential growth cells would be better able to incorporate foreign DNA. As well, since the number of cells used in the transformation was important; with more cells leading to an increase in transformation frequency, harvesting the cells at a later stage of growth, such as when the culture had grown to approximately 5 X 10^7 cells/ml, made it easier to obtain a large number of cells for use in electroporation.

Transformants were only obtained if the harvested cells were washed at least three times; twice in water and once in the buffer used for electroporating the cells. Many strains of *P. polymyxa*, including *P. polymyxa* PKB1, tend to produce extracellular polysaccharides that may impede transfer of foreign DNA into the electrocompetent cells when the cells are at a high concentration.

The concentration of the DNA needed to be large for transformation to occur and it was not necessary to purify the plasmid from excess salts. This is unlike electrotransformation of DNA into *E. coli*, where a low concentration of pure plasmid ensures successful transformation. Routinely, about 0.5 to 1.0 μg of DNA in a 30 μl volume was combined with approximately 4 X 10^9 *P. polymyxa* PKB1 electrocompetent cells in an 800 μl volume in a 0.4 cm gapped cuvette and incubated on ice for 5 min. The mixture was pulsed with an electric charge of 6250 V/cm and 25 μF. The rate of transformation of *P. polymyxa* PKB1 with pC194, using the adapted protocol described in Section III.2.7, was on average 4.3 X 10^3 transformants/μg of pC194.

The *P. polymyxa* PKB1 transformants containing pC194 remained chloramphenicol resistant after successive cycles of growth on non-chloramphenicol containing solid media suggesting that the plasmid was stable within the *P. polymyxa* PKB1 genetic background.

III.3.7.2 Transformation of *P. polymyxa* PKB1 with a non-replicating *Bacillus* vector carrying a *P. polymyxa* PKB1 genomic DNA fragment

The plasmid pJH101 is an *E. coli* to *Bacillus* spp. shuttle vector that confers tetracycline resistance to *E. coli* transformants and chloramphenicol resistance to *Bacillus* spp. transformants. It is able to replicate in *E. coli* because it contains an *E. coli* origin of replication, but cannot replicate in *Bacillus* spp. Therefore chloramphenicol resistant transformants can only occur if the plasmid can integrate into the genome. Integration of the plasmid into the genome is dependant on the presence of DNA in the plasmid that is homologous to DNA in the genome. The integration of pJH101 *into B. subtilis* has been found to occur by a Campbell-type integration, where a single cross-over event occurs between the sites of homology on the plasmid and the genome, resulting in the entire plasmid inserting into the genome at that site (Ferrari *et al.*, 1983).

This integrating plasmid was used in attempts to disrupt the production of the antifungal material by *P. polymyxa* PKB1, in order to determine if the PS genetic fragments generated by PCR from the *P. polymyxa* PKB1 genome or screened from the *P. polymyxa* PKB1 genomic phage library were involved with production.

The approximately 400 bp sized PS-PCR products 4, 16, 17, 26, and 31 were ligated into pJH101 and called pPB4 to 31 (Table III.2.2). These plasmids were electroporated into *P. polymyxa* PKB1 electrocompetent cells, under the conditions used

successfully to introduce pC194 into *P. polymyxa* PKB1, however no chloramphenicol resistant transformants were produced. However, the same electrocompetent cells could be transformed to chloramphenicol resistance when electroporated with pC194. The PS-PCR products were originally amplified from the *P. polymyxa* PKB1 genome therefore there should be sites of homology between the recombinant pJH101 plasmids and the genomes of the electrocompetent cells. This suggests that the size of the homologous site supplied by the inserts were too small for Campbell-type integration to occur, therefore not allowing for the plasmid to integrate into the genome and confer chloramphenicol resistance.

The digested DNA fragments from rp19, the λ phage screened from the *P. polymyxa* PKB1 genomic library by hybridization to the PS-PCR product 17, provided a source of DNA fragments larger than 400 bp to test for integration of pJH101 into *the P. polymyxa* PKB1 genome. The fragments from rp19 were known by sequencing and the results of BLAST searches to contain portions of a PS encoding gene

J. Taylor, a project student in the Department of Biological Sciences, University of Alberta, ligated the 2 kb insert from pSac8A, that stretched from core sequences D to Q, into the plasmid pJH101 (plasmid was called pJT1), and used it in electrotransformation of *P. polymyxa* PKB1 (Table III.2.2). This DNA fragment was chosen due to its size and also because the sequence suggested that it may be encoding part of an ornithine activating module in a PS. The amino acid compositional analysis of the antifungal material produced by *P. polymyxa* PKB1 suggested that one of the amino acids in the antifungal antibiotic was citrulline (Section II.3.3.5), a carbamoylated derivative of ornithine (Hash, 1975).

