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# A Synthetic Biology Approach to Engineering New Anticancer Agents

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# A SYNTHETIC BIOLOGY APPROACH TO ENGINEERING NEW ANTICANCER AGENTS

by

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ABSTRACT
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Histone deacetylase (HDAC) inhibitors are becoming increasingly valuable therapeutic agents in treatment of several types of malignancies. FK228 is a depsipeptde anticancer compound produced by *Chromobacterium violaceum* no. 968 through a nonribosomal peptide synthetase (NRPS)-polyketide synthase (PKS) hybrid assembly line. In the present study, reconstitution of the biosynthetic pathway responsible for the production of FK228 revealed cross-talk between modular PKS and fatty acid synthase. This pathway contains two PKS modules on the DepBC enzymes that lack a functional acyltransferase (AT) domain, and no apparent AT-encoding gene exists within the gene cluster or its vicinity. We reported through heterologous expression of the FK228 biosynthetic pathway in *E. coli* cells, two essential genes, *fabD1* and *fabD2*, both encoding a putative malonyl CoA acyltransferase component of the fatty acid synthase complex, are positively identified to be involved in FK228 biosynthesis. Either gene product appears sufficient to complement the "AT-less" PKS modules on DepBC for polyketide chain elongation. Concurrently a gene (*sfp*) encoding a putative Sfp-type phosphopantetheinyltransferase was identified to be necessary for FK228 biosynthesis as well. Importantly, engineered *E. coli* strains carrying variable genetic components produced significant levels of FK228 under both aerobic and anaerobic cultivation

conditions. Discovery of the *trans* complementation of modular PKSs by housekeeping ATs reveals natural product biosynthesis diversity. Moreover, demonstration of anaerobic production of FK228 by an engineered facultative bacterial strain validates our effort toward engineering of novel tumor-targeting bio-agents. The second part of the dissertation focused on the engineering of cell penetrating peptides (CPPs) with anticancer activity. A fusion protein combining the transmembrane activity of a bacterial CPP with a eukaryotic mitochondrial disrupting protein (MDP) was constructed to become a new CPP-MDP protein with anticancer activity. A new *E. coli* BL21(DE3)-based expression system that facilitates the secretion of CPP fusion proteins into the growth media for rapid and efficient protein purification was also created. This protein secretion system utilizes induced L-form bacterial spheroplasts to generate a yield of 32 mg/L of CPP-MDP directly from the media. Construction of an L-form based *E. coli* BL21 (DE3) protein secretion system and a modular CPP expression system allows future rapid CPP-fusion protein production with new fusion protein partners to develop new CPP-based anticancer agents.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

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CHAPTER 1

# INTRODUCTION

## 1.1 Targeting Cancer

Cancer continues to be a major public health problem and the development of effective cancer treatments with no or few side effects remains one of the most difficult scientific challenges in medicine. Cancer occurs in many tissue types (such as breast cancer, skin cancer, or leukemia) and the effectiveness of conventional treatments (surgical removal, radiotherapy and chemotherapy) varies (1). Consequently, there have been intensive efforts to develop alternative cancer therapies including bone marrow transplantation, immunotherapy, gene therapy, hormone therapy, and inhibition of angiogenesis (formation of blood vessels) or specific protein targets (mostly kinases) in key signaling pathways, with mixed success (2). Many current therapies using radiation, platinum and nucleic acid analogs have activity against cancer cells but are also toxic to normal tissue. Furthermore, many cancer cell lines have developed resistance to one or all of these treatments, limiting their effectiveness (3). Current goals in the development of new anticancer agents combine high activity toward cancer cells while limiting toxicity to preserve healthy tissue. In order to overcome issues of toxicity and resistance, research has focused on developing new therapies that target specific cellular pathways in cancer cells such as apoptosis, cell cycle regulation, mitochondrial growth and repair, endoplasmic protein folding and transport and reactive oxygen species (ROS) repair.

The trademark of cancer cells is the development of pro-cancer genetic and epigenetic changes that uncouple the regulatory controls of both cellular division and cell death. These genetic mutational and transcriptional activities impart new survival advantages over normal tissue. The aberrant cancer cells gain the ability to infinitely divide and out-compete surrounding cells for nutrients. Furthermore, the new genetic

influences lead to the rapid growth of tumors and the physiologic changes that preserve the abnormal cells.

## 1.2 Epigenetics of Cancer

Traditional cancer research focuses on genetic regulation and the respective mutations that lead to the various cancer phenotypes. The field of epigenetics relates to the inherited genetic expression patterns associated with environmental adaptation (4). Both the genetic and epigenetic changes are important determinants of cancer development and progression, and epigenetic changes may preclude mutational changes leading to cancer. In comparison to inherited genetic changes, inherited epigenetic gene transcription levels are environmentally linked adaptations and are reversible chromosomal structure changes that exert significant control on chromosomal activation and gene regulation. Epigenetic chromosomal changes can favor genetic mutation, and enhance gene mutation effects by deactivating a chromosomal region related to the activation of oncogenes and tumor suppressor genes (5). Current epigenetic research therefore focuses on determining the effect of gene mutations or DNA sequence changes from epigenetic changes with regard to onset and progression of cancer. As with genetic mutations, epigenetic changes may alter more than one pathway leading to the development of a pro-cancerous state or cancer. In many cases, frequent epigenetic changes may create a cancer susceptible cell which is prone to develop genetic mutation leading to a cancerous state (4).

The reversible chromosomal modifications are capable of converting a “malignant state” of a cell to a “normal state” either through intrinsic factors or with extrinsic factors

targeting epigenetic chromosomal modifications. Two major epigenetic changes in the cell targeted to return a malignant cell to a normal gene expression state are DNA methylation and modifications of the histone tails including acetylation (4, 6) have been elucidated. It is hypothesized that both DNA methylation and histone acetylation result in similar changes in the control of regional chromosomal activation. DNA methylation is controlled by DNA methyl transferases (DMT), while histone tail modifications are either methylated or acetylated. The methyl regulation of the histone tails are coordinated by reversible histone methyl transferases (HMT) that adds or removes methyl groups from the N terminal histone tails. Alternatively, the histone acetyl transferases (HAT) add acetyl groups onto lysine residues on histone tails and histone deacetylases (HDAC) catalyze removal of acetyl groups from lysine residues on histone tails.

The major epigenetic changes associated with cancer are related to the regulatory activities of HAT and HDAC which usually determine chromosomal activity states. DNA exists in one of two structural states; euchromatin (open for transcription) or heterochromatin (closed for transcription). Increased euchromatin based transcription activity is related to HAT activity by increasing the acetylation of histone tail lysine residues, which decreases the affinity of DNA to histones, resulting in an open transcription state. In contrast, HDAC activity removes acetyl groups from histone tails, increasing the affinity of DNA to histones causing an increase in chromatin compaction preventing transcription (4, 6) (**Figure 1**). The reversion of deactivated chromosomal regions is the focus of epigenetic therapy aiming to reactivate transcription of tumor suppressor genes and other genes that are related to a normal cell gene expression state (7). Two common epigenetic therapies currently on the market involve inhibitors of

DMTs and HDACs. The correlation between histone tail lysine acetylation and chromatin remodeling producing an open transcription state was demonstrated to be an effective epigenetic anticancer therapy where HDAC inhibition resulted in an increased open chromatin structure (6). The effectiveness of HDAC inhibition has led to the continued search and development of new compounds targeting the specific HDACs to elucidate the chromosomal remodeling pathways.

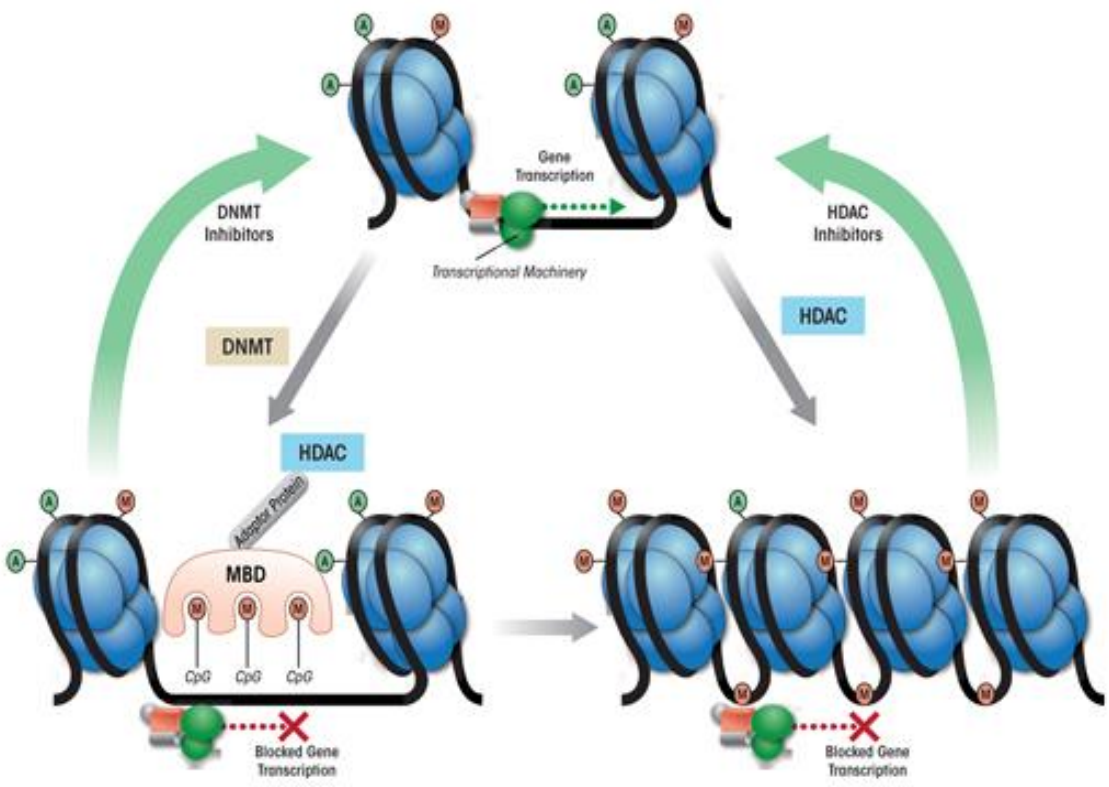


Figure 1. The methylation and acetylation epigenetic modifications of DNA and chromosomes regulate the gene transcriptional processes. Normally, the conversion of genetic information encoded in the DNA into gene products via transcription, requires the DNA to be in an "open" state where histone proteins (light blue) are modified by the addition of acetyl groups (green). Acetylation of the histones tails results in a relaxed chromatin configuration allowing the transcriptional machinery access to the DNA.

DNA methyltransferases (DNMTs), can add methyl groups (red) to the DNA at specific sequences of DNA causing the recruitment of methyl binding domain (MBD) protein complexes that includes repressor proteins, such as histone deacetylases (HDAC). The HDACs remove acetyl groups from histone proteins resulting in condensed chromatin that blocks the transcriptional machinery. Therefore, both DNMT and HDAC cause a reduction in gene transcription and may deactivate key tumor suppressor genes. New therapies targeting DNMTs and HDACs that inhibit these enzymes can return the chromatin to a relaxed state, allowing gene transcription. Adapted from Moonat and Pandey, 2012 (8).

## 1.3 Human HDAC Classes and Function

The discovery of chromosomal inactivation associated with tumor suppressor gene silencing has identified HDACs as a potential target in epigenetic cancer therapy (9). The human HDAC superfamily is divided into classes I, II, III and IV based on structure, phylogeny and biological activity (10). Classes I, II and IV are termed "classical HDACs" containing zinc as a cofactor in the catalytic pocket. The classical HDACs reaction cleaves the amide bond between ε-amino group on lysine residues and the terminal acetyl group in the N-terminal tail. Class III HDACs are also called the sirtuins and contain nicotine adenine di-nucleotide (NAD)-dependent enzymes, including the silent information regulator (Sir)-2 family (11). Currently, only the classical HDAC family has been targeted for epigenetic therapy and many inhibitors of this family have progressed through clinical trials and on to the market as therapeutic agents (12, 13).

Currently, the human genome contains 18 HDACs belonging to the four superfamily classes. The Class I HDACs contains HDAC1, HDAC2, HDAC3 and HDAC8 and are localized only in the nucleus. While, Class II consists of HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10 and are found in the cytoplasm but can also migrate to the nucleus. Class IV consists of the hybrid HDAC11, that shares similarity with both class I and class II HDACs. The remaining seven HDAC members are zinc-independent and NAD-dependent enzymes belonging to Class III HDACs.

Structural examination of Class I HDACs identified the catalytic mechanism associated with acetyl transferase activity and inhibition. The catalytic pocket responsible for hydrolysis of acetyl-lysine amide bond contains a $Zn^{2+}$ ion. The ion is bound to two negatively charged aspartic acid residues and one histidine residue, leaving open two coordination sites for acetyl-lysine interactions from histone tails (14). The crystal structure of the HDAC8 catalytic pocket was shown to resemble a long tunnel largely composed of hydrophobic residues. This hydrophobic tunnel was postulated to make contact with the lysine residues from histone tails and guide the terminal acetyl group to the catalytic core. Also, the amino acids surrounding the entrance to the catalytic pocket of HDACs are responsible for surface recognition interactions critical to selectivity. The structural information of the catalytic center and surrounding pocket provide important information related to the design of HDAC inhibitors (14, 15).

