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Title: Growth and Secondary Metabolite Production of a Historic Collection of *Sorangium cellulosum*

Abstract approved: Scott S. Cipper

Epothilones are a novel class of microtubule stabilizers produced by the bacterium *Sorangium cellulosum*. Although distinct from paclitaxel (Taxol®), they possess a similar mode of action. Epothilones have several advantages as compared to Taxol®, which makes them the likely successors of Taxol®. Unfortunately, *S. cellulosum* is a very hard organism to work with and it produces only about 20 mg/L of epothilones. The isolation of *S. cellulosum* strains that overproduce epothilones would be a tremendous advantage to the production of these important compounds. Unfortunately, the production of epothilones among *S. cellulosum* strains is rare. Currently, the only way to determine if a strain produces epothilones is to grow the organism and examine extracts by mass spectrometry, a costly and time-consuming process. In this study, we developed culture conditions for the growth of *S. cellulosum* and prepared extracts of secondary metabolites from 45 different strains. Also, data are presented in the development of a PCR-based approach to the identification of the epothilone gene cluster.

Growth and Secondary Metabolite Production of a Historical Collection of *Sorangium cellulosum*

A Thesis

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PREFACE

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TABLE OF CONTENTS

LIST OF TABLES

PAGE

LIST OF FIGURES

PAGE

Introduction

The myxobacteria are a group of bacteria whose name is derived from the slimy mucus-like secretion which they produce. The prefix "myxo" is comparable to the word mucus; hence the name, myxobacteria. Currently, there are 12 genera, containing a total of 40 different species (19). They have been isolated from a wide assortment of natural environments, including many different types of topsoils and decaying plant material (24), and in all climates, vegetation belts, and altitudes (23). While these organisms seem to prefer soils rich in organic material and microbial life, the exact number of myxobateria in any particular habitat is difficult to estimate because the organism tends to stick to foreign particles and each other (34).

Several features of the myxobacteria have provoked the interest of researchers worldwide. Most investigations involve the examination of social interactions between individual cells. Myxobacterial colonies exhibit a well coordinated swarming motility, which allows them to swarm the topsoil with a "wolf-pack" behavior as they prey on other bacteria. This predatory life-style results in the production of lytic exoenzymes which lyse the bacterial prey. The most astonishing aspect of their social behavior, however, occurs upon starvation when tens of thousands of cells aggregate to begin a series of coordinated movements leading to the production of a fruiting body. Inside the fruiting body, vegetative cells convert into dormant myxospores, which are resistant to the harshest of environmental conditions (37). When myxospores are transported to a more favorable location, they germinate and become vegetative cells.

The most notable member of the myxobacteria is *Myxococcus xanthus*. This organism is easily culturable, genetically amenable, and has a doubling time of

approximately 5 hours (38). Furthermore, it exhibits many interesting features, such as gliding motility, a large genome of approximately 9,500 Kb, the ability to form fruiting bodies, and a sophisticated intercellular communication system.

One member of the myxobacteria is characterized by its ability to decompose cellulose. This organism is classified in the taxon *Polyangium* and is referred to as *Sorangium cellulosum.* It is a Gram negative, aerobic, mesophilic bacterium with a doubling time of 16 hours (16). It has the largest bacterial genome described to date with an estimated size of 12.2 million base pairs (16). Typical plate cultures of *S. cellulosum* consist of rod shaped, orange colored cells with swarming radiating veins throughout the agar. In liquid media, some strains grow as a homogenous cell suspension, whereas others form cell aggregates. *Sorangium cellulosum* also possess gliding motility similar to that of *M. xanthus* (12).

Microbial metabolites, especially secondary metabolites, are continuously exploited by chemical and pharmaceutical companies as a rich source of novel therapeutic drugs (22). To date, most newly discovered drugs are the result of the organism's secondary metabolism. Although secondary metabolites are not essential for growth and their production is sporadic, they give the producing organism a competitive edge over other organisms (29). Secondary metabolites have been isolated predominantly from bacteria comprising the order Actinomycetales. On the whole, bacteria appear to represent an inexhaustible source of novel secondary metabolites (35).

Previous studies have also established that myxobacteria produce a vast variety of novel secondary metabolites as well. Many of them are completely novel chemical structures and some show rare and even unique mechanisms of action. It is estimated that

approximately 50% of all myxobacterial strains produce secondary metabolites. For some myxobacteria, such as the cellulose degrader *S. cellulosum*, it is estimated that nearly 100% of strains posses some novel bioactive property (25).

Although many novel compounds have been isolated from *S. cellulosum*, the most notable secondary metabolites produced are the epothilones (8). These compounds have been shown to be a promising anticancer drug with a high economic potential (18). They were first discovered in *S. cellulosum* strain Soce90, which was isolated in 1985 from top soil obtained from the banks of the Zambesi River in South Africa by the Reichenbach laboratory in Germany (8). This strain possessed a unique inhibitory activity against the fungus *Mucor hiemalis*. The compounds responsible for this antifungal activity were isolated and named epothilones. In 1995, researchers at Merck Research Laboratories also discovered a strain of *S. cellulosum* that produced epothilones (3, 13). This strain, termed *S. cellulosum* SMP44, was isolated in 1963 from top soil in a cotton field located in southern Missouri by Dr. John Peterson, Emporia State University, Emporia, KS (personal communication with Dr. Peterson). Interest by the scientific community peaked when it was noted that these compounds inhibited cancerous cells by a mechanism similar to that of the commonly used anti-cancer drug Taxol®.