Electroporation of 50 μl of pJT1 into 800 μl of *P. polymyxa* PKB1 resulted in the production of 78 chloramphenicol resistant transformants, suggesting that Campbell-type integration of the plasmid occurred at the site of homology between the recombinant plasmid and the *P. polymyxa* PKB1 genome. However, these transformants were still able to inhibit *L. maculans* when patched onto agar plates spread with the fungus, as seen by zones of inhibition around the *P. polymyxa* transformant colonies.

Four representative transformants, numbered 17, 40, 68, and 72, were selected for further investigations. All four transformants were able to sporulate on solid media and in liquid culture, although the endospores from cultures of these four developed approximately 48 h after wild type *P. polymyxa* PKB1 produced endospores when grown in the same conditions. The ability of these four transformants to inhibit the Gram

positive bacterium, *M. luteus*, was measured in order to determine if the inhibition of the fungus by the transformants was due to another factor other than the antifungal material. The transformant number 68 did not appear to be able to inhibit the Gram positive bacterium, while the other three were able to.

The genomic DNA from these four transformants was isolated to be used in a Southern to determine if pJH101 had integrated into the genome. Radiolabelled pJH101 was used as a probe of these digests. The four genomic preparations were digested to completion with the restriction enzyme *Hind* III (Figure III.3.7.2). This enzyme was chosen because the pJH101 plasmid did not contain a site for *Hind* III digestion because it was a part of the multiple cloning site that was excised from the plasmid when the pSac8A insert was ligated into the plasmid, as a result, the Southern analysis of the genomic digests were expected to show a single hybridizing band. As well, if the plasmid had integrated into the same position in the genomes of the four transformants under study, it would be seen because the size of the hybridizing DNA fragment would be the same in all cases. The genome from transformant number 17 was not digested with *Hind* III, probably due to the presence of inhibitory contaminants within the genomic preparation. Hybridization of the radiolabelled pJH101 vector with the digested genomes of the other three transformants showed two of them, numbers 40 and 72, had one DNA fragment each that hybridized. The hybridizing DNA fragment from both of the digested genomes were the same size as each other (Figure III.3.7.2).

This suggested that although the ability of the transformants to produce antifungal material capable of inhibiting *L. maculans* was not disrupted, integration of pJH101 into the genome of *P. polymyxa* PKB1 was successful. It is possible to integrate pJH101 into the *P. polymyxa* PKB1 genome using a sufficiently large region of homology between the plasmid and the genome, therefore this method could be used to disrupt production of the antifungal material.