## 1.4 Effects of HDAC Inhibition

HDAC inhibition has been connected with broad epigenetic changes with various anticancer effects such as increase in genome wide gene expression, anti-angiogenesis,

cell cycle arrest, cellular differentiation, apoptosis and tumor regression (**Figure 2**) (16, 4). The therapeutic action of HDACs inhibition leads to hyper-acetylation of histones which reduces the affinity of DNA to histones because of the neutralized positive charge on lysine residues. The euchromatin open structure is thought to increase the gene expression of tumor suppressor genes which is one of the various mechanisms suggested for anticancer effects stemming from HDAC inhibition (17). In particular, acetylation of histones has been shown to direct chromatin assembly, DNA recombination and repair (18). Also, inhibition of HDACs may affect both histone HDACs and non-histone substrates leading to similar results (16). While there is considerable difference in the mechanisms proposed for the anticancer effects, new evidence has shown HDAC inhibition increases the expression of pro-apoptosis signals suppressed in cancer cells (19). Also, anticancer chromosomal changes are dependent on which HDACs they inhibit. HDAC inhibitors may be termed as pan-HDAC inhibitors which inhibit all classical HDACs, or as isoform-selective inhibitors which inhibit only a certain class (20). There is evidence suggesting that pan-HDAC inhibitors affect multiple cellular pathways that are dependent on acetylation of histone tails and are not related to onset or progression of cancer. Events resulting from nonspecific HDAC inhibition may be circumvented with application of isoform-specific HDACi (17). This evidence suggests isoform-specific HDAC inhibitors may provide critical insight into the regulation of epigenetic chromosomal changes and identifies the need for new HDAC inhibitors that limit broad changes in expression patterns.

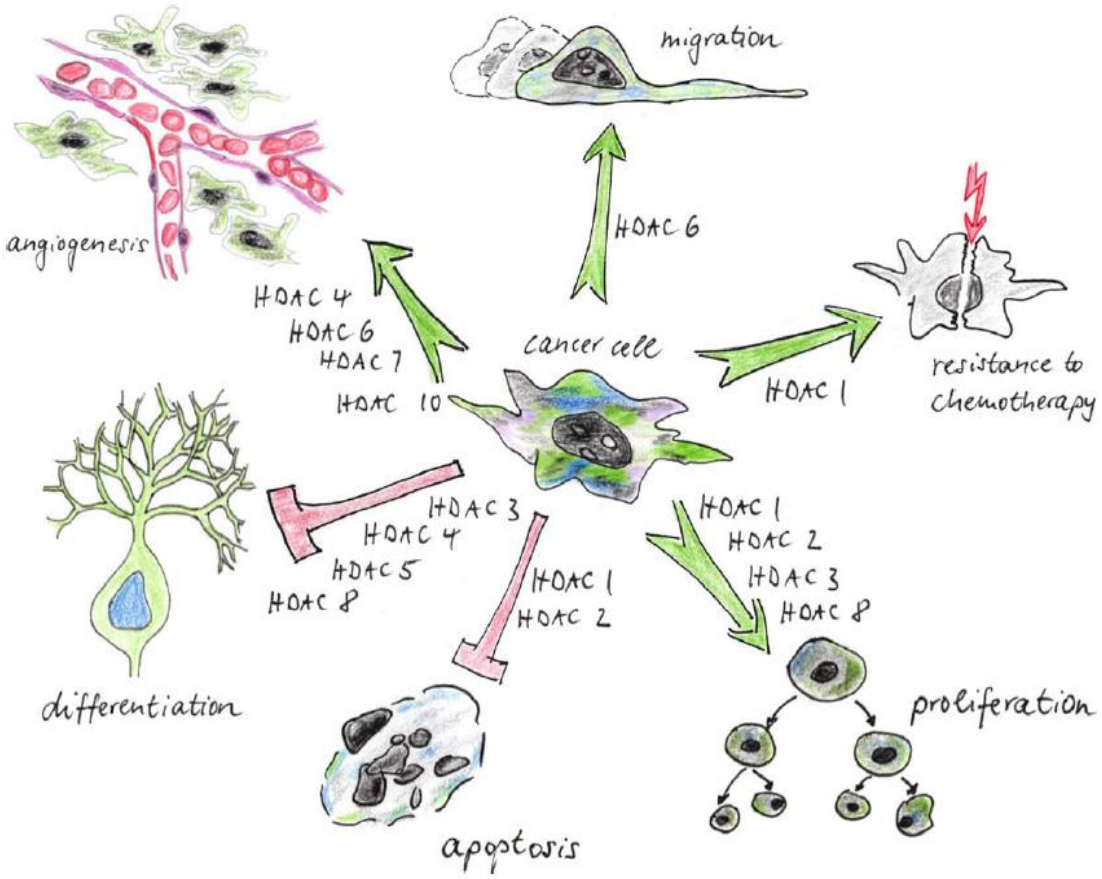


Figure 2. Pathway specificity and expression levels of class I and Class II HDACs lead to cancer phenotypes. Decreased expression (red lines) of specific HDACs result in apoptosis, cell cycle arrest and growth inhibition. In contrast, overexpression (green arrows) of specific HDACs increases proliferation, resistance to chemotherapy, cancer cell migration and angiogenesis. FK228 has strong inhibitory action on class I HDAC 1 and HDAC 2, reversing the HDAC specific cancer phenotypes. Adapted from Witt, O. et al. 2009 (21).

## 1.5 Natural Product Biosynthesis by NRPS and PKS Systems

The broad class of natural products (secondary metabolites) has shown to be a source of new HDAC inhibitors. Natural products are considered to be non-essential to

the growth and development of bacteria but may provide selective survival advantages. In bacteria, secondary metabolites are often biosynthesized starting in middle to late logarithmic growth and into the stationary growth suggesting a physiological role in survival and maintenance. A majority of secondary metabolites are produced by three common biosynthetic systems; nonribosomal peptide synthetases (NRPS), polyketide synthases (PKS) and hybrid NRPS-PKS systems (22).

Natural product biosynthesis from modular NRPS and PKS systems are produced in a similar "assembly line" fashion where NRPS and PKS multifunctional enzyme complexes are arranged in modules. Typically, one substrate is added per module over the biosynthetic pathway with successive substrate addition until the product reaches full length. The full length product is released by cyclization or hydrolysis. Structure of the biosynthetic product depends on the number of modules, the nature of substrates incorporated, accessory enzyme modification and the length of the final product. Modules are further defined by the individual protein domains with specific function integrated into the module. The unique nature of the protein domains within the module dictates the length, substrate specificity and reactions that occur at each module. A minimal NRPS module is composed of three distinct domains: adenylation (A) domain responsible for recognizing a specific amino acid substrate and adding it to the thiolation (T, also known as peptidyl carrier protein, PCP) domain that carries the substrate and moves it to the condensation (C) domain which is responsible for peptide bond formation between two successive substrates. In contrast, a minimal PKS module consists of domains performing analogous functions to the NRPS module. It consists of an acyl transferase (AT) domain similar to A domain that determines acyl-CoA substrate

specificity, a thiolation (T, also known as acyl carrier protein, ACP) domain, and ketosynthase (KS) domain catalyzing the formation of a C-C bond through a claisen condensation reaction between successive substrates (22,23). The hybrid NRPS-PKS system combine modules from each biosynthetic system connected by thiolation domains that allow the addition of substrates by upstream KS or C domains.

A requirement for all PKS and NRPS biosynthetic pathways is the conversion of the *apo*-carrier proteins, ACP and PCP respectively, to the active *holo* form through the attachment of a 4'-phosphopantetheinate (Ppant) prosthetic group. The transfer of the prosthetic group is catalyzed by a family of $Mg^{2+}$ dependent 4'-phosphopantetheinyl transferases to a conserved serine within the consensus sequence GX(D/E/H)S motif on the ACP/PCP (24, 25). The newly introduced thiol (-SH) group of the Ppant prosthetic group acts as a nucleophile for acylation by a substrate, which may be acyl-CoA or malonyl-CoA derivatives for the fatty acid and PKS, or aminoacyl-AMPs for the peptide and depsipeptide synthetases. Therefore, the Ppant modification is a critical feature required by both NRPS and PKS modules that allows substrates to be tethered to a carrier protein (PCP or ACP) via a thioester bond.

The mechanism of chain elongation of both NRPS and PKS systems occurs in an assembly line fashion while substrates are bound as thioesters to the carrier protein. In NRPS systems, the nucleophilic attack from the downstream aminoacyl-*S*-T on the upstream peptidyl-*S*-PCP results in chain elongation at that condensation domain between the modules. While in PKS systems, nucleophilic attack from the downstream thioester enolate on the upstream acyl-*S*-ACP leads to chain elongation by the upstream KS domain. Therefore in accordance with the colinearity rule, the number of modules in an

enzymatic assembly line of NRPS and PKS systems usually determines the chain length and the identity of the module A or AT domains define the substrates incorporated. The identification of the substrates by A and AT domains dictates the structure of the secondary metabolite. Once the biosynthetic product reaches its full length, it can either be cyclized or hydrolyzed by a (type I) terminal thioesterase (TE) to release the product. In many secondary metabolite biosynthetic systems, tailoring reactions or post biosynthetic modifications like glycosylation, hydroxylation and oxidative cyclization occur after assembly line synthesis while in other systems the presence of embedded accessory domains may modify the final structure of the natural product (22, 23).

Interestingly, PKS domains share an ancestral relationship to fatty acid biosynthetic domains and the PKS core domain structure can be modified to possess similar tailoring enzymes. The fatty acid biosynthesis pathway consists of a core set of enzymes including a ketosynthase (KS), an acyl carrier protein (ACP), a keto-reductase (KR), a dehydrogenase (DH), enoyl reductase (ER), an acetyl-CoA acyl transferase (AT), and a malonyl-CoA:ACP acyl transferase (MAT). In comparison to the minimal PKS domains consisting of a ketosynthase (KS), acyl carrier protein (ACP) and an acyl transferase (AT/MAT) for complete polyketide biosynthesis, the polyketide intermediates can also be modified by tailoring enzymes derived within the PKS gene cluster or from fatty acid biosynthesis, such as KRs, DHs, ERs, aromatases (AROs), and cyclases (CYCs) to yield a diverse array of natural products (26). Additional modifications by other tailoring enzymes such as dimerases, P450 monooxygenases, methyltransferases, and glycosyltransferases can further elaborate the structure of the natural product. New

modifications can contribute to significantly increase structure diversity and function of a molecule and has been shown to increase antibiotic activity (27).

Whereas the NRPS and PKS multimodular and multienzyme assembly lines produce nonribosomal peptides and polyketides respectively, there are also examples of mixed NRPS-PKS systems in nature which biosynthesize hybrid NRPS-PKS natural products. The modular structure of NRPS and PKS biosynthetic systems and the compatible chemistry at the NRPS-PKS interface permits the biosynthesis of these hybrid natural products (22, 23).

The dedicated function of biosynthetic pathways requires their streamlined genetic organization and this may reflect their coordinated regulation. Thus it is not surprising that genes required for the biosynthesis, tailoring and export of natural products are usually clustered (28, 29).

## 1.6 Discovery and Bioactivities of the Natural Product FK228 as an HDAC Inhibitor

The search for new cyclic depsipeptides with increased anticancer activities shows great promise for epigenetic anticancer treatment (17, 30, 31, 32). The FK228-family of class specific HDAC inhibitors, consisting of FK228, thailandepsins and spiruchostatins, are natural products produced by Gram-negative bacteria. The function of cyclic depsipeptide within these bacteria is not clearly understood. The characteristic features of the FK228-family of compounds contain a bicyclic depsipeptide architecture, presence of an ester linkage (depsipeptide bond) and a disulfide bond to close the second ring structure **(Figure 3)**.

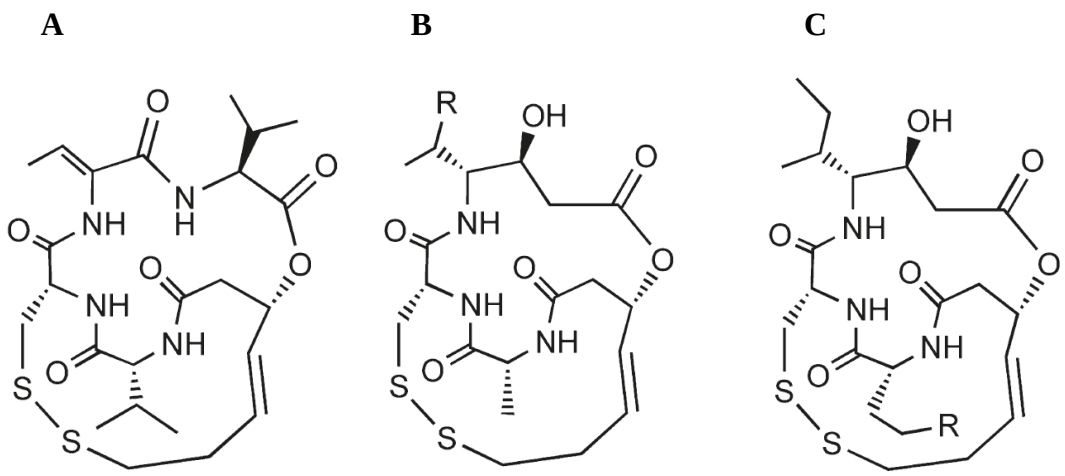


**Figure 3**. Structures of FK228-family of HDAC inhibitors. (**A**) FK228, (**B**) Spiruchostatin A ($R=CH_3$) and Spiruchostatin B ($R=CH_2CH_3$), (**C**) Thailandepsin A ($R=SCH_3$) and Thailandepsin B ($R= CH_2CH_3$).