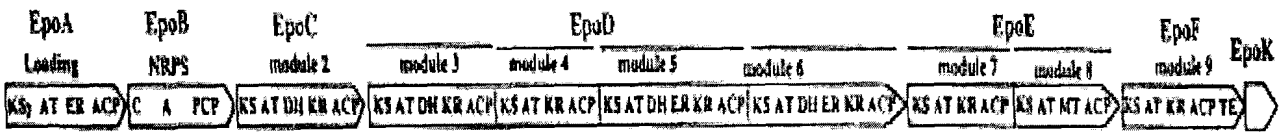
Taxol®, which was first isolated from the Pacific Yew Tree (*Taxus brevifolia*), has shown considerable promise in treating cancer of the ovaries (20), breast (15), head and neck (6), melanoma (4), and the lung (5). Its anti-cancer properties reside in its ability to stabilize cellular microtubules in rapidly dividing cells. Unfortunately, Taxol® has limited solubility and is delivered clinically in conjunction with Cremophor to increase its solubility. The Cremophor portion of this therapeutic agent causes

undesirable side effects (26) when administered to patients and attempts to increase its solubility have failed (33). Also, Taxol® resistance occurs in some cancerous cells as it is a substrate for P-glycoprotein, an efflux pump that removes cytotoxic compounds from cells (3, 17). Cells in which this pump is activated show drug resistance to Taxol®, which poses a major obstacle in effectively treating various types of cancers.

Epothilones produced by *S. cellulosum* are structurally different from Taxol®, but posses a similar mode of action in the stabilization of microtubules (3). Additionally, they are not a substrate for P-glycoprotein, are more soluble than Taxol®, and are effective against Taxol®-resistant cancer cell lines. Due to these properties, the scientific community considers epothilones the likely successor of Taxol® in the fight against cancer. Unfortunately, the availability of epothilones for clinical evaluation has been severely limited by the poor productivity and slow doubling time of the producing organism *S. cellulosum*. Artificial synthesis of epothilones is not cost-effective because of its structural complexity.

The epothilone gene cluster has been cloned (16, 21) and shown to have an association typical of type I- polyketide synthases (PKS), which are large multifunctional complexes possessing a modular organization (14). Its genetic organization is shown in Figure 1. The gene cluster is located on a 56 kb fragment which is divided into nine PKS-like modules that contain the genetic information for *epoA, epoC, epoD, and epoF* (16). Also, the *epoB* gene codes for a protein that is a non-ribosomal peptide synthetase (NRPS) involved in catalyzing the formation of a thiazole found in the epothilones.

Figure 1. Genetic organization of the epothilone gene cluster.


EpoA
Loading
KS AT ER ACP
EpoB
NRPS
C A PCP
EpoC
module 2
KS AT DH KR ACP
EpoD
module 3
KS AT DH KR ACP
module 4
KS AT KR ACP
module 5
KS AT DH ER KR ACP
module 6
KS AT DH ER KR ACP
EpoE
module 7
KS AT KR ACP
module 8
KS AT MT ACP
EpoF
module 9
KS AT KR ACP TE
EpoK

Finally, the product of the *EpoK* gene codes for P450 epoxidase that helps in conversion of epothilone D into epothilone B. The gene cluster has been expressed in *Streptomyces coelicolor* (36), which yields the production of epothilones, but not in great quantities. Finding a strain of *S. cellulosum* that is an overproducer of epothilones is the best chance to increase its yields.

In lieu of the problems faced in obtaining enough epothilones or cytotoxic compounds for study, the objectives of this research are as follows:

1. To screen approximately 45 different strains of *S. cellulosum* for cytotoxic activity against various cancerous cell lines.
2. To develop a Polymerase Chain Reaction (PCR) - based approach for identification of the epothilone gene cluster by targeting a rare methyltransferase domain.

Methods and Materials

Propagation of *Escherichia coli*

Escherichia coli DH5α was propagated in Luria-Bertani (LB) liquid media or on agar plates (20 g/L) for preparation of competent cells. Cultures were typically incubated overnight at 37° C or 30° C.

Propagation of *Sorangium cellulosum* from Lyophilized Vials

Lyophilized vials of *S. cellulosum* were obtained from Dr. John Peterson, Department of Biological Sciences, Emporia State University, Emporia, Kansas. Briefly, the lyophilized vials were surface disinfected with 95% ethyl alcohol. A steel file was used to score the surface of the vial to aid in breaking it open. The lyophilized samples were rehydrated by the addition of 1 ml of Brain Heart Infusion (BHI) media. A 100 µl sample of the rehydrated microbial culture was placed on the surface of a Cellulose Nitrate Salt (CNS) agar plate (Table 1) containing cycloheximide (100 µg/ml) and a piece of sterile filter paper (Whatman 3M) on its surface. Inoculated plates were incubated at 30° C for approximately 4-6 weeks or until growth was evident. Using a sterile spatula, a piece of the partially digested filter paper was cut out and placed on a Glucose Nitrate Salt (GNS) agar plate (Table 1) containing cycloheximide (100 µg/ml). Growth was allowed to occur for approximately 1 month at 30° C or until the culture was well established. This culture was used to inoculate a liquid culture of GNS (25 ml) followed by incubation with continuous shaking at room temperature. Growth generally became evident in 1 week.

Table 1. Composition of media used for the propagation of *Sorangium cellulosum*.

Media Composition	Amounts/Liter
GNS	
Glucose (Dextrose)	15.0 g
Yeast extract	1.0 g
Potassium nitrate (KNO_3)	1.5 g
HEPES buffer	2.4 g
Sodium phosphate (Na_2HPO_4) 0.1M	2.0 ml
Magnesium sulfate ($MgSO_4$) 0.1M	1.0 ml
Calcium chloride ($CaCl_2$) 0.1M	100 µl
Iron-molybdate stock solution *	1.0 ml
A5 trace element stock solution *	1.0 ml
CNS	
Potassium nitrate (KNO_3)	0.5 g
Sodium phosphate diabasic (Na_2HPO_4) 0.1M	800 µl
Magnesium sulfate ($MgSO_4$) 0.1M	4.0 ml
HEPES buffer	2.4 g
Ferric chloride ($FeCl_2$)	0.01 g

* Iron-Molybdate stock solution- 3g Ferric citrate, 0.25g Na_2HPO_4/500ml H_2O

*A5 trace element stock solution- 2.86g H_3BO_3, 0.222g $ZnSO_4$, 1.81g $MnCl_2$, 0.079g $CuSO_4$, 0.049g CO (NO_3)/1L of H_2O

Preparation of Extracts from *S. cellulosum*

Liquid cultures (25 ml) of *S. cellulosum* in GNS media were used to inoculate 250 ml of GNS (contained in a 1 L flask) with 20 g/L of Amberlite XAD-16 resin (Sigma, St. Louis, MO). Growth was allowed to occur for approximately 4-5 weeks at room temperature with shaking. The resin was separated from the cells by pouring the culture into a 1 L graduated cylinder. Within a few minutes, all of the resin settled to the bottom of the cylinder. The supernatant and cells were removed by gently decanting the mixture. After washing the resulting resin three times with water, it was placed in a 500 ml beaker and extracted overnight with three volumes of ethyl acetate by continuous stirring. Once completed, a separatory funnel was used to separate the resin from the ethyl acetate phase. The bottom aqueous layer containing the resin was discarded and the remaining organic ethyl acetate phase was taken to dryness using a model 78860-00 Labconco Rotary Evaporator (Kansas City, Missouri). The precipitate was rehydrated in a minimal volume of ethyl acetate and stored at -20° C.