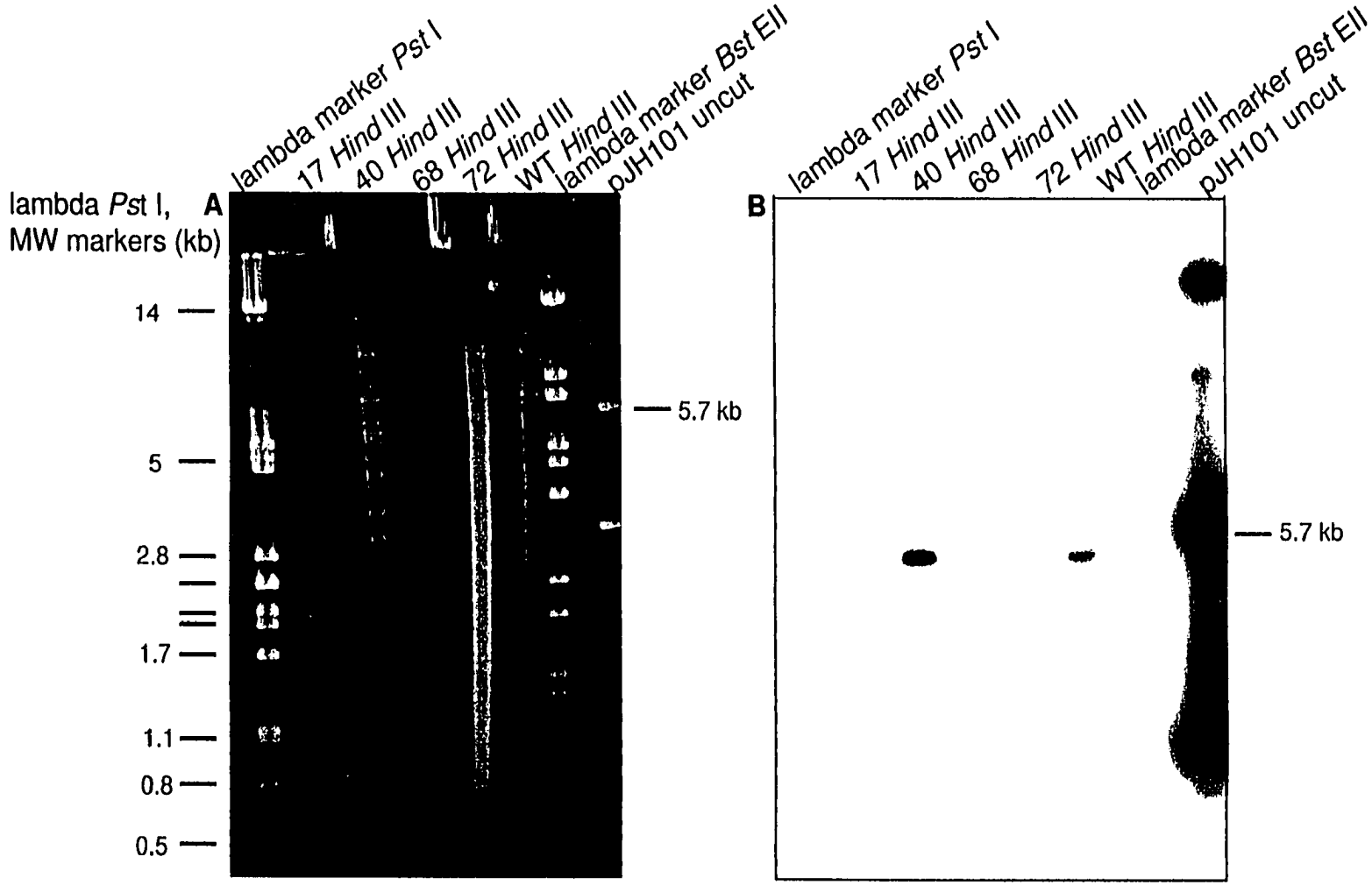


Figure III.3.7.2. A. Genomic preparations of the four *P. polymyxa* PKB1 putative transformants and wild type *P. polymyxa* PKB1 digested with the restriction enzyme, *Hind* III. B. Southern hybridization of the digested genomes with radiolabelled pJH101. The apparent size of the pJH101 vector is indicated on the right of both the pictures.

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IV. Summary, Conclusions and Avenues for Further Investigations

IV.1 Summary and conclusions

P. polymyxa is known as a plant growth promoting rhizosphere bacterium. Strains of *P. polymyxa* have been isolated from the rhizosphere of many different plant species and have been found to produce cell-wall degrading enzymes, siderophores, and antibiotics that all contribute to protection of plants from disease causing fungi. *P. polymyxa* has also been found to promote the growth of plants by fixing nitrogen and increasing the porosity of soil around the plant roots.

An environmental bacterium was isolated from canola stubble that was able to inhibit *L. maculans*, a fungus that causes a disease of canola called blackleg. This bacterium was determined by colony and cellular morphology, biochemical tests, FAME analysis and 16S rRNA similarity to be *P. polymyxa* and was given the strain designation PKB1.

Material was extracted from cultures of this bacterium with methanol that was able to inhibit *L. maculans*. Further preliminary studies on the antifungal material, such as measurement of apparent size after electrophoresis on SDS-PAGE and passage through ultrafiltration membranes along with acid hydrolysis of the material and subsequent positive reaction of the hydrolysate with ninhydrin, suggested that the nature of the methanol soluble antifungal material was peptidic. It is very common for species of *P. polymyxa* and other spore-forming bacteria to produce peptides that have antibiotic characteristics.