FK228 was discovered as a fermentation product of *Chromobacterium violaceum* no. 968, a Gram-negative cocco-bacillus opportunistic pathogen. Furumai discovered FK228 while screening for agents that could reverse malignant phenotype of Ha-*ras* cells to normal (33). In addition to significant anticancer activity against a variety of cell lines, FK228 also showed weak antibiotic activities (30, 33). FK228 exists in two forms. First, it acts as an inactive prodrug with its intact disulfide bond to diffuse across membranes. This is a key property that affects its bioavailability. Upon entry into the cell, intracellular reduction of the disulfide bond results in the formation of the active form with free sulfhydryl groups that can react with the active site zinc of class I HDACs (30). HDAC inhibition ultimately results in a cascade of cellular changes that produce the anticancer effects and regression of cancer (34). Furthermore, FK228 was granted FDA approval for the treatment of cutaneous T-cell lymphoma (CTCL) and peripheral T-cell lymphoma

(PTCL). This signifies the important role of cyclic depsipeptides in anticancer therapy and validates research towards developing and engineering structural analogs with improved therapeutic properties (34).

## 1.7 Discovery and Function of Cell Penetrating Peptides

Cell-penetrating peptides (CPPs) are composed of relatively short basic peptides of 5 to 40 amino acids and possess the ability to cross the cell membrane through a number of mechanisms. The membrane translocation process appears to be dependent on the cell type and the cargo that may be conjugated to the CPP (35). Recent studies suggest that most CPPs enter eukaryotic cells through endocytosis uptake mechanisms, however small arginine-rich CPPs have been shown to cross the plasma membrane in an energy independent process (36). CPPs can utilize both the clathrin-dependent and clathrin-independent endocytosis pathways to gain entrance into the cell and are able to escape from the endosomes to the cytoplasm. Translocation from the endosome appears to be facilitated by endosome acidification allowing the CPP to cross the lipid bilayer (35, 37, 38). Recently, YopM, a protein associated with *Yersinia enterocolitica* infection was shown to have cell translocation capabilities. Normally, YopM and other pathogenic proteins are passed through the cell membrane via the type III secretion system. This activity was attributed to two highly cationic amino acid regions within two alpha helices in the N-terminus that demonstrated the ability to translocate a fused protein cargo across the cellular membrane (39).

Table 1. Common CPP sequences

| Names | Sequences |
|---|---|
| Penetratin | RQIKIWFQNRRMKWKK |
| Tat | GRKKRRQRRRPPQ |
| Transportan | GWTLNSAGYLLGKINLKALAALAKKIL |
| MAP | KLALKLALKALKAALKLA |
| KALA | WEAKLAKALAKALAKHLAKALAKALKACEA |
| P1 | MGLGLHLLVLAAALQGAWSQPKKKRKV |
| MPG | GALFLGFLGAAGSTMGAWSQPKKKRKV |
| Pep-1 | KETWWETWWTEWSQPKKKRKV |
| Arg9 | RRRRRRRRR |
| hCT | LGTYTQDFNKFHTFPQTAIGVGAP |

The membrane translocation activity of CPPs and CPP fusion proteins represents new engineering opportunities with vast therapeutic applications. CPPs have been shown to deliver a variety of cargo including short inhibiting RNA, plasmids, proteins, liposomes, drugs and fluorescent markers, and represent a powerful strategy for drug delivery. This strategy can be used to increase cancer cell activity of these anticancer agents by facilitating their delivery into tumor cells. Furthermore, the CPP transmembrane activity showed that drug resistant cancer cells could be reverted back to a drug sensitive phenotype by conjugating CPP with Taxol and other known anticancer drugs by increasing intracellular drug concentrations (40). Also, CPPs conjugated with protein or protein domains can be used to specifically target pathways associated with a pro-cancer cell state or apoptosis pathways leading to cell death of cancer cells. While cell targeting with most CPPs is ambiguous, recent studies identified CPPs with tumor targeting capabilities (41). Finally, fluorescent labeled-CPP conjugates have been developed for surgical cancer therapy. The fluorescent-CPP with an antibody specific for the cancer cells facilitates the endosomal escape of the conjugate preserving the

fluorescent signal within the cancer cell cytoplasm for easy surgical removal of only tumor tissue. Therefore, these unique peptides offer a variety of engineering options for the development of new anticancer agents and many other therapeutic applications.

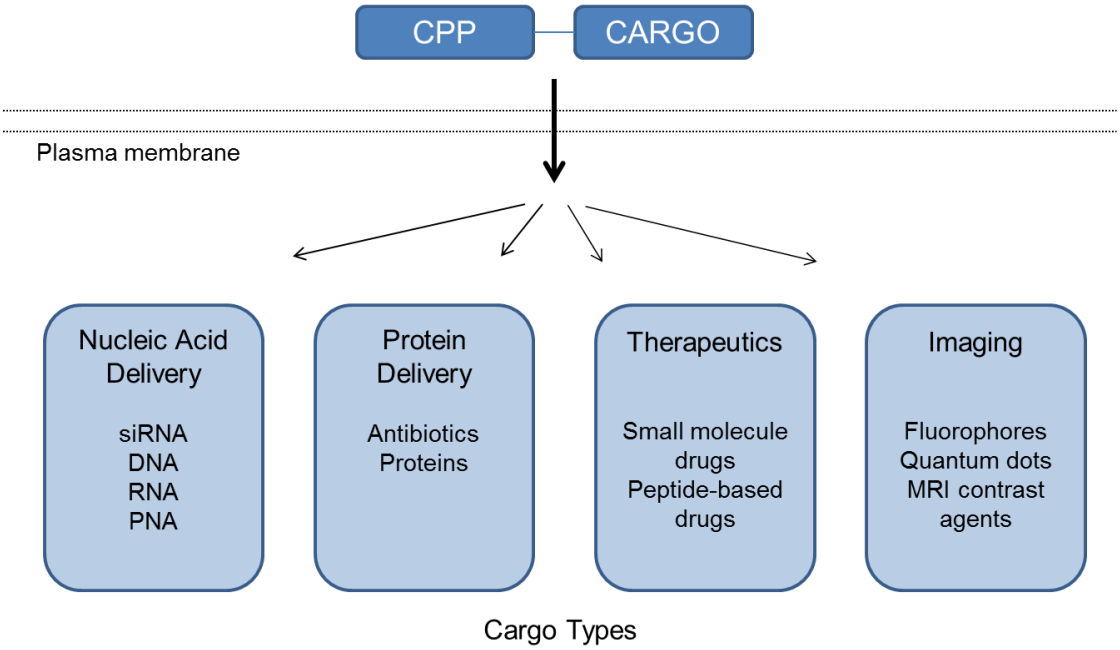


Figure 4. Applications of CPP-cargo constructs. Cell penetrating peptides are capable of transporting a variety of cargo across the cell membrane. The variety of cargo makes CPPs a valuable shuttle targeting multiple cellular structures and pathways. These characteristics have shown valuable in gene silencing, protein delivery, drug delivery and cellular imaging. Adapted from Koren and Torchilin, 2012 (42).

## 1.8 Targeting Mitochondrial Apoptosis Pathway for Cancer Therapy

The development of CPP anticancer peptides requires the identification of cellular targets capable of inducing cell death. The translocation of CPPs to the cancer cell cytoplasm permits the targeting of the mitochondrial apoptotic pathway or downstream caspases associated with the death receptor pathway. Normally cell death can be

induced by the binding of apoptosis-inducing ligands (such as FasL) to specific death receptors (such as FAS) located at the cell surface. The transmembrane receptor FAS, contains a cytoplasmic death domain where FADD (Fas-associated death domain) can bind in presence of FasL, and recruit pro-caspase 8. This recruitment results in the activation of caspase 8 that initiates caspase 3 activation and subsequent activation of caspase activated DNase (CAD). Caspase 3 cleaves I-CAD, the inhibitor of CAD releasing it to enter the nucleus and degrade DNA. Caspase 8 also cleaves Bid protein, resulting in a truncated Bid (tBid) (43). Upon cleavage, tBid can dimerize with either Bax or Bad causing the release of cytochrome c from mitochondria (44). However, the mechanisms leading to mitochondrial membrane disruption and release of pro-apoptotic factors remain unclear. The disruption of the mitochondrial membrane leads to the release of cytochrome c, Smac/DIABLO (second mitochondrial-derived activator of caspase; Diablo: direct IAP-binding protein with low pI), AIF (apoptosis inducing factor) and procaspases. The inhibitor Bcl2, prevents the release of cytochrome C and AIF in the cytoplasm. Once in the cytoplasm, cytochrome c binds to Apaf-1 (apoptosis protease activating factor) forming the apoptosome, which activates caspase 9 and downstream effector caspases. Smac/DIABLO binds to IAP (inhibitors of apoptosis) and prevent them from inhibition of the caspase 9 and caspase 3 activation (43). AIF has an indirect role in chromosome degradation as it activates endonuclease G, a DNase that moves from the mitochondria to the nucleus during apoptosis. Interestingly to note, the mtDNA is not fragmented during apoptosis.

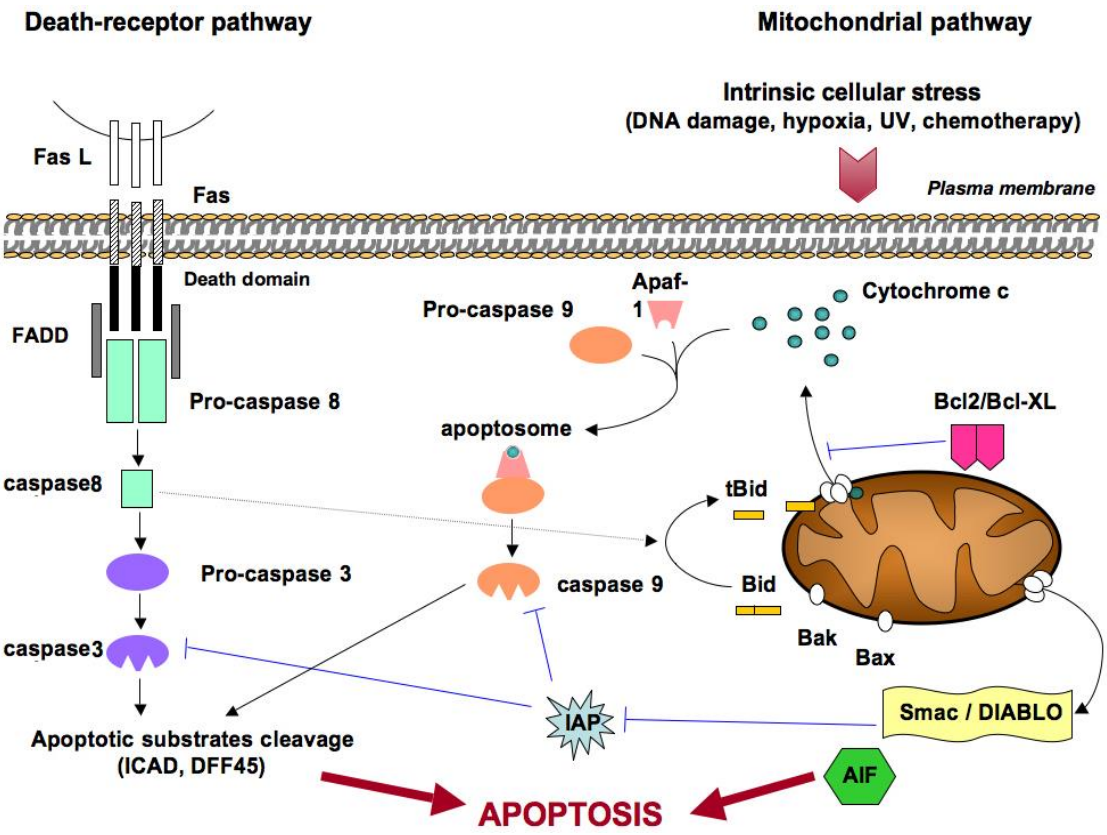


Figure 5. Death-receptor and mitochondrial apoptosis pathways. Exploration of the two apoptosis pathways identified a number of intracellular targets to induce apoptosis with CPP constructs. Adapted from Bayir and Kagan, 2008 (43).

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# CHAPTER 2

# RECONSTITUTION OF FK228 BIOSYNTHETIC PATHWAY REVEALING CROSS-TALK BETWEEN MODULAR POLYKETIDE SYNTHASES AND FATTY ACID SYNTHASE

Content of this chapter is a reformatted version of **Wesener, S. W., Potharla, V. Y., and Y. Q. Cheng.** 2011**.** Reconstitution of FK228 Biosynthetic Pathway Revealing Cross-Talk between Modular Polyketide Synthases and Fatty Acid Synthase. Appl Environ Microbiol **77**: 1501-1507.