Cytotoxic Assay

Extracts prepared from each *S. cellulosum* strain were analyzed for the presence of cytotoxic activity in the laboratory of Dr. Richard Himes, Department of Molecular Biosciences, University of Kansas, Lawrence, KS. Each extract was examined for its ability to inhibit the proliferation of a breast cancer (MCF-7), a Taxol®-resistant breast cancer (MCF-7ADR), and a colon cancer (HCT-116) cell line. Briefly, a standardized concentration of cells (2,500 cells/well in a 96-well plate) was established and incubated with various dilutions of the extracts to be tested. After a period of time, protein

synthesis was arrested upon the addition of trichloroacetic acid. An indicator solution was added and the absorbance determined at 570 nm to measure the amount of protein in the culture. Control cultures in which no extract was added were considered 100% cell growth. The amount of protein detected in experimental cultures was determined by measuring the difference in absorbance as compared to the control cultures.

Chromosomal DNA Isolation from *S. cellulosum*

Chromosomal DNA was isolated from liquid cultures of *S. cellulosum* using a modification of an established procedure for *E. coli* (28). Briefly, cells were obtained from 10 ml of liquid culture by centrifugation at 4000 rpm for 5 minutes in a Sorvall GLC-1 tabletop centrifuge (Newton, CT). Following resuspension of the cells in 10 ml of Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA; pH 8), cell lysis was initiated by the addition of 400 μl (10 mg/ml) of lysozyme at 37° C for 30 min. Cell lysis was completed by the addition of 500 μl of 20% sodium dodecyl sulfate (SDS), followed by incubation at 60° C for 2 hours. Degradation of RNA and protein was carried out by the addition of 50 μl of RNase (1 mg/ml) and subsequent incubation at 37° C for 20 minutes, followed by the addition of 75 μl of proteinase K (1 mg/ml) and incubation at 60° C for 1 hour, respectively. Phenol extraction was performed by adding 10 ml of phenol (pH 8), with gentle mixing of the solution by inversion for 2-3 minutes. Following centrifugation at 4000 rpm for 5 minutes, the clear upper (aqueous) layer was separated carefully from the lower (organic) layer and pipetted into a 15 ml conical tube and the phenol extraction step was repeated once more as described above. An equal amount of chloroform/isoamyl alcohol (24:1) was added to the solution with subsequent

centrifugation and extraction as indicated above. Ice-cold 95% ethanol (2.5 volumes) was added to precipitate the DNA. Visible strands of DNA were removed using a sterile pipette tip and placed into a 1.5 ml microcentrifuge tube. The DNA was dried using a Savant ISS 110 Integrated Speed Vac System (Holbrook, NY) and resuspended in 500 µl of TE buffer. The DNA was stored at 4°C until needed.

Plasmid DNA Isolation

Plasmid DNA was isolated from recombinant *E. coli* cultures using a QIAprep Spin Miniprep kit (Qiagen Inc; Valencia, CA) according to the manufacturers' recommendations. Quantity and purity was determined as described below, followed by storage at 4°C.

Quantification and Purity of DNA

To determine the quantity and purity of DNA, a 1/50 dilution of the DNA sample in question was prepared by mixing 490 µl of TE buffer and 10 µl of DNA in a Fisherbrand quartz cuvette (Fisher; St. Louis, MO). A Beckman DU530 spectrophotometer (Fullerton, CA) was used to determine the absorbance at 260 nm and the absorbance ratio at 260 nm and 280 nm, respectively. The quantity of DNA was determined using the following equation: (260 nm reading) (dilution factor) (50µg/ml) = µg/ml DNA.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to visualize DNA according to standard conditions (28). A 0.7% agarose gel was prepared by dissolving 0.21 g of agarose in 30 ml of 1X TAE, which was prepared from a 50X stock of 242 g Tris, 57.1 ml acetic acid, and 4 ml 0.5 M EDTA/1L. One microliter of a 10 mg/ml solution of ethidium bromide was added and the mixture heated in a microwave for 1 minute to dissolve the agarose prior to placing it into a gel mold for solidification. For electrophoresis, gels were placed into a Minicell EC370M electrophoretic chamber (Fisher) powered by a Bio-RAD model 250/2.5 power supply (Bio-RAD; Hercules, CA). DNA containing 1X gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) was loaded into wells contained in the agarose. After electrophoresis, DNA was visualized using a UV Intensity Transilluminator (Fisher). Results were recorded using a Panasonic CCD Ultra Lum camera and Scion Image software (Ultra Lum; Paramount, CA).

Purification of DNA from Agarose Gel

DNA was extracted from agarose gels and purified using the QIAEX®II Gel Extraction Kit (Qiagen) according to the manufacturers' recommendations.

Polymerase Chain Reaction

Chromosomal DNA was used as template to amplify desired regions of DNA in a polymerase chain reaction (PCR) using a MJ Research Minicycler (Watertown, Mass). Specific primers used are listed in Table 2. Typical PCR mixtures consisted of 100-500 ng of DNA, 200 μM deoxynucleotide triphosphates (dNTPs), 1.5 μM $MgCl_2$, 1.0 μl *Taq*

Table 2. Primers used in PCR.