Growth studies on *P. polymyxa* PKB1 showed that the bacterium produces the antifungal material just prior to, or at the onset of, sporulation. Production of peptide antibiotics at the onset of sporulation is also common among the spore-forming bacteria. Attempts to optimize production of the antifungal material by *P. polymyxa* PKB1 illustrated that an increase in growth rate does not correlate to an increase in antifungal material production. The conditions that gave the highest production of the antifungal material, reproducibly, were determined to be; growth of starter culture for 24 h in TCSS broth, inoculation of 200 ml of PDB in a 500 ml flask at 1%, grown at 28°C, shaken at 200 rpm. These were referred to as the production conditions. The bacterium was also found to be able to grow and produce antifungal material at 10°C; the growth cycle showed a large lag phase before onset of exponential growth but the production of the

antifungal material was higher than from *P. polymyxa* PKB1 grown in the same conditions except for the temperature. *P. polymyxa* PKB1 was grown in both 10 L and 50 L bioreactors where they were able to produce antifungal material in quantities similar to when the bacterium is grown under production conditions. The inhibitory spectrum of the antibiotic was found to be against fungi and Gram positive bacteria. The production of the antifungal material by other strains of *P. polymyxa*, such as; a Brazilian soil isolate; *P. polymyxa* SCE2, a subspecies of *P. polymyxa* called *colistinus koyama* that was originally isolated from Japanese soil, and a culture collection strain of *P. polymyxa*, NCIMB 8468 were investigated. *P. polymyxa* SCE2 and *P. polymyxa* subsp. *colistinus koyama* were both able to inhibit *L. maculans* but the NCIMB strain was not. The source of antifungal activity of *P. polymyxa* SCE2 had not been characterized at this time, however, there was an antibiotic, called gatavalin, that was active against fungus, from *P. polymyxa* subsp. *colistinus koyama*, that had been partially characterized.

The antifungal material that was extracted by methanol from sporulating *P. polymyxa* PKB1 culture was purified by size exclusion chromatography and reverse-phase chromatography based on polarity, and traced by activity against *L. maculans* measured by a well bioassay, 16.5% acrylamide tricine gel electrophoresis and bioautography. The antifungal material extracted from *P. polymyxa* subsp. *colistinus koyama* was purified in the same manner, the purification profile was found to be the same as for the antifungal material from the environmental bacterium isolated from canola stubble.

The antifungal material isolated from *P. polymyxa* PKB1 was very similar in amino acid composition and MW to these other antibiotics isolated from strains of *P. polymyxa*; gatavalin, LI-F04, LI-F03, and fusaricidins A to D (Section I.1.4). FAB-MS of a mixture of the LI-F series of antibiotics contained parent MW ions of the same mass as those found in the MS spectra of the antifungal material from the present study. The MS data for LI-F04 in particular showed the presence of two molecular ions at 883 and 897 Da; which are the same mass as the molecular ions of the antifungal material from the present study, as seen in MS spectra of the antifungal material. The MS data for LI-FO3 showed the presence of two molecular ions at 947 and 961 Da, which are also present in the MS spectra of the antifungal material from the present study. Fusaricidin A, B, C, D have MW of 883, 897, 947 and 961 Da, respectively, all four of these MW structure are seen in MS spectra of the antifungal material, as they also were in the MS spectra of LI-F03 and LI-F04.

Sequencing of the partially hydrolysed antifungal material by Edman degradation determined a sequence of thr-val-val-X-glu-ala for the peptide that represents a structure similar to fusaricidin B, of 897 Da. Fusaricidin B has a D-allothreonine where the unknown residue was found in the antifungal material of this study, suggesting that these two structures may differ by the identity of one residue. The amino acid composition of the antifungal material correlated with the sequence from the partially hydrolysed antifungal material and with the possibility of the presence of two peptides differing in composition by an asx/glx switch. This is also seen for fusaricidin A and B.

The fusaricidin antibiotics all carry a 15-guanidino-3-hydroxypentadecanoic acid side chain that, after acid hydrolysis of the fusaricidin peptide structure, is thought to undergo dehydration, which would release the moiety from the cyclic peptide to form an αβ hydroxy fatty acid with an attached guanidino group of 298 Da.

Tandem MS of the antifungal material and of an abundant fragment ion at 256 Da, suggested that there was a guanidino group-containing fatty acid bound to the peptide ring structure of the antifungal material in this study.

Based on the similarity of amino acid composition, the identical molecular weights and the MS fragmentation pattern of the 256 mu side chain moiety, the antifungal material of the present study was determined to be a mixture of cyclic depsipeptides with a possible 15-guanidino-3-hydroxypentadecanoic acid side chain, similar to the known structures of fusaricidin A, B, C, and D. Antifungal material that was hydrolysed and sequenced suggested that there may, or may not, be a difference of one amino acid between the peptides found in this material and the fusaricidins.