## 2.1 Abstract

Functional cross-talk between fatty acid biosynthesis and secondary metabolism has been discovered in several cases in microorganisms, none of them however involves a modular biosynthetic enzyme. Previously we reported a hybrid modular nonribosomal peptide synthetase (NRPS)-polyketide synthase (PKS) pathway for the biosynthesis of FK228 anticancer depsipeptide in *Chromobacterium violaceum* no. 968. This pathway contains two PKS modules on the DepBC enzymes that lack a functional acyltransferase (AT) domain, and no apparent AT-encoding gene exists within the gene cluster or its vicinity. We report here that, through reconstitution of the FK228 biosynthetic pathway in *E. coli* cells, two essential genes, *fabD1* and *fabD2*, both encoding a putative malonyl CoA acyltransferase component of the fatty acid synthase complex, are positively identified to be involved in FK228 biosynthesis. Either gene product appears sufficient to complement the "AT-less" PKS modules on DepBC for polyketide chain elongation. Concurrently a gene (*sfp*) encoding a putative Sfp-type phosphopantetheinyltransferase was identified to be necessary for FK228 biosynthesis as well. Most interestingly, engineered *E. coli* strains carrying variable genetic components produced significant levels of FK228 under both aerobic and anaerobic cultivation conditions. Discovery of the *trans* complementation of modular PKSs by housekeeping ATs reveals natural product biosynthesis diversity. Moreover, demonstration of anaerobic production of FK228 by an engineered facultative bacterial strain validates our effort toward engineering of novel tumor-targeting bio-agents.

## 2.2 Introduction

Polyketide synthases (PKSs) share many similarities with fatty acid synthases (FASs) and can be largely classified as type I, II and III, according to their architecture and mode of catalysis; yet many variants of PKSs exit in nature (5, 27, 29). Type I PKSs are multifunctional enzymes organized into modules, each of which harbors a set of distinct domains responsible for the catalysis of one cycle of polyketide chain elongation. A prototypical type I PKS elongation module contains minimally three integral domains – a ketosynthase (KS), an acyltransferase (AT) and an acyl carrier protein (ACP) – that together catalyze one round of polyketide chain elongation. Optional domains (such as ketoreductase [KR], dehydratase [DH], enoylreductase [ER]) are found between the AT and ACP domains, which carry out variable steps of reductive modifications on polyketide intermediate. Type II PKSs are multienzyme complexes that carry a single set of catalytic domains acting iteratively. Type III PKSs are chalcone synthase (CHS)-like enzymes that essentially are iteratively acting condensing enzymes. FASs, and type I and type II PKSs all use ACPs to tether acyl CoA substrates during fatty acid or polyketide biosynthesis, whereas type III PKSs directly condense acyl CoA substrates without carrier proteins. Prior to biosynthesis, apo form of ACPs must be activated to holo form by attaching a 4'-phosphopantetheinyl moiety from CoA onto a conserved serine residue; this posttranslational modification reaction is catalyzed by phosphopantetheinyltransferases (PPTases) that can be largely classified into two groups, AcpS-type and Sfp-type (12, 18, 22).

Integral AT domains in the prototypical type I PKSs were termed cognate ATs (8). A distinct variant of the type I PKSs contains no intact cognate ATs but a short

segment of remnant AT sequence in some or all modules. This subclass of type I PKSs was named the "AT-less" type I PKSs and the remnant AT segment the AT docking domain (7, 8, 31). The essential AT activities are provided *in trans* by discrete AT enzyme encoded by genes that are physically separated from the PKS genes, but nevertheless within the same gene cluster.

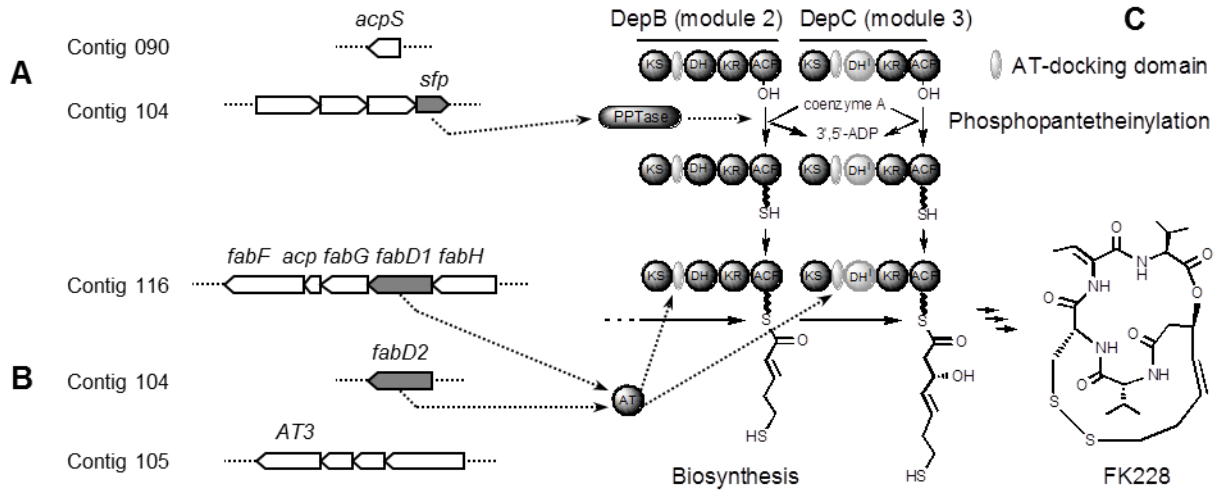


**Figure 6**. Identification of missing genes necessary for FK228 biosynthesis in *Chromobacterium violaceum* no. 968. (**A**) Local genetic map of two candidate PPTase-encoding genes, with the *sfp* gene product postulated to participate in phosphopantetheinylation of carrier proteins of the FK228 biosynthetic pathway. (**B**) Local genetic map of three candidate AT-encoding genes, with the *fabD1 and fabD2* gene products postulated to provide AT activities to complement the "AT-less" PKS modules on DepBC proteins for FK228 biosynthesis. (**C**) Scheme of two "AT-less" PKS modules on DepBC proteins that require a PPTase for carrier protein phosphopantetheinylation and a *trans* AT for polyketide chain elongation in FK228

biosynthesis. An inactive DH domain is drawn in light gray and labeled as $DH^{i}$. KS, AT, ACP, DH, KR and PPTase are standard abbreviations of domain/enzyme names that have been described in the text.

Our studies of the biosynthesis of FK228, a potent histone deacetylase inhibitor recently approved by FDA for the treatment of cutaneous T-cell lymphoma (CTCL) (1, 15), revealed that a 12-gene *dep* gene cluster encodes a hybrid modular NRPS-PKS pathway (9, 23), and that an FAD-dependent oxidoreductase is responsible for a critical disulfide bond formation in FK228 biosynthesis in *Chromobacterium violaceum* no. 968 (32). Nevertheless there are still several unanswered questions regarding the FK228 biosynthetic pathway. In particular, the two PKS modules on DepBC enzymes do not contain any cognate AT domain but a remnant AT docking domain (Fig. 7C), and there is no AT-encoding gene anywhere in the defined *dep* gene cluster (9, 23). Where does the AT activity necessary for FK228 biosynthesis come from? In this work we report the identification of two essential genes, *fabD1* and *fabD2*, both encoding a putative malonyl CoA acyltransferase (MCAT; also known as FabD, encoded by the *fabD* gene) component of the FAS II complex, that are positively involved in FK228 biosynthesis. Concurrently we identified a gene (*sfp*) encoding a putative Sfp-type PPTase necessary for FK228 biosynthesis as well. Furthermore, we show that, through reconstitution of the FK228 biosynthesis pathway, FK228 could be produced by recombinant *E. coli* strains under both aerobic and anaerobic cultivation conditions. Our studies revealed, for the first time, that modular PKSs recruit FabD components of the primary metabolism for the biosynthesis of a secondary metabolite. Discovery of the *trans* complementation of

modular PKSs by housekeeping ATs reveals natural product biosynthesis diversity. Moreover, demonstration of anaerobic production of FK228 by an engineered facultative bacterial strain validates our effort toward engineering of novel tumor-targeting bio-agents (6).

## 2.3 Materials and Methods

### 2.3.1 Bacterial strains, plasmids, culture conditions, and general molecular biological manipulations.

The bacterial strains and plasmids used in this study are listed in Table 1. Culture conditions and general molecular biological manipulations were performed as described (9, 32), or according to standard protocols (25).

### 2.3.2 Rapid genome sequencing and gene identification.

Genomic DNA of the wild type *C. violaceum* no. 968 strain was prepared from an overnight culture with an UltraClean Microbial DNA Isolation kit (MO BIO Labs, Carlsbad, CA), and was submitted for shotgun single-end and paired-end shotgun sequencing on a G20 FLX platform (454 Life Science, Branford, CT) at the Research Technology Support Facility of Michigan State University (East Lansing, MI). *de novo* assembly of sequence reads by instrument software was performed and resulted in a

quality draft genome sequence. Candidate genes were identified by using known protein sequences as bait to search the draft genome sequence of *C. violaceum* by Blastx algorithm (3).

2.3.3 General strategies for targeted gene deletion in *C. violaceum* no. 968.

A multiplex PCR method, as described elsewhere (10, 23, 32), was used for all intended gene deletion experiments. This method utilized a broad host-range Flp-*FRT* recombination system for site-specific gene replacement/deletion and subsequent marker removal (10, 16). Primers used for making gene deletion constructs and for detection of genotypes are listed in Table 2.

2.3.3 Reconstitution of FK228 biosynthetic gene cluster in engineered *E. coli* strains.

To probe whether individual candidate AT-encoding genes or PPTase-encoding genes are involved in FK228 biosynthesis, a three-plasmid system was utilized for gene cluster reconstitution and FK228 biosynthesis in bacterial strains derived from *E. coli* BL21(DE3) (Fig. 7A). First, all candidate genes were amplified by high fidelity PCR from the genomic DNA of *C. violaceum* with primer sets carrying designed restriction sites. Second, two candidate PPTase-encoding gene amplicons were double-digested with BamHI/HindIII and individually cloned into the first multiple cloning site (MCS) of pCDFDuet-1 (Novagen, Madison, WI) to create two intermediate constructs, pCDFDuet-1-*acpS* and pCDFDuet-1-*sfp*. Third, two candidate AT-encoding gene amplicons were double-digested with NdeI/KpnI and individually cloned into the second multiple cloning site of pCDFDuet-1 to generate pCDFDuet-1-*fabD1* and pCDFDuet-1-*fabD2*, or into the two previously made intermediate constructs to create a combination of 4 final

constructs, pCDFDuet-1-*acpS-fabD1*, pCDFDuet-1-*acpS-fabD2*, pCDFDuet-1-*sfp-fabD1*, pCDFDuet-1-*sfp-fabD2* (Table 1). Finally, the Cosmid 18, which carries the original incomplete FK228 biosynthetic gene cluster that lacks any AT-encoding gene or PPTase-encoding gene (9), and pBMTL-3-*depR*, which was created to complement a *depR*-deletion mutant of *C. violaceum* (23), were used in combination with the above expression constructs for the transformation of *E. coli* BL21(DE3) cells to create a series of bacterial strains (Table 2). Kanamycin at 25 µg/ml, chloramphenicol at 25 µg/ml, and streptomycin at 25 µg/ml were used individually or in combination for selection and maintenance of respective *E. coli* strains.

2.3.4 Bacterial fermentation and quantification of FK228 production by liquid chromatography-mass spectrometry (LC-MS).

Wild type *C. violaceum* strain and recombinant *E. coli* strains were fermented aerobically for 4 days at 30ºC under constant agitation (200 rpm) in 50 ml of LB media supplemented with 1% (w/v) Diaion HP-20 resin (Sigma-Aldrich, St. Louis, MO) and appropriate antibiotics where necessary. Gene expression was induced with 0.5% (w/v) lactose and 0.1 mM IPTG when bacterial culture reached an $OD_{600}$ of 0.4. Strict anaerobic fermentation of bacterial strains was carried out similarly for 5 days at room temperature in a Coy anaerobic chamber (Grass Lake, MI) with occasional manual agitation, except for that 0.05% (w/v) thioglycolate was added to the media to capture any oxidative species generated during fermentation. Extraction of metabolites, and detection and quantification of FK228 by LC-MS were performed as described (23).

2.3.5 RNA extraction and reverse transcription (RT)-PCR.

Recombinant *E. coli* BL21(DE3) strains were grown in LB media supplemented with appropriate antibiotics at 30ºC under constant agitation (200 rpm) to an $OD_{600}$ of 0.4. Five ml of each pre-induction sample was collected and the remaining cultures were induced with 0.5% (v/v) lactose and 0.1 mM IPTG. Aliquots of sample were collected at 60 min and again at 120 min post induction. Preservation of sample aliquots, extraction of total RNA, and RT-PCR experiments were performed as described (23). Primers used for detection of individual gene expression are listed in Table 4.

2.3.6 Nucleotide sequence accession numbers.

The nucleotide sequences of *C. violaceum* no. 968 genes reported in this paper have been deposited in the GenBank database under accession numbers HM449690 for 16S rRNA gene, HM449691 for *fabD1*, HM449692 for *fabD2*, HM449693 for *AT3*, HM449694 for *acpS*, and HM449695 for *sfp*, respectively.