Primer Name	Primer sequence 5'-3'
MT1 (Forward)	ATC TTG GAG ATC GGA GCA GGG ACG
MT2 (Reverse)	GCC TCC GGG CGC GAG CAA CGA CAG
MT3 (Forward)	CTG CCT GAC CGG ACA GAA TAC CAT
MT4 (Reverse)	CGA CAG GAG ACG CTT CGC CGT GGC
MT5 (Reverse)	CCG AGG ATC CCC GCC GGA GAT CCG

polymerase, and 50 nM each of the forward and reverse primers, in buffer containing 50 mM TrisCl, 50 mM KCl and 0.01% Triton-X100 in a final volume of 100 μl. For amplifications using *S. cellulosum* chromosomal DNA, dimethyl sulfoxide (DMSO) was added to 5 % of the final reaction volume. A typical amplification consisted of an initial denaturation step at 94° C for 5 min, followed by 30 cycles of DNA denaturation at 94° C for 1 min, annealing at 60° C for 1 min, and elongation at 72° C for 30 seconds. Agarose gel electrophoresis was used to analyze the amplified DNA as described above.

DNA Ligation and Transformation

Gel purified PCR products were incubated with an end conversion mix (Novagen; Madison, WI) containing T4 DNA kinase and ATP at 22° C for 15 min. Following inactivation of the DNA kinase at 75° C for 5 min, the reaction was incubated on ice for 2 min. Ligation was accomplished by adding 1 μl of T4 DNA ligase (4 U/μl) and 50 ng of *EcoR*V digested pT7Blue-3 (Novagen). Reaction mixtures were incubated for 2 h at 22° C. Competent *E. coli* cells were prepared and transformed according to a standard protocol (28). Briefly, 100 μl of competent cells were incubated with ligation reactions on ice for 15 min followed by a heat shock at 42° C for 90 seconds. After incubation on ice for two minutes, 900 μl of LB was added. Transformed cells (100 μl) were spread plated on LB plates supplemented with 100 μg/ml ampicillin and 20 μg/ml of X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside). Plates were incubated overnight at 37° C.

PCR Based Screening of Recombinants

A modified PCR-based procedure was used to screen bacterial colonies containing potential recombinant plasmids. Briefly, with a sterile toothpick, potentially recombinant colonies were scraped from LB/AMP plates and placed into 10 μl of sterile water. The cell solution was heated at 95° C for 5 min to lyse the cells. This lysed cell suspension was used in a PCR to amplify the cloned insert using gene specific primers (Table 2). Amplicons were visualized by agarose gel electrophoresis as described above.

DNA Sequencing and Sequence Analysis

Plasmid DNA was sent to a commercial facility for DNA sequencing. Sequence data obtained were compared to known *S. cellulosum* genes using the Basic Local Alignment Search Tool (BLAST) (1).

Results

Propagation of *Sorangium cellulosum*

Different media compositions and growth conditions were examined for the propagation of *S. cellulosum* from lyophilized vials (data not shown). In liquid GNS media, *S. cellulosum* often clumps together as shown in Figure 2. When placed on solid GNS media, *S. cellulosum* grows as radiating veins throughout the media (Figure 3). Forty-five strains of *S. cellulosum* were propagated from lyophilized vials in this study (Table 3). Many cultures demonstrated unique characteristics, such as color variations and clumping.

Preparation and Screening of *S. cellulosum* Extracts for Bioactive Material

Extracts prepared from 45 different *S. cellulosum* strains were examined for cytotoxic activity against three different cancerous cell lines. As shown in Table 3, the 45 different extracts varied considerably in their cytotoxic activity. In general, the extracts were more cytotoxic to MCF-7 (breast cancer) cells versus the HCT-116 (colon cancer) and MCF-7ADR (Taxol® resistant breast cancer) cell lines.

Development of a PCR-Based Method to Detect the Epothilone Gene Cluster

The epothilone gene cluster from *Sorangium cellulosum* SMP44 has been cloned (16) and its genetic organization is shown in Figure 1. Since one of the modules contained a rare methyltransferase domain, it was hypothesized this region in the DNA may serve as a potential target for the identification of *S. cellulosum* strains containing the epothilone gene cluster. Thus, DNA primers were designed to specifically amplify

nucleic acid regions in the methyltransferase domain of the epothilone gene cluster as indicated in Figure 4. To facilitate the development of a PCR-based method to identify *S. cellulosum* strains containing the epothilone gene cluster, overlapping cosmid clones containing the gene cluster were obtained from Kosan Biosciences (Hayward, CA) as described previously. As shown in Figure 5, primers with sequences based on the methyltransferase domain were able to specifically amplify the desired segment of DNA. Amplicons of approximately 500 bp (primers MT1- MT5), 450 bp (primers MT3- MT5), 300 bp (primers MT1-MT2, MT1-MT4), and 250 bp (primers MT3-MT2, MT3-MT4) were observed. To determine if these primers could specifically amplify this sequence of DNA in *S. cellulosum*, chromosomal DNA was prepared from both epothilone positive and negative strains and used in a PCR. As shown in Figure 6, the primer pair MT1 and MT2 was able to amplify a 300 bp DNA segment only in the epothilone positive strain.

Cloning and DNA Sequencing of MT1- MT2

To determine if the 300 bp amplicon generated with the MT1- MT2 primer pair was indeed from the rare methyltransferase domain in the epothilone gene cluster, the DNA fragment was ligated into pT7Blue3 and transformed into *E. coli* DH5α. A generalized flow chart demonstrating the entire cloning scheme is shown in Figure 7. Recombinant plasmids were purified and subjected to automated nucleotide sequencing. BLAST analysis of the cloned insert confirmed it was from the epothilone cluster (data not shown).

Table 3. Cytotoxicity analysis of *S. cellulosum* extracts. Dilution inhibiting ≥ 50% of cells.