It is suggested that since the antifungal material in this present study may be a mixture of cyclic peptides closely related to (or exactly like) the fusaricidins, that due to the similarity in amino acid composition of LI-FO4, –F03 and gatavalin to the antifungal material, and the identical MW, they are all closely related structures (or exactly like) to fusaricidins, A, B, C, and D.

The peptide antibiotics from spore-forming bacteria such as *P. polymyxa* PKB1 tend to be constructed by peptide synthetase (PS) enzymes instead of ribosomally as proteins are synthesized. PS enzymes are highly structured, containing an enzymatic module that is responsible for the activation and addition of each amino acid residue in the peptide under construction. The modules contain several separate amino acid sequences called core sequences that are conserved throughout the PS modules from bacteria and fungi. These core sequences were used as the basis for the design of

oligonucleotides that were used as primers in a PCR reaction with the *P. polymyxa* PKB1 genome to amplify sections of PS enzyme encoding genes. Five different 400 bp sized fragments that were similar to PS enzymes were amplified from the genome. The DNA similarity between these five sequences ranged from 94% (16 and 31) to 46% (4 and 17). A LambdaGem®11 *P. polymyxa* PKB1 genomic library was screened for recombinant phage carrying PS enzyme encoding DNA using three of the PS-PCR products as radiolabelled probes. Many phage hybridized with these PS-PCR products, and so representative phage that hybridized with each of these probes were analysed further. The recombinant phage were digested with a series of restriction enzymes and hybridized with each radiolabelled PS-PCR product on the same gel, separately. This showed that the *P. polymyxa* PKB1 genomic inserts from two of the three recombinant phage were possibly related and the third was not. One recombinant phage was chosen for partial restriction mapping and sequence analysis and a partial map of the recombinant phage insert was proposed.

A DNA fragment from the recombinant phage insert appeared to be part of an ornithine activating module. Since initial amino acid composition data suggested that the antifungal material of this study may contain an ornithine, or ornithine related residue, this DNA fragment was chosen for use in gene disruption studies. A method to transfer vector DNA into *P. polymyxa* PKB1 had been developed, therefore a DNA fragment from this putative ornithine activating module was ligated into a *Bacillus* spp. integration vector and used to transform *P. polymyxa* PKB1. The integration vector can only confer resistance to chloramphenicol if it is able to integrate into the genome at a point of similarity between the vector and the genome by a single cross over event that places the entire vector into the genome at the point of similarity. The vector with the DNA fragment of a part of a putative ornithine activating module encoding gene, was able to integrate into the genome of *P. polymyxa* PKB1, and integration was confirmed by Southern hybridization of genomic preparations from the transformants. However the integration of the pJH101 vector into the *P. polymyxa* PKB1 genome did not disrupt the inhibitory activity of the antifungal material against *L. maculans*. Since this study, it has been determined that the identity of the unknown residue of the antifungal material is neither ornithine nor citrulline, which correlates with the lack of effect on production of the antifungal material when the integrating vector was inserted into the *P. polymyxa* PKB1 genome at the putative ornithine activating module.

IV.2 Avenues for further investigation

Further investigation into the structure of the antifungal material of this study is warranted in order to identify the nature of the unknown residue of the antifungal material by Ion-trap MS. The identity of the fragment ion of 256 Da could also be confirmed by this same type of analysis. Although separation of the depsipeptides is not necessary to investigate the sequences if tandem MS is used, further experimentaion into separation of the peptides in the antifungal material would allow for confirmation of the sequences by Edman degradation after partial hydrolysis to remove the side chain moiety and to linearize the peptides at the ester bond. The researchers studying the fusaricidin antibiotics were able to separate fusaricidins A and B but not C and D.

Further investigations into the PS enzyme encoding genes for this antifungal material could be done by analysing the recombinant phage identified with PS-PCR product 4. This core four to core six PS DNA fragment was the most dissimilar to PS-PCR product 17: the one that was determined not to be a part of the putative PS encoding genes of the antifungal material of this study. Since PS-PCR product 4 is quite different from PS-PCR 17 it may be a part of a separate PS gene cluster. Five recombinant phage were identified by PS-PCR product 4 in the second screening of the LambdaGEM®-11 *P. polymyxa* PKB1 genomic library and these could be investigated further. As well, since the amino acid sequence of one of the peptides in the antifungal material has now been elucidated, this information can aid in the determination of whether a PS enzyme encoding gene fragment encodes the PS enzymes of interest.