2.4.1 Results

2.4.2 Draft genome sequencing of *Chromobacterium violaceum* no. 968.

Shotgun sequencing of *C. violaceum* genomic DNA on a GS20 FLX Sequencer generated a total of 163,954,650 input bases, which were assembled into 122 contigs; among them 82 are large contigs (>500 nt) with an average contig size of 59,514 bps. Those contigs were further aligned into 15 scaffolds with a total length of 4,909,141 bps.

The sequence coverage for this draft bacterial genome was thus calculated at 33.4 folds, which exceeded the desired 30-fold oversampling of raw sequence for the 454 pyrosequencing and *de novo* assembly technology platform (13). Compared to the published 4.75-Mb complete genome of a type strain of *C. violaceum* ATCC 12472 with a (G + C) content of 64.8% (11), the 4.91-Mb draft genome sequence of *C. violaceum* no. 968 with an overall (G + C) content of 61.9% obtained in this study appears to be near complete. The quality of this draft genome sequence was assessed by the following analyses. First, a comparison of the 16S rDNA sequences of two *C. violaceum* strains revealed an overall 96% identity without a single gap (Fig. 9), indicating a high quality of the draft genome sequence and a taxonomical relatedness of the two strains. Second, a homology search by Blastn algorithm of the draft genome sequence using our previously published FK228 biosynthetic gene cluster sequence (GenBank no. EF210776) (9) as bait identified two contigs that carry the gene cluster with a 100% sequence identify and with a 1298-bp sequence gap (data not shown). This gap was artificially created due to two highly homologous regions within the gene cluster that were assembled into a single copy of sequence.

2.4.3 Identification and initial characterization of candidate genes.

Sequences of three bait proteins, FabD of *E. coli* K-12 (GenBank no. AAC74176), FabD of *C. violaceum* ATCC 12472 (GenBank no. NP_903085) and LnmG of *Streptomyces atroolivaceus* S-140 (GenBank no. AAN85520), were used to search the draft genome sequence and identified three candidate genes, *fabD1*, *fabD2* and *AT3*, that encode putative AT enzymes (FabD, MCAT or AT) (Table 3; Fig. 7B). Based on

bioinformatic analysis, the *fabD1* gene lies within an apparent FAS II gene cluster that also includes *fabH*, *fabG*, *acp* and *fabF*, whereas *fabD2* is a standalone gene whose function cannot be predicted *a priori*. The *AT3* gene lies within a putative gene cluster that may be involved in cell-surface O-antigen biosynthesis.

Similarly, sequences of five bait proteins, AcpS of *E. coli* K-12 (GenBank no. P24224), Sfp of *Bacillus subtilis* (GenBank no. P39135), AcpS of *C. violaceum* ATCC 12472 (GenBank no. NP_901742), EntD of *C. violaceum* ATCC 12472 (GenBank no. NP_902320) and PcpS of *Pseudomonas aeruginosa* PAO1 (GenBank no. AAG04554), were used to search the draft genome sequence and identified two candidate genes, *acpS* and *sfp*, that encode putative PPTase enzymes (AcpS; Sfp) (Table 3; Fig. 7A). The *acpS* gene appears to be a standalone gene likely involved in primary metabolite (e.g. fatty acid) biosynthesis. The *sfp* gene is located at the end of an apparent NRPS gene cluster, therefore it is likely involved in secondary metabolite (e.g. nonribosomal peptide or hybrid molecule) biosynthesis.

Individual candidate genes were subjected to targeted gene deletion by a well-established multiplex PCR procedure to probe whether they play any roles in FK228 biosynthesis. Only *AT3* was successfully mutated and the mutant strain did not show any notable difference from the wild type strain (data not shown), suggesting that *AT3* is disposable and is independent of FK228 biosynthesis. Thus *AT3* was not further tested. In contrast, four other candidate genes, *fabD1*, *fabD2*, *acpS* and *sfp*, could not be mutated despite numerous attempts, indicating that they are all essential to the bacterial physiology and survival. They were then subjected to tests by a different strategy described below.

2.4.4 Reconstitution of the FK228 biosynthetic pathway in *E. coli*.

A series of recombinant *E. coli* strains were created herein (Table 2; Fig. 7A) and the relative levels of FK228 production by these strains were examined by LC-MS (Fig. 8; Table 5 and Fig. 10). When Cosmid 18, the originally identified large construct that carries the *dep* gene cluster which definitely lacks an AT-encoding gene and a PPTase-encoding gene (9), was introduced into *E. coli* BL21(DE3) cells, the recombinant strain SW01 failed to produce FK228. When a newly defined pathway regulatory gene, *depR* (23), was introduced into SW01 via a broad-host range construct pBMTL-3-*depR*, the resulting strain SW02 still did not produce any detectable level of FK228. When the candidate *sfp* gene was introduced into SW02 via a compatible construct pCDFDuet-1-*sfp*, the resulting strain SW03 produced a moderate level of FK228. When either the candidate *fabD1* or *fabD2* gene was added in tandem with *sfp* on pCDFDuet-1-*sfp-fabD1* or pCDFDuet-1-*sfp-fabD2* construct, the resulting strains of SW07 and SW08 produced significant levels of FK228. All other strains that received a single candidate gene of either *acpS* (strain SW04), *fabD1* (strain SW05) or *fabD2* (strain SW06), or two genes with *acpS* in tandem with either *fabD1* (strain SW9) or *fabD2* (strain SW10) did not show production of detectable level of FK228. Those observations led to the following conclusions: (1) the *sfp* gene but not the *acpS* gene is capable of converting all ACPs (and presumably peptidyl carrier proteins – PCPs) in the FK228 biosynthetic pathway from their inactive apo form to their active holo form; (2) indigenous *E. coli* AcpS cannot promiscuously act on those heterologous carrier proteins from a secondary metabolic pathway; (3) either gene product of the essential *fabD1* or *fabD2* gene of *C. violaceum*

no. 968 is able to complement the "AT-less" PKS modules on DepBC for polyketide chain extension; (4) even *E. coli* FabD (in the background in case of strain SW03) is able to provide the necessary AT activities for FK228 biosynthesis albeit at a lower rate; and (5) at this point we believe that genes necessary for FK228 biosynthesis have all been identified.

2.4.5 RT-PCR verification of gene expression.

To verify whether key biosynthetic and regulatory genes for FK228 biosynthesis were adequately expressed in the SW07 recombinant strain under normal aerobic conditions, aliquots of bacterial culture were collected at three time points, total RNA samples were prepared and subjected to semi-quantitative RT-PCR analysis (Fig. 7B). Prior to chemical induction, all examined genes including two representative structural genes (*depA* and *depJ*) but excluding the 16S rDNA control were not expressed. This suggests that the *depR* gene in its native position on Cosmid 18 is not functioning in *E. coli* cells, likely due to a lack of proper external or internal stimulus, or signal transduction pathway, or other regulatory components; expression of the *dep* gene cluster carried by Cosmid 18 in *E. coli* cells requires an ectopic copy of *depR* driven by lactose-inducible promoter on a pBMTL-3 vector. Upon induction by lactose and IPTG for the

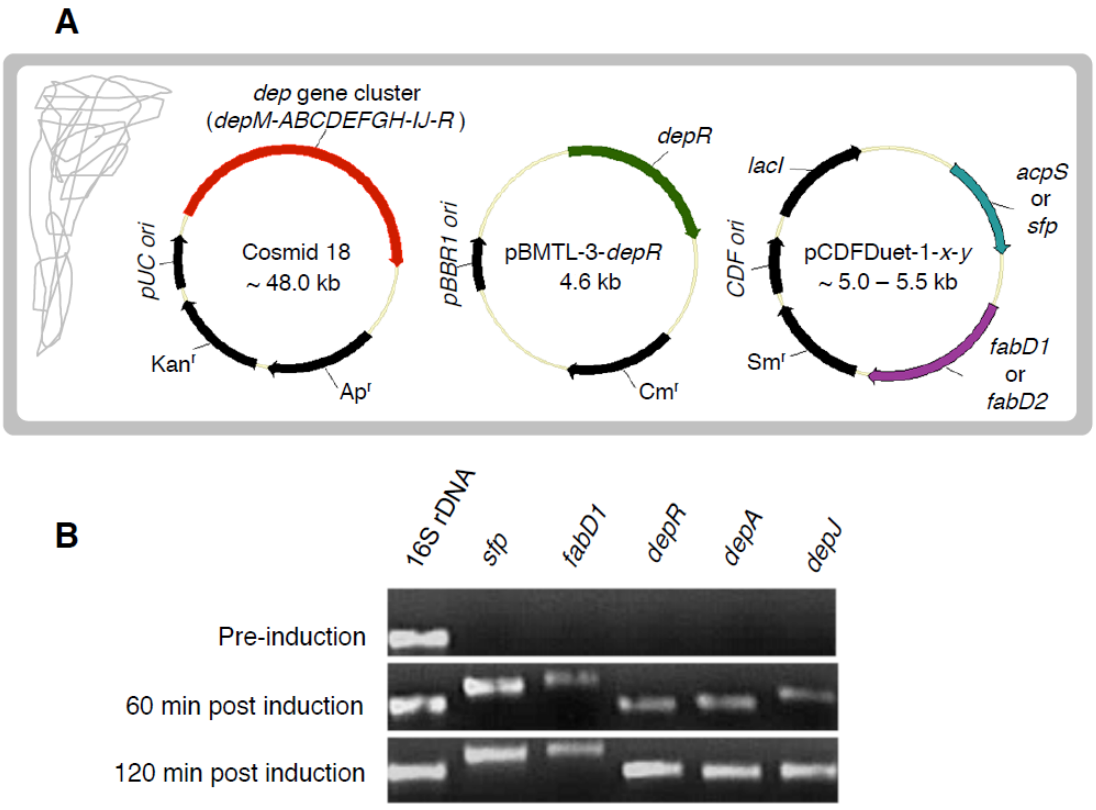


**Figure 7.** Reconstitution of FK228 biosynthesis in *E. coli* cells. (**A**) Scheme of a three-plasmid approach for reconstitution of FK228 biosynthetic pathway in *E. coli* BL21(DE3) cells. Only one cell containing one copy of each plasmid is drawn into picture for simplicity. A series of engineered strains were generated with different combinations of plasmids or different genes on plasmids (Table 2). (**B**) Examination of gene expression by semi-quantitative RT-PCR in engineered strain SW07 cultivated under aerobic conditions. 16S rDNA was amplified as an internal control.

expression of *depR* from pBMTL-3-*depR* and for the expression of *sfp* and *fabD1* from pCDFDuet-1-*sfp*-*fabD1*, those genes and two representative structural genes (*depA* and

*depJ*) were found to have expressed at 60 min post induction; and the gene expression reached higher levels at 120 min post induction.

2.4.6 Production of FK228 under anaerobic fermentation conditions.

As a pilot study toward engineering of novel tumor-targeting bacterial agents that may effectively infiltrate, multiply and continuously produce an anticancer drug inside the hypoxic core of solid tumors (6), we examined whether the engineered SW07 strain may produce FK228 under anaerobic fermentation conditions. To our delight the SW07 strain produced c.a.14.1% as much FK228 as did by the wild type *C. violaceum* strain or 15.3% by the SW07 strain itself under aerobic conditions (Fig. 8; Table 5 and Fig. 10); this relative level of FK228 production was translated into 0.40 mg/L of actual yield or c.a. 741 nM concentration when normalized by FK228 standard. As a control, the wild type *C. violaceum* strain was also found to have produced a lower level of FK228 under anaerobic conditions. This Gram-negative bacterial species appeared to be able to survive and grow slightly during the first 24 hr under the strict anaerobic conditions tested.

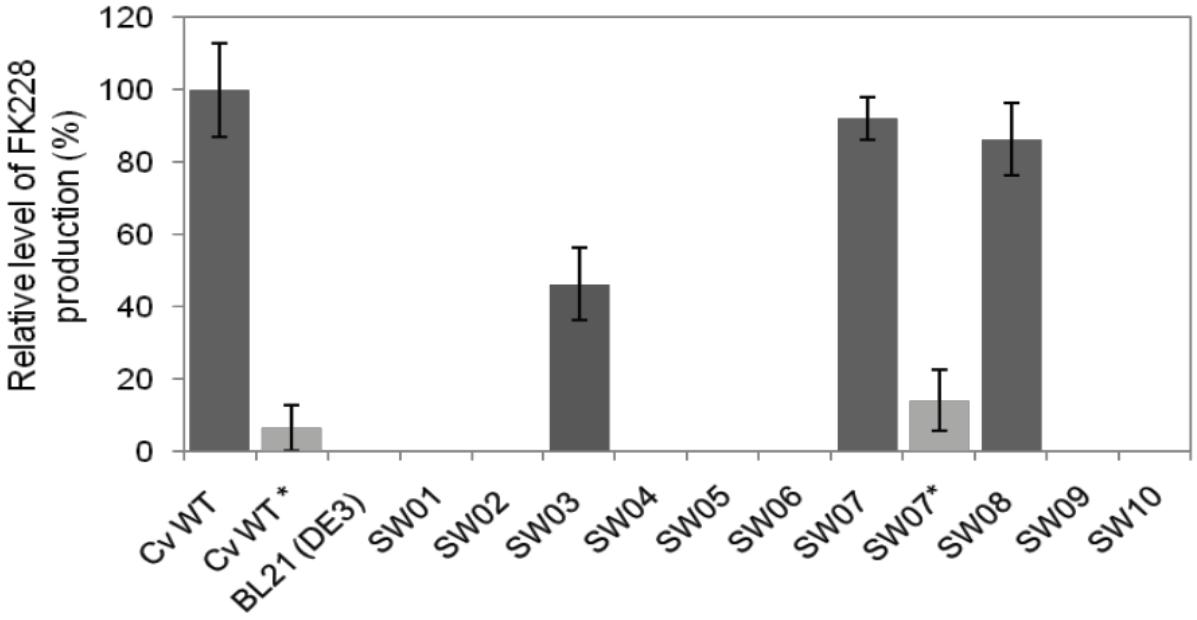


**Figure 8.** Levels of FK228 produced by recombinant *E. coli* strains relative to the wild type strain of *Chromobacterium violaceum* no. 968 cultivated under aerobic conditions unless indicated by * for under anaerobic conditions. Data are mean values of duplicate experiments with error bars indicating standard deviation. Detailed strain information and FK228 levels are provided in Table 2 and Table 5.