Strain	MCF 7	MCF 7 ADR	HCT-116
1. SMP 785	100,000	1,800	10,000
2. SMP 789	100,000	-	-
3. SMP 751	-	NT	1,000
4. SMP 750	10,000	100	-
5. SMP 788	1,000	100	-
6. SMP 760	1,000	100	-
7. SMP 779	100	100	100
8. SMP 754	1,000	100	100
9. SMP 776	1,000	100	100
10. SMP 762	1,000	100	-
11. SMP 784	1,000	100	-
12. SMP 777	1,000	NT	-
13. SMP 753	1,000	1,000	1,000
14. SMP 763	1,000	1,000	100,000
15. SMP 759	1,000	NT	1,000
16. SMP 786	1,000	NT	1,000
17. SMP 778	1,000	100	100
18. SMP 764	1,000	NT	100
19. SMP 780	1,000	NT	100,000
20. SMP 756	1,000	100	100,000
21. SMP 758	1,000	NT	100,000
22. SMP 548	32,000	32,000	4,000
23. SMP 591	16,000	16,000	8,000
24. SMP 599	16,000	32,000	16,000
25. SMP 579	8,000	16,000	8,000
26. SMP 597	8,000	8,000	4,000
27. SMP 533	64,000	32,000	16,000
28. SMP 581	32,000	16,000	8,000
29. SMP 596	16,000	16,000	16,000
30. SMP 542	64,000	64,000	4,000
31. SMP 594	64,000	64,000	16,000
32. SMP 587	>64,000	16,000	8,000
33. SMP 513	>64,000	32,000	32,000
34. SMP 574	>64,000	32,000	32,000
35. SMP 598	>64,000	32,000	32,000
36. SMP 508	>64,000	32,000	32,000
37. SMP 543	>64,000	64,000	32,000
38. SMP 578	>64,000	64,000	32,000
39. SMP 599	64,000	16,000	16,000
40. SMP 529	64,000	16,000	16,000
41. SMP 537	64,000	32,000	16,000

Strain	MCF 7	MCF 7 ADR	HCT-116
42. SMP 585	32,000	16,000	8,000
43. SMP 582	32,000	32,000	16,000
44. SMP 548	32,000	16,000	8,000
45. SMP 586	32,000	32,000	32,000

* NT = Not Tested
* MCF 7 = Breast cancer cell lines
* MCF 7 ADR = Taxol-resistant breast cancer cell lines
* HCT-116 = Colon-cancer cell lines
* - = No response

Figure 2. Growth of *Sorangium cellulosum* (SMP 44) in GNS liquid media.

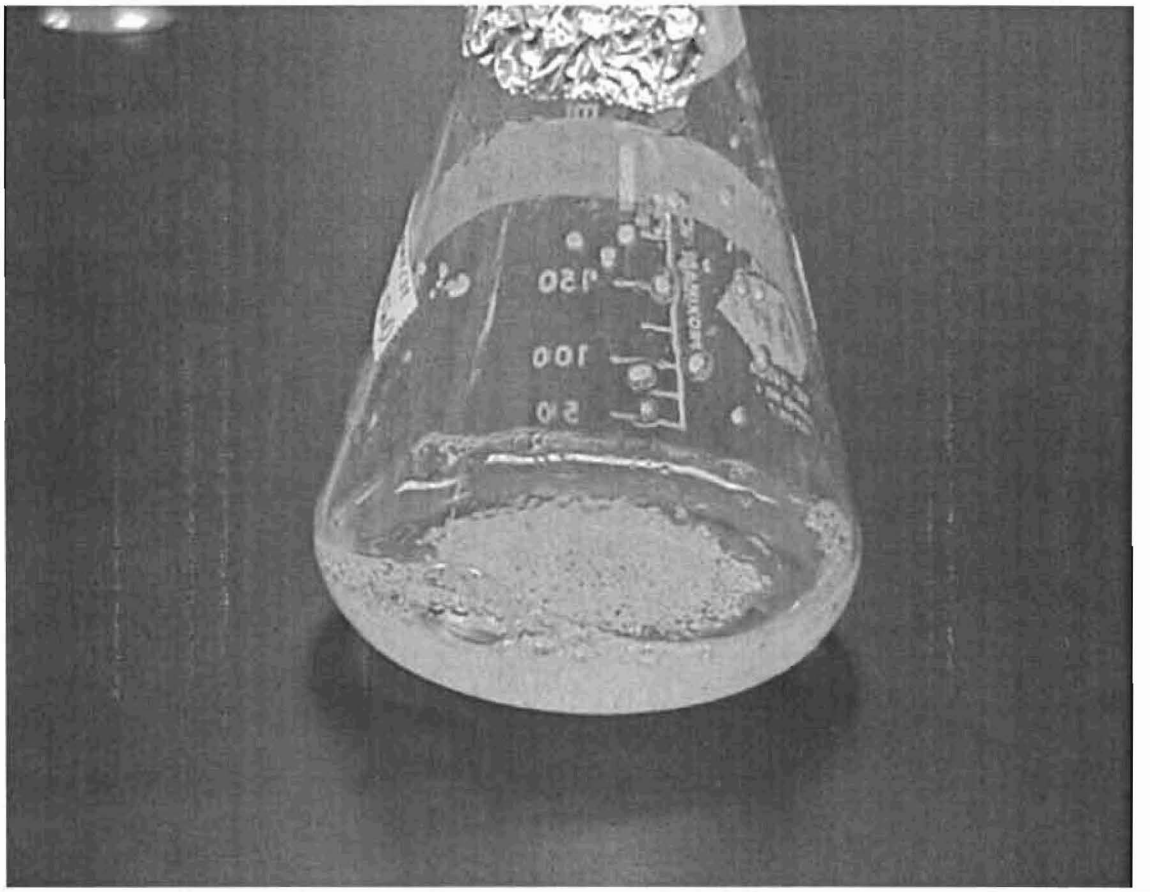

150
100
50

Figure 3. Growth of *S. cellulosum* on GNS agar media. *Escherichia coli* (plate on right) is included for comparison purposes.