### 2.5.1 Discussion

Organizational and functional variation of PKSs leads to diverse structure of polyketide natural products (5, 27). The modular "AT-less" PKSs represent a severe deviation from the canonical type I PKSs (7, 8) and this phenomenon appears to be a transition state of complex enzyme evolution from FAS II to type II PKSs (28). Nevertheless in most "AT-less" PKS pathways the AT activities required for polyketide chain elongation are often encoded by discrete genes within the respective biosynthetic gene clusters. In the present work, for the first time, we uncovered an extreme case of

"AT-less" PKS system where the AT activity is encoded by *fabDs*, the essential bacterial genes involved in fatty acid biosynthesis.

As more and more modular "AT-less" PKSs are identified (7), it was not so surprising to find that the two PKS modules on DepBC do not contain an intact cognate AT domain but remnant of AT-docking segment (Fig. 7C). However a failure to identify any AT-encoding gene within or in the vicinity of the defined *dep* gene cluster was intriguing (9, 23). To address the mystery about where the necessary AT activities would come from, the genome of *C. violaceum* no. 968 was decoded by a rapid genome sequencing platform, revealing three candidate genes that may encode the AT activities for FK228 biosynthesis. However, attempts to identify the exact AT-encoding gene by a direct gene deletion approach was not successful, because two candidate genes (*fabD1* and *fabD2*) could not be mutated due to their apparent essential roles in bacterial viability. A third gene (*AT3*) could be mutated but the mutant did not show any obvious sign of physiological defect or decrease in FK228 production.

We then engineered a series of recombinant *E. coli* strains that harbor one to three compatible vectors that carry either the previously reported incomplete *dep* gene cluster or candidate genes identified in this work with three objectives in mind (Fig. 7A). First, we hoped to identify the missing AT-encoding gene and PPTase-encoding gene necessary for FK228 biosynthesis. Second, we hoped to reconstitute a functional FK228 biosynthetic pathway in a heterologous host such as *E. coli* cells. Third, we hoped to demonstrate that FK228 could be produced by a recombinant *E. coli* strain under both aerobic conditions and particularly anaerobic conditions as a pilot experiment for genetic engineering of novel cancer-targeting bio-agents (6).

Three engineered *E. coli* strains, SW03, SW07 and SW08, were subsequently found to produce variable amounts of FK228 under normal (aerobic) fermentation conditions. In addition to the Cosmid 18 and the *depR* gene on a vector, all these three strains received the *sfp* gene; while other *E. coli* strains, regardless of having the *acpS* gene or not, did not produce FK228. It was thus clear that the AcpS-type PPTase from either *E. coli* host or from *C. violaceum* cannot activate the carrier proteins from the FK228 biosynthetic pathway. Therefore it was concluded that the *sfp* gene, which was predicted to encode a broad substrate-range Sfp-type PPTase, is involved in FK228 biosynthesis. This PPTase should have other essential function as well, otherwise the *sfp* gene could have been mutated in the first place. In addition, two of the three strains, SW07 and SW08, which also received either *fabD1* or *fabD2* gene of *C. violaceum*, produced much higher levels of FK228 than did by SW03. Those observations suggested that either *fabD1* or *fabD2* of *C. violaceum* is involved in FK228 and their involvement is interchangeable; it is also possible that both genes are redundantly involved in FK228 biosynthesis. Surprisingly, the indigenous *fabD* of *E. coli* appeared to function as well for FK228 biosynthesis in the SW03 strain which received the *sfp* gene but not *fabD1* or *fabD2*.

Sequence alignment of the *C. violaceum* FabD1 and FabD2, and *E . coli* FabD shows that FabD1 and FabD share a much higher amino acid sequence identity than other two sets of comparison (Fig. 11), suggesting that FabD1 is very likely the housekeeping FAS II component for bacterial fatty acid biosynthesis. The role of FabD2 is less certain. Besides a positive role in FK228 biosynthesis, FabD2 should have other essential

functions; otherwise the *fabD2* gene could have been mutated in early experimental attempts.

Now it is proven that an MCAT component of FAS II is recruited by modular type I PKS modules on DepBC for FK228 biosynthesis, which establishes a unique functional cross-talk between bacterial primary metabolism and secondary metabolism, and adds new evidence to the phenomenon of complex enzyme evolution from FAS II to type II PKSs (28). Previously two type II PKS systems responsible for the biosynthesis of actinorhodin (24) or tetracenomycin (4, 14, 30) were found to recruit FabD of FAS II. Additional variants of cross-talk between bacterial primary metabolism and secondary metabolism have also been reported. For example, in the biosynthesis of quinoxaline antibiotics in two *Streptomyces* strains, an ACP from the primary FAS II is recruited by a standalone condensation domain of type II NRPS to form an initiation module of the biosynthetic pathway (26). Furthermore, cross-talk between a type I FAS (FAS I) for primary metabolism and a secondary FAS I for HC-toxin biosynthesis had also be postulated in the fungal species *Cochliobolus carbonum* where only one gene encoding the $\beta$-subunit of FAS I was found in the secondary metabolic gene cluster, while the $\alpha$-subunit of FAS I necessary for HC-toxin biosynthesis might be recruited from the primary FAS I (2). Recently an opposite example was reported in the apicidin biosynthetic pathway in *Fusarium semitectum* where a gene encoding the $\alpha$-subunit of FAS I is present in the apicidin biosynthetic gene cluster while the $\beta$-subunit of FAS I necessary for apicidin biosynthesis is speculated to be recruited from the primary FAS I (17).

Lastly, the ability by which the engineered SW07 strain produced 0.4 mg/L of

actual yield or c.a. 741 nM concentration of FK228 under strict anaerobic conditions is worth highlighting. This accomplishment represents an important milestone toward the goal of engineering of tumor-targeting bio-agents (6). The next step will be undertaken to introduce the FK228 biosynthetic capacity into one or more selected nonpathogenic anaerobic bacterial species, such as *Bifidobacterium longum* or *Clostridium oncolyticum* (21, 33), so that when the engineered bacteria infiltrate and multiply inside the necrotic region of solid tumors, FK228 will be produced *in situ*. Because FK228 is a potent histone deacetylase inhibitor and a new anticancer drug effective in the lower nM range (19, 34), the synergistic efforts of bacterial oncolysis of tumor tissue and the anticancer activity of FK228 could potentially be clinically effective against many types of cancer.

## 4. 6. Supplementary Figures

```
Cv_No968_16S_rDNA          1 AACTGAAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTA 50
Cv_ATCC12472_16S_rDNA      1 AACTGAAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTA 50
                             **************************************************

Cv_No968_16S_rDNA         51 CACATGCAAGTCGAACGGTAACAGGGTGCTTGCACCGCTGACGAGTGGCG 100
Cv_ATCC12472_16S_rDNA     51 CACATGCAAGTCGAACGGTAACAGGGTGCTTGCACCGCTGACGAGTGGCG 100
                             **************************************************

Cv_No968_16S_rDNA        101 AACGGGTGAGTAATGCATCGGAATGTACCGTGTAATGGGGGATAGCTCGG 150
Cv_ATCC12472_16S_rDNA    101 AACGGGTGAGTAATGCGTCGGAATGTACCGTGTAATGGGGGATAGCTCGG 150
                             *************** ********************************

Cv_No968_16S_rDNA        151 CGAAAGCCGGATTAATACCGCATACGCCCTGAGGGGGAAAGTGGGGGACC 200
Cv_ATCC12472_16S_rDNA    151 CGAAAGCCGGATTAATACCGCATACGCCCTGAGGGGGAAAGCGGGGGATC 200
                             ****************************************** ****** *

Cv_No968_16S_rDNA        201 GTAAGGCCTCACGTTATACGAGCAGCCGATGTCTGATTAGCTAGTTGGTG 250
Cv_ATCC12472_16S_rDNA    201 GAAAGACCTCGCGTTATACGAGCAGCCGACGTCTGATTAGCTAGTTGGTG 250
                             * *** **** ****************** ********************

Cv_No968_16S_rDNA        251 GGGTAAAGGCTCACCAAGGCTTCGATCAGTAGCGGGTCTGAGAGGATGAT 300
Cv_ATCC12472_16S_rDNA    251 AGGTAAGAGCTCACCAAGGCGACGATCAGTAGCGGGTCTGAGAGGATGAT 300
                              *****  ************  ****************************

Cv_No968_16S_rDNA        301 CCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG 350
Cv_ATCC12472_16S_rDNA    301 CCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG 350
                             **************************************************

Cv_No968_16S_rDNA        351 TGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGT 400
Cv_ATCC12472_16S_rDNA    351 TGGGGAATTTTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGT 400
                             ********************* **** **********************
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Cv_No968_16S_rDNA        401 CTGAAGAAGGCCTTCGGGTTGTAAAGGACTTTTGTCCGGGAGCAAATCCC 450
Cv_ATCC12472_16S_rDNA    401 CTGAAGAAGGCCTTCGGGTTGTAAAGGACTTTTGTCAGGGAGGAAATCCC 450
                             ************************************ ***** *******

Cv_No968_16S_rDNA        451 AGTGGTTAATACCTACTGGGGCTGAGAGTACCGGAAGAATAAGCACCGGC 500
Cv_ATCC12472_16S_rDNA    451 GCTGGTTAATACCCGGCGGGGATGACAGTACCTGAAGAATAAGCACCGGC 500
                               ***********    **** *** ****** *****************

Cv_No968_16S_rDNA        501 TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCG 550
Cv_ATCC12472_16S_rDNA    501 TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCG 550
                             ************************************** ***********

Cv_No968_16S_rDNA        551 GAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTGTGCAAGTCTGATGTGA 600
Cv_ATCC12472_16S_rDNA    551 GAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTGTGCAAGTCTGATGTGA 600
                             ************************************************

Cv_No968_16S_rDNA        601 AAGCCCCGGGCTTAACCTGGGAACGGCATTGGAGACTGCACGACTAGAGT 650
Cv_ATCC12472_16S_rDNA    601 AAGCCCCGGGCTTAACCTGGGAACGGCATTGGAGACTGCACAGCTAGAGT 650
                             ****************************************  *******

Cv_No968_16S_rDNA        651 GCGTCAGAGGGGGGTAGAATTCCGCGTGTAGCAGTGAAATGCGTAGAGAT 700
Cv_ATCC12472_16S_rDNA    651 GCGTCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGAT 700
                             ********************** *************************

Cv_No968_16S_rDNA        701 GCGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGATGACACTGACGCT 750
Cv_ATCC12472_16S_rDNA    701 GTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGATGACACTGACGCT 750
                             * ************************************************

Cv_No968_16S_rDNA        751 CATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA 800
Cv_ATCC12472_16S_rDNA    751 CATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA 800
                             ************************************************

Cv_No968_16S_rDNA        801 CGCCCTAAACGATGTCAACTAGCTGTTGGGGGTTTGAATCCTTGGTAGCG 850
Cv_ATCC12472_16S_rDNA    801 CGCCCTAAACGATGTCAACTAGCTGTTGGGGGTTTGAATCCTTGGTAGCG 850
                             ************************************************

Cv_No968_16S_rDNA        851 AAGCTAACGCGAGAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAA 900
Cv_ATCC12472_16S_rDNA    851 TAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAA 900
                              ********** ***************************************

Cv_No968_16S_rDNA        901 ACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTA 950
Cv_ATCC12472_16S_rDNA    901 ACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTA 950
                             ************************************************

Cv_No968_16S_rDNA        951 ATTCGATGCAACGCGAAAAACCTTACCTGGTCTTGACATGTAACGAACGC 1000
Cv_ATCC12472_16S_rDNA    951 ATTCGATGCAACGCGAAAAACCTTACCTGCTCTTGACATGTACGGAACTT 1000
                             ***************************** ************  ****

Cv_No968_16S_rDNA       1001 CGCAGAGATGTGGTGGTGCCCGAAAGGGAGCGTTAACACAGGTGCTGCAT 1050
Cv_ATCC12472_16S_rDNA   1001 GCCAGAGATGGCTTGGTGCCCGAAAGGGAGCCGTAACACAGGTGCTGCAT 1050
                               ********   ******************  ******************

Cv_No968_16S_rDNA       1051 GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG 1100
Cv_ATCC12472_16S_rDNA   1051 GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG 1100
                             ************************************************

Cv_No968_16S_rDNA       1101 CGCAACCCTTGCCATTAGTTGCCATCATTAAGTTGGGCACTCTAATGGGA 1150
Cv_ATCC12472_16S_rDNA   1101 CGCAACCCTTGTCATTAGTTGCCATCATTCAGTTGGGCACTCTAATGAGA 1150
                             *********** ***************** ***************** **

Cv_No968_16S_rDNA       1151 CTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGG 1200
Cv_ATCC12472_16S_rDNA   1151 CTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGG 1200
                             ************************************************

Cv_No968_16S_rDNA       1201 CCCTTATGACCAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGGTC 1250
Cv_ATCC12472_16S_rDNA   1201 CCCTTATGAGCAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGGTT 1250
                             ********* ************************************

Cv_No968_16S_rDNA       1251 GCGAAGCCGCGAGGTGGAGCCAATCTCATAAAACCGATCGTAGTCCGGAT 1300
Cv_ATCC12472_16S_rDNA   1251 GCCAAGCCGCGAGGTGGAGCTAATCTCAGAAAACCGATCGTAGTCCGGAT 1300
                             ** ***************** ******* ********************
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```
Cv_No968_16S_rDNA       1301 CGCACTCTGCAACTCGAGTGCGTGAAGTCGGAATCGCTAGTAATCGCAGA 1350
Cv_ATCC12472_16S_rDNA   1301 CGCACTCTGCAACTCGAGTGCGTGAAGTCGGAATCGCTAGTAATCGCAGA 1350
                             **************************************************

Cv_No968_16S_rDNA       1351 TCAGCATGCTGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTC 1400
Cv_ATCC12472_16S_rDNA   1351 TCAGCATGCTGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTC 1400
                             **************************************************

Cv_No968_16S_rDNA       1401 ACACCATGGGAGTGAGTTTCACCAGAAGTGGGTAGGCTAACCGTAAGGAG 1450
Cv_ATCC12472_16S_rDNA   1401 ACACCATGGGAGTGAGTTTCACCAGAAGTGGGTAGGCTAACCGCAAGGAG 1450
                             ****************************************** ******

Cv_No968_16S_rDNA       1451 GCCGCTTACCACGGTGGGATTCAT 1474
Cv_ATCC12472_16S_rDNA   1451 GCCGCTTACCACGGTGGGATTCAT 1474
                             ************************
```

**Figure 9.** Alignment of 16S rDNA sequences of *Chromobacterium violaceum* no. 968 (GenBank no. HM449690; result of the current work) and *Chromobacterium violaceum* ATCC 12472 (GenBank no. AE016825) by CLUSTAL W showing 96% sequence identity and without any gap between the two sequences.

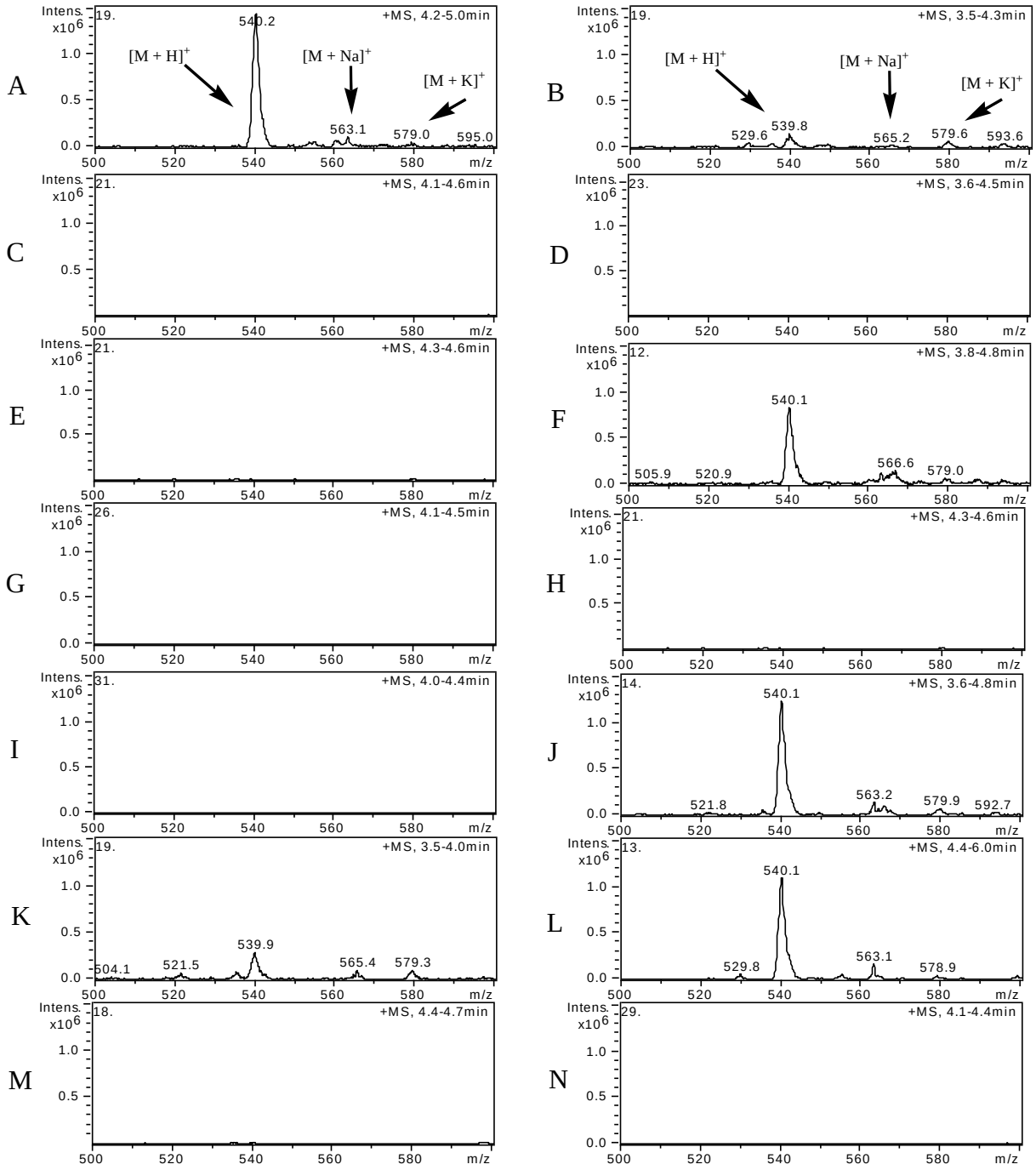


**Figure 10.** Examination and quantification of FK228 production by LC-MS. A, strain Cv WT; B, strain Cv WT under anaerobic conditions; C, strain BL21(DE3); D, strain SW01; E, strain SW02; F, strain SW03; G, strain SW04; H, strain SW05; I, strain SW06;

J, strain SW07; K, strain SW07 under anaerobic conditions; L, strain SW08; M, strain SW09; N, strain SW10. Detailed strain information is listed in Table 2.

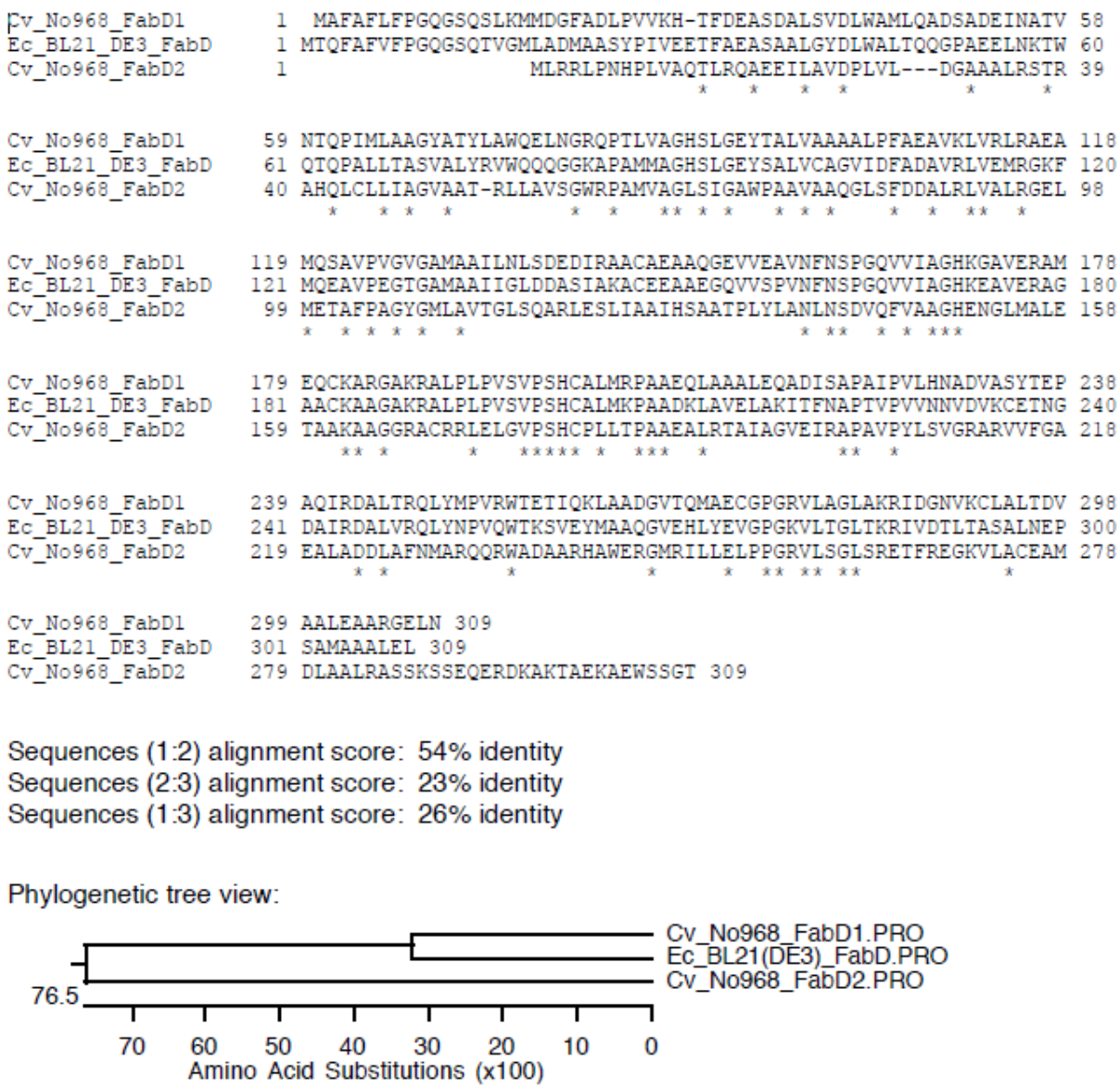


**Figure 11.** Alignment of FabD sequences of *Chromobacterium violaceum* no. 968 (GenBank no. HM449691 for FabD1, GenBank no. HM449692 for FabD2; result of the current work) and *Escherichia coli* BL21(DE3) FabD (GenBank no.ACT42983) by CLUSTAL W.

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**TABLE 2.** Bacterial strains and plasmids used in this study

| Strains or plasmids | Description[a] | Source or reference |
|---|---|---|
| *Chromobacterium violaceum* no. 968 (=FERM BP-1968) | Wild type strain, FK228-producing, $Ap^r$ $Thio^r$ | IPOD[b] |
| *Escherichia coli* | | |
| DH5α | General cloning host | Lab stock |
| S17-1 | Host strain for interspecies conjugation | Lab stock |
| BL21(DE3) | Host strain for heterologous gene expression | Novagen |
| SW01 | BL21(DE3) harboring Cosmid 18 | This work |
| SW02 | BL21(DE3) harboring Cosmid 18 and pBMTL-3-*depR* | This work |
| SW03 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*sfp* | This work |
| SW04 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*acpS* | This work |
| SW05 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*fabD1* | This work |
| SW06 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*fabD2* | This work |
| SW07 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*sfp-fabD1* | This work |
| SW08 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*sfp-fabD2* | This work |
| SW09 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*acpS-fabD1* | This work |
| SW10 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*acpS-fabD2* | This work |
| Plasmids | | |
| pGEM-3Zf | $Ap^r$, general cloning vector | Promega |
| pGEM-T Easy | $Ap^r$, general cloning vector | Promega |
| Cosmid 18 | $Ap^r$, $Kan^r$, cosmid clone containing the FK228 biosynthetic gene cluster (*dep*) and flanking DNAs, shotgun sequenced | (9) |
| pBMTL-3 | $Cm^r$, pBBR1 *ori*, broad host-range vector | (20) |

| | | |
|---|---|---|
| pBMTL-3-*depR* | Cm$^r$, *depR* (with RBS from pET29a) cloned into pBMTL-3 | (23) |
| pCDFDuet-1 | Sm$^r$, CDF *ori*, dual expression vector | Novagen |
| pCDFDuet-1-*sfp* | *sfp* cloned into the MCS1 region of pCDFDuet-1 | This study |
| pCDFDuet-1-*acpS* | *acpS* cloned into MCS1 region of pCDFDuet-1 | This study |
| pCDFDuet-1-*fabD1* | *fabD1* cloned into MCS2 region of pCDFDuet-1 | This study |
| pCDFDuet-1-*fabD2* | *fabD2* cloned into MCS2 region of pCDFDuet-1 | This study |
| pCDFDuet-1-*sfp-fabD1* | *sfp* cloned into MCS1, *fabD1* cloned into MCS2 of | This study |
| pCDFDuet-1-*sfp-fabD2* | pCDFDuet-1 | This study |
| pCDFDuet-1-*acpS-fabD1* | *sfp* cloned into MCS1, *fabD2* cloned into MCS2 of | This study |
| pCDFDuet-1-*acpS-fabD2* | pCDFDuet-1 | This study |
| | *acpS* cloned into MCS1, *fabD1* cloned into MCS2 of pCDFDuet-1 | |
| | *acpS* cloned into MCS1, *fabD2* cloned into MCS2 of pCDFDuet-1 | |

[a] Ap$^r$, ampicillin resistance; Cm$^r$, chloramphenicol resistance; Kan$^r$, kanamycin resistance; Sm$^r$, streptomycin resistance; Thio$^r$, thiostrepton resistance; MCS, multiple cloning site.