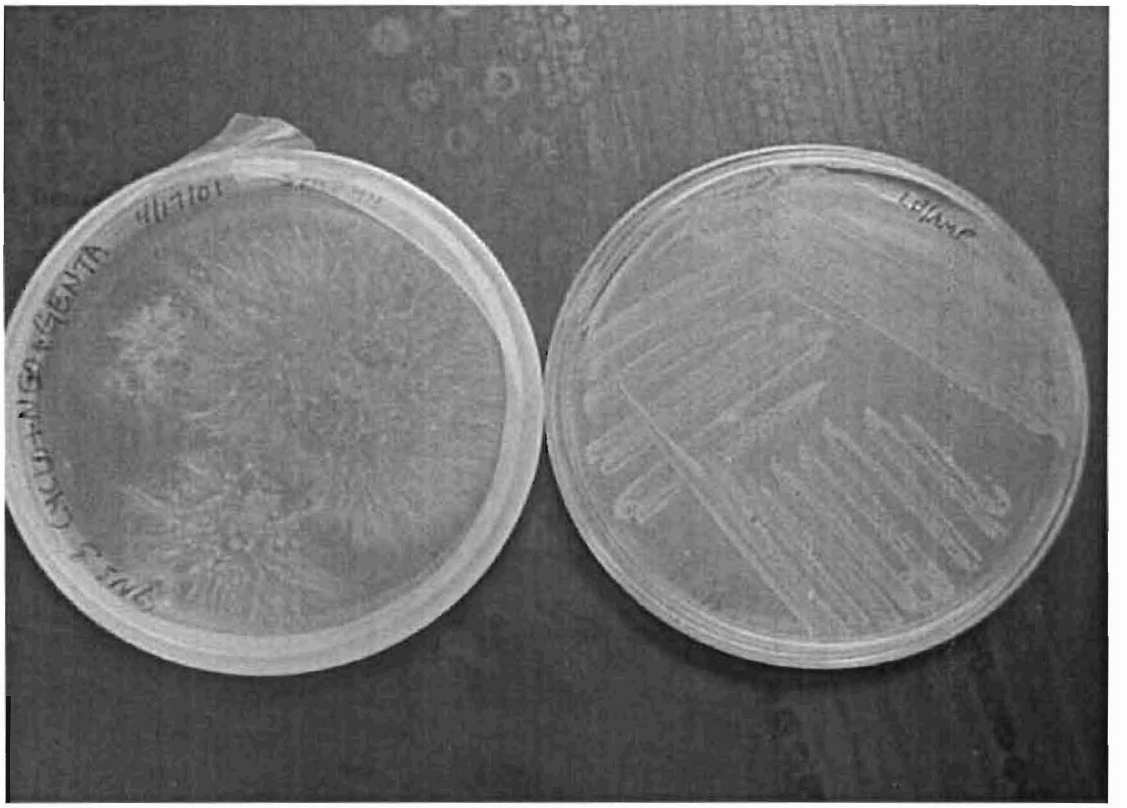


Figure 4. Illustration of where DNA primers target the methyltransferase domain of the epothilone gene cluster.


MT 1
MT 3
MT Domain
MT 4
MT 2
MT 5

Figure 5. Amplification of methyltransferase domain using designed primers. Lane 1, molecular weight markers; lane 2, MT1- MT4=300 bp amplicon; lane 3, MT1-MT2=300 bp amlicon; lane 4, MT1-MT5=500 bp amplicon; lane 5, MT3-MT2=250 bp; lane 6, MT3- MT4=250 bp amplicon; lane 7, MT3- MT5=450 bp amplicon.

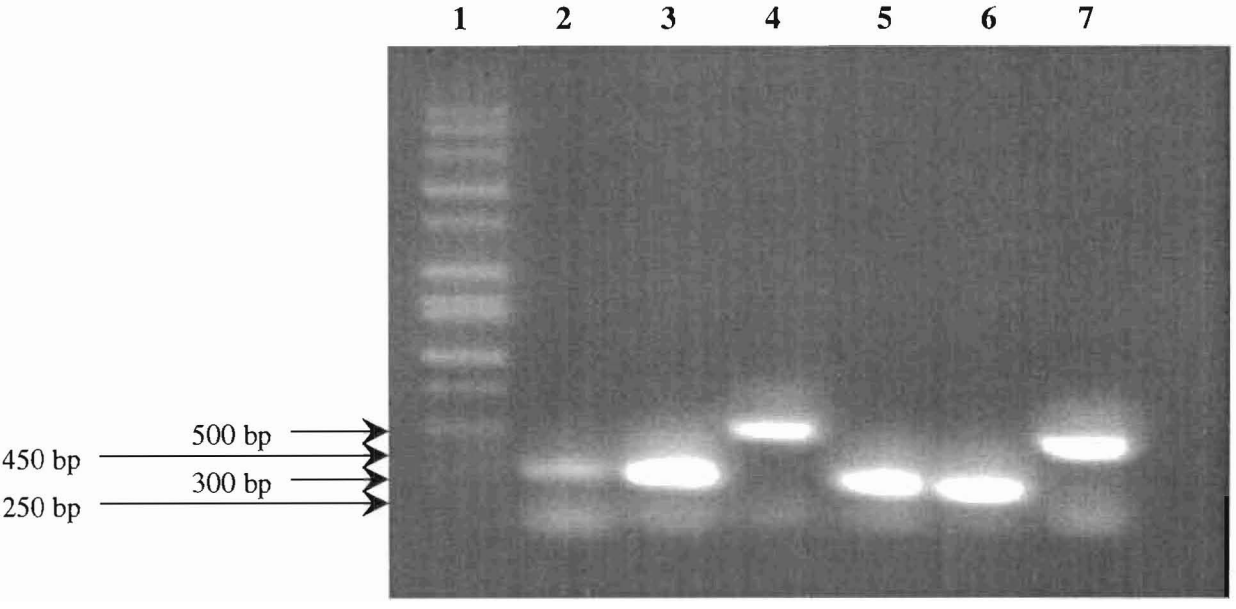

1
2
3
4
5
6
7
500 bp
450 bp
300 bp
250 bp

Figure 6. Primer pairs MT1 and MT2 amplified 300 bp DNA segment in epothilone positive strain. Lane 1, molecular weight markers; lane 2-3, 5-8, 300 bp amplicon from epothilone positive strains.

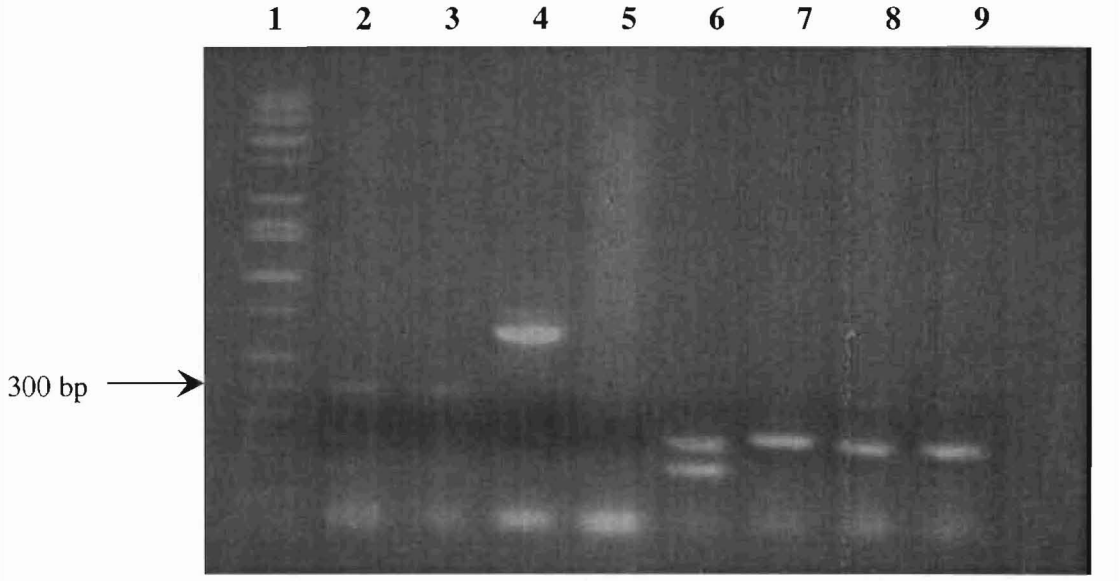
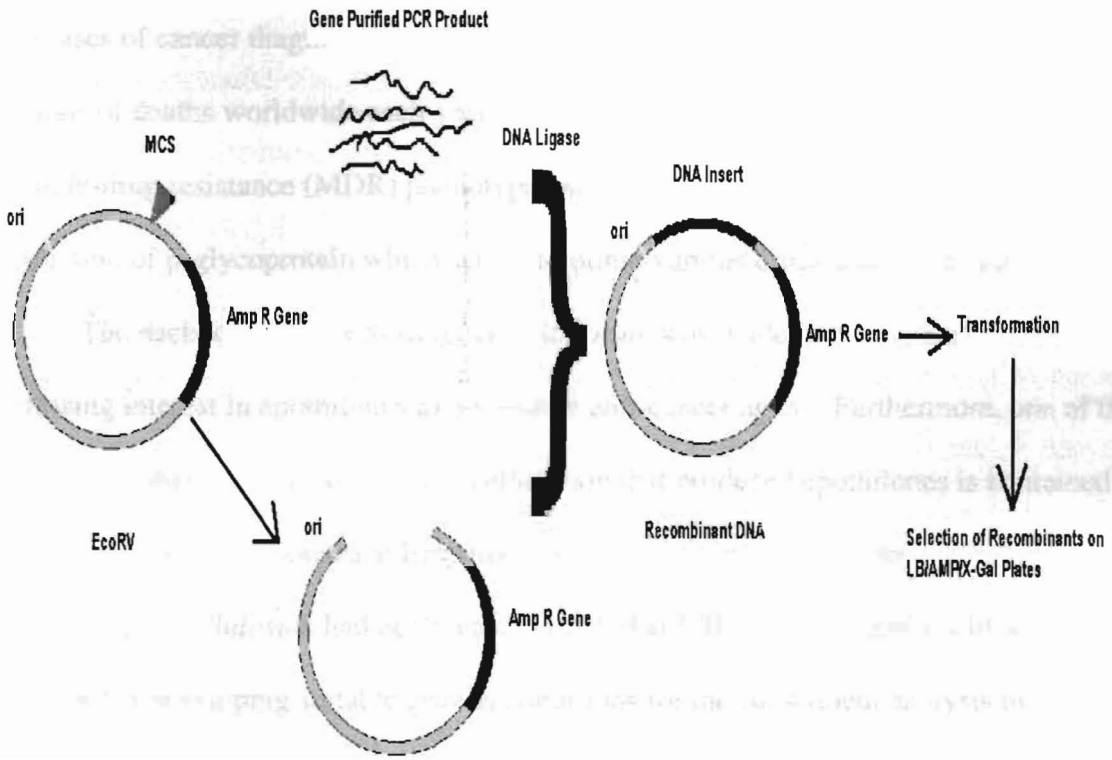

1
2
3
4
5
6
7
8
9
300 bp

Figure 7. General cloning scheme using the cloning vector pT7Blue3 and gel purified PCR products.


Gene Purified PCR Product
MCS
ori
Amp R Gene
EcoRV
ori
Amp R Gene
DNA Ligase
DNA Insert
ori
Amp R Gene
Recombinant DNA
Transformation
Selection of Recombinants on
LB/AMP/X-Gal Plates

Discussion

Cancer is an important health concern in the world today. It is the second most common cause of death in the United States, accounting for almost 25% of all deaths in 1998 (http://www3.cancer.gov/public/factbook98/relcan.htm). There are over 1 million new cases of cancer diagnosed in the USA each year, resulting in 500,000 deaths. Of the millions of deaths worldwide each year due to cancer, it is estimated that 90% exhibit a multiple-drug resistance (MDR) phenotype, which is characterized by the over production of p-glycoprotein which serves to pump various drugs out of the cell.

The decision to study *Sorangium cellulosum* was made primarily due to an increasing interest in epothilones as a valuable anti-cancer agent. Furthermore, one of the two original documented strains of *S. cellulosum* that produced epothilones is contained in a culture collection housed at Emporia State University (ESU). Since the parameters for growing *S. cellulosum* had not been established at ESU, considerable effort was expended in developing suitable growth conditions for the subsequent analysis of epothilones and other secondary metabolites produced by this organism. The most satisfactory method of initially isolating *S. cellulosum* employs the use of sterile filter paper as the sole carbon source. Since the only cellulolytic form of myxobacteria is the genus *Sorangium*, this media is selective for these organisms. Growth of a swarming-type organism on this media is likely *S. cellulosum*, but the possibility of cellulytic fungi cannot be ruled out.

Lyophilized organisms were propagated on a CNS plate containing filter paper as the sole carbon source. Once growth was established, strains were placed on a GNS agar plate. *Sorangium cellulosum* grows faster on this media due to the incorporation of

glucose as a sole carbon source, but still at a pace far behind other bacteria, such as *E. coli*. Each strain, however, takes on an individual appearance, which has contributed to the difficulties describing this genus. Currently, *S. cellulosum* is the only species described in this genus. Although many researchers agree that there are likely other species, difficulties encountered propagating these organisms have precluded such taxonomic differentiations. Due to the slow growth of these organisms, contamination by fungi is often problematic. Inclusion of cycloheximide into the media inhibits some, but not all, contaminating fungi.

A cellular suspension obtained from GNS agar media was used to inoculate 25 ml of liquid GNS media. Growth generally became evident after one week of incubation at room temperature with continuous shaking but varied depending on the strain. A pure *S. cellulosum* GNS culture (verified by gram-staining) was used to inoculate 250 ml of the same media containing 2% of XAD-16 absorber resin. This resin continuously adsorbs lipophilic metabolites as they are secreted by growing cultures. The uses of absorber resins of this type are common in obtaining metabolites from aqueous solutions (2, 9, 10, 11). Specifically, XAD-16 has been used previously to collect metabolites that contained antifungal activity against *Mucor hiemalis* from *S. cellulosum* liquid cultures (10). Since I did not know exactly when the majority of secondary metabolites would be secreted by each strain, I allowed cultures to reach well beyond stationary phase before harvesting the absorber resin. This methodology ensured that I would allow each strain ample opportunity to produce its battery of metabolites. After growth for approximately 4 weeks at room temperature with continuous shaking, both cells and the XAD-16 resin were harvested to ensure I collected all metabolites (both secreted and cell bound)

produced by the cells. Ethyl acetate was used as an elution agent as it ensures complete release of all lipophilic metabolites from the absorber resin and its use for these types of experiments is well established. Subsequently, the extraction method was changed to use methanol as an eluent instead of ethyl acetate. This was done at the request of a collaborator at the University of Kansas in order to provide extracts in a solvent more comparable with subsequent purification steps. Methanol, like ethyl acetate, releases all the lipophilic compounds from the resin. The development of the growth and extraction procedures used to collect the secondary metabolites, although time consuming, were instrumental to the successful completion of this thesis.

Myxobacteria as a group is well know producers of many bioactive secondary metabolites (27). I analyzed extracts prepared from various *S. cellulosum* strains for cytotoxic compounds inhibitory to a breast cancer cell line (MCF-7), a Taxol®-resistant breast cancer cell line (MCF-7ADR), and a colon cancer cell line, designated HCT-116. The cell lines MCF-7 and MCF-7ADR have previously been used as a model system to examine the clinical progression of human breast cancer (6, 7, 30, 31, 32). Analysis of the data obtained against MCF-7 cell line demonstrates extracts prepared from several different strains are cytotoxic at very high dilutions (> 64,000). Overall, this cell line was the most sensitive to the extracts. The MCF-7ADR cell line, which is derived from the MCF-7 cell line, demonstrated considerable more resistance to the extracts. The HCT-116 cell line was generally the most resistant of the three cell lines examined, although exceptions were noted. Interestingly, some extracts were extremely bioactive against one cell type, with minimal amounts of activity noted against the remaining two. Although I do not know the nature of the bioactive compound responsible for the cytotoxicity, I can

conclude from the data that many of the extracts do indeed posses cytotoxic compounds. *S. cellulosum* is known to produce a wide array of cytotoxic compounds, the nature and mechanism of action of most, however, remain unidentified (K. Gerth, personal communication). These data also do not confirm or deny the existence of epothilones. The results are, however, very encouraging as there is a potential for finding overproducers of epothilones or the discovery of a new drug.

There is considerable interest in identifying new strains of *S. cellulosum* which over produce epothilones. This is also especially relevant since the chemical synthesis of epothilones is not efficient. Currently, the only way to determine if a strain produces epothilones is to examine fermentation extracts by mass spectrometry, a costly and lengthy process. To investigate a more efficient approach to identify producers of epothilones, I attempted to develop a PCR-based approach to the identification of the epthilone gene cluster. Specifically, we targeted a rare methyltransferase (MT) domain present within module 8 of the epothilone gene cluster. This is an attractive target because it is known to be present in only three other polyketide synthase genes, of which the epothilone gene cluster is a member (14). Using a variety of primers designed to target within and adjacent to the MT domain, I initially determined optimal amplification conditions using cosmid clones containing pieces of the epothilone gene cluster (14). These conditions were subsequently used to specifically amplify a 300bp DNA segment contained within the epothilone gene cluster in an epothilone positive strain, but not in an epothilone negative strain. Results obtained were very promising, nonetheless, many more *S. cellulosum* strains need to be examined by this PCR-based approach to conclusively demonstrate the utility of this method. Strains in which amplicons are

present would be good candidates for further evaluation to determine if they are indeed producers of epothilones.

In summary, I have succeeded in examining 45 different strains of *S. cellulosum* from lyophilized cultures. Extracts were prepared from each culture and examined for the presence of cytotoxic compounds. Although the results appear promising, the nature of the cytotoxic compounds remains unidentified. The PCR-based screening approach to target MT domains of the epothilone gene cluster could revolutionize the way the initial screening is conducted for epothilones. Thus, experience gained in these investigations has provided the basic foundation for the analysis of subsequent strains of *S. cellulosum.*

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NOV. 19, 2004
Date

GROWTH AND SECONDARY METABOLITE PRODUCTION OF A HISTORICAL COLLECTION OF Sorangium cellulosu
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