[b] IPOD, International Patent Organism Depositary, Tsukuba, Japan.

**TABLE 3.** Candidate genes identified through rapid genome sequencing and their involvement in FK228 biosynthesis

| Gene Name[a] | Deduced Product and Predicted Function | Gene Disposability | Involvement in FK228 Biosynthesis |
|---|---|---|---|
| AT-encoding genes | | | |
| *fabD1* | FabD1, likely involved in fatty acid biosynthesis | No | Positive |
| *fabD2* | FabD2, function unpredictable *a priori* | No | Positive |
| *AT3* | AT3, likely involved in cell-surface O-antigen biosynthesis | Yes | No |
| PPTase-encoding genes | | | |
| *acpS* | AcpS-type PPTase, likely involved in primary metabolite biosynthesis | No | No |
| *sfp* | Sfp-type PPTase, likely involved in secondary metabolite biosynthesis | No | Yes |

[a] AT, acyltransferase; PPTase, phosphopantetheinyltransferase.

**TABLE 4**. Primers used for gene deletion, genotype detection and detection of gene expression

| Name (primers sorted by gene) | Sequence (5' → 3')[a] | Gene Deletion |
|---|---|---|
| KpnI-NdeI-fabD1-UpF | AGGTACCCATATGgcgtttgcatttctgttcc | Not successful |
| FRT-F-fabD1-UpR | TCAGAGCGCTTTTGAAGCTAATTCGttcaactcctgccaggcc | |
| FRT-R-fabD1-DnF | AGGAACTTCAAGATCCCCAATTCGccagctacaccgagcc | |
| BamHI-fabD1-DnR | CTGGATCCtcagttcagttcgccgcgag | |
| fabD1-RT-PCR-FP | (Same as KpnI-NdeI-fabD1-UpF) | |
| fabD1-RT-PCR-RP | (Same as FRT-F-fabD1-UpR) | |
| KpnI-NdeI-fabD2-UpF | AGGTACCCATATGctgcgccgcttgccgaa | Not successful |
| FRT-F-fabD2-UpR | TCAGAGCGCTTTTGAAGCTAATTCGatggaaagccccgccac | |
| FRT-R-fabD2-DnF | AGGAACTTCAAGATCCCCAATTCGcgtgggaacgcggcatg | |
| HindIII-fabD2-DnR | CTAAGCTTcatgtaccacttgaccattcc | |
| fabD2-RT-PCR-FP | (Same as KpnI-NdeI-fabD2-UpF) | |
| fabD2-RT-PCR-RP | (Same as FRT-F-fabD2-UpR) | |

| | | |
|---|---|---|
| KpnI-NdeI-AT3-UpF<br>FRT-F-AT3-UpR<br>FRT-R-AT3-DnF<br>BamHI-AT3-DnR<br>AT3-RT-PCR-FP<br>AT3-RT-PCR-RP | AGGTACCCATATGgcgtttgcatttctgttcc<br>TCAGAGCGCTTTTGAAGCTAATTCGc gtagaatgccttgaagtc<br>AGGAACTTCAAGATCCCCAATTCGtcg cctgcaaattgttcag<br>CTGGATCCttagcgttcggcgcttcttgc<br>(Same as KpnI-NdeI-AT3-UpF)<br>(Same as FRT-F-AT3-UpR) | Successful |
| KpnI-NdeI-acpS-UpF<br>FRT-F-acpS-UpR<br>FRT-R-acpS-DnF<br>HindIII-acpS-DnR<br>acpS-RT-PCR-FP<br>acpS-RT-PCR-RP | AGGTACCCATATGatttacggcattggcactg<br>TCAGAGCGCTTTTGAAGCTAATTCGa aacgcttggccaggaag<br>AGGAACTTCAAGATCCCCAATTCGctt agccccgaattggaag<br>CTAAGCTTctagccgggcgcggagcg<br>(Same as KpnI-NdeI-acpS-UpF)<br>(Same as FRT-F-acpS-UpR) | Not successful |
| KpnI-NdeI-sfp-UpF<br>FRT-F-sfp-UpR<br>FRT-R-sfp-DnF<br>HindIII-sfp-DnR<br>sfp-RT-PCR-FP<br>sfpRT-PCR-RP | AGGTACCCATATGgaactgaacgctttgatcaag<br>TCAGAGCGCTTTTGAAGCTAATTCGc caacagccgtcgtagtg<br>AGGAACTTCAAGATCCCCAATTCGga gcagaccttgctgcc<br>CTAAGCTTcagccctccgcccaggc<br>(Same as KpnI-NdeI-sfp-UpF)<br>(Same as FRT-F-sfp-UpR) | Not successful |
| 16S-RT-PCR-F<br>16S-RT-PCR-R | gactcctacgggaggcagc<br>gtattaccgcggctgctggc | N/A |
| depA-RT-PCR-F<br>depA-RT-PCR-R | cacctcggggtccacgcg<br>gggaaaaccgcattgcaaggg | N/A |
| depJ-RT-PCR-F<br>depJ-RT-PCR-R | tgctttccgtatgcgggcgg<br>ctcgcgcccatgctgtgccc | N/A |
| depR-RT-PCR-F<br>depR-RT-PCR-R | gaacttcgctatatcgtcgcg<br>gctgcgattgctcgatgatgc | N/A |

[a]Sequences in capital letters are designed either to provide restriction sites (underlined) for directional cloning, or to provide extra base(s) in front of restriction sites for efficient restriction enzyme digestion, or to provide overlapping sequences for *FRT* cassette assembly during multiplex PCR. Lower-case letters indicate gene-specific sequences used for amplification of individual genes.

**Table 5**. Quantification of FK228 production by recombinant *E. coli* strains relative to the wild type strain of *Chromobacterium violaceum* no. 968 cultivated under aerobic conditions unless indicated by * for under anaerobic conditions. Experiments were performed in duplicate and the data are presented as mean values with standard deviation.

| Strains | Description[a] | Relative level of FK228 Production (%) |
|---|---|---|
| *Chromobacterium violaceum* no. 968 (=FERM BP-1968) | Wild type, FK228 producing | 100.0 |
| no. 968* | (Same as above) | 6.7±6.2 |
| *Escherichia coli* | | |
| SW01 | BL21(DE3) harboring Cosmid 18 | 0.0 |
| SW02 | BL21(DE3) harboring Cosmid 18 and pBMTL-3-*depR* | 0.0 |
| SW03 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*sfp* | 46.3±10.2 |
| SW04 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*acpS* | 0.0 |
| SW05 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*fabD1* | 0.0 |
| SW06 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*fabD2* | 0.0 |
| SW07 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*sfp-fabD1* | 92.2±5.8 |
| SW07* | (Same as above) | 14.1±8.4 |
| SW08 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*sfp-fabD2* | 86.3±10.1 |
| SW09 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*acpS-fabD1* | 0.0 |
| SW10 | BL21(DE3) harboring Cosmid 18, pBMTL-3-*depR* and pCDFDuet-1-*acpS-fabD2* | 0.0 |

# CHAPTER 3

# ENGINEERING A NOVEL SECRETION SYSTEM FOR PRODUCTION AND DIRECT PURIFICATION OF ANTICANCER CELL PENETRATING PEPTIDES FROM THE MEDIA

Content of this chapter is a reformatted version of a manuscript to be submitted to the journal of Applied and Environmental Microbiology.

## 3.1. Abstract

Cell penetrating peptides (CPPs) are short peptides that possess the unique capacity to translocate across cell membranes. Recently, a CPP derived from a virulence protein associated with the type III secretion system of *Yersinia enterocolitica* was identified and shown to be important in bacterial pathogenicity. Here, we show the construction of a fusion protein combining the transmembrane activity of a bacterial CPP with a eukaryotic mitochondrial disrupting protein (MDP) to produce a new CPP-MDP protein with anticancer activity. We also show the creation of a new *E. coli* BL21(DE3)-based expression system that facilitates the secretion of CPP fusion proteins into the growth media for rapid and efficient protein purification. This protein secretion system utilizes induced L-form bacterial spheroplasts to generate a yield of 32 mg/L of CPP-MDP directly from the media. Construction of an L-form based *E. coli* BL21 (DE3) protein secretion system and a modular CPP expression system allows future rapid CPP-fusion protein production with new fusion protein partners to develop new CPP-based anticancer agents.

## 3.2. Introduction

Protein secretion directly into the surrounding growth media offers many advantages for recombinant protein production. However, traditional laboratory strains of *E. coli* do not typically secrete proteins prompting development of new strategies for protein secretion. Currently, four strategies have been developed to release recombinant proteins into the media. The first involves the engineering of pathogenic strains of *E. coli* to link recombinant protein expression with one of the six dedicated protein secretion pathways, with the Type I secretion pathway most often targeted (1). The second strategy involves the use of carrier proteins like the IgG binding domain fused with recombinant protein A, but the mechanism for release is unknown (2). The third strategy exploits cell wall structural deficits produced by mutations targeting the gene regulating cell wall biosynthesis and division (3). One drawback to this approach is the decrease in cell growth limiting the overall protein production. Finally, the fourth strategy involves cell lysis through the co-expression of Kil protein with the recombinant protein (4). However, this strategy utilizing cell membrane disruption lowers the cell density and does not insure the proper folding of recombinant proteins.

One of the major inhibitors of recombinant protein secretion in *E. coli* is the presence of the cell wall. While not critical for cell survival, the bacterial cell wall plays many important roles in a bacterial growth and survival. Not only is the cell wall important in protection, but also functions in cell morphology, cellular adhesion and cell to cell communication. However, under certain circumstances bacteria will lose all or part of their cell wall producing osmo-sensitive variants. These cell wall defective bacteria (CWDB) can be generated *in vitro* and *in vivo* in many species and has been

suggested to be a survival strategy utilized by bacteria to negate cell wall-targeting antibiotics and evade recognition by the immune system (5,6). CWDB can be classified into four groups (stable vs. unstable, spheroplast vs. protoplast) based on their ability to grow on specialized osmotically balanced media (7). Stable L form bacteria are formed through genetic mutation resulting in a physiologically different offspring incapable of reverting back to a classical parental form possessing a cell wall (8). Therefore, stable L form CWDB are genetically different from their parent strain. In contrast, unstable L forms retain their ability to revert back to the parental form and are considered genetically identical to the parent strain (9). Also, L forms can be further classified as spheroplasts or protoplasts. Cells that retain a residual cell wall are defined as spheroplasts, while those cells completely lacking a cell wall are protoplasts. Finally, L form bacteria exhibit a classic "fried egg" appearance when grown on solid media but L form manipulation may also be performed in liquid media.

Currently, renewed interest in L form bacteria has occurred due to their association with antibiotic resistance and certain disease states. L form bacteria gain many advantages including resistance to cell wall-targeting antibiotics, resistance to bacteriophage infection and faster growth in nutrient rich environments at the cost of losing mobility (lacking fimbrae and flagella) (7-13). In contrast, L form bacteria develop the disadvantage of osmotic sensitivity and lose genetic transfer ability by conjugation. In *E. coli*, the *relA*, *spoT*, *creC* and *metB* mutations were identified in the formation of the stable L form strain LW1655F+ (7). Further research showed that mutations also existed in critical cell division genes in the *dcw* cluster in stable L form bacteria. Therefore, L form bacteria represent a unique convertible phenotype that may

offer the bacteria various advantages to adapt to a changing environment, including oxidative stress and DNA damage. *Staphylococcus aureus* was shown to form unstable L forms and developed beta-lactam resistance in revertants (8). Genetic analysis of *E. coli* unstable L forms undergoing cell division identified regulatory and structural colanic acid mutants and an *mrcB* penicillin-binding protein (PBP) 1B mutant defective in L form development (9). Furthermore, preliminary whole genome transcriptome analysis of *E. coli* mutants defective in L form development showed these mutations were also critical in DNA repair, oxidative stress response and acid tolerance pathways (13).

Since the discovery of cell penetrating peptides in 1988, new attributes and functions have been identified demonstrating broad therapeutic applications. Structurally, cell penetrating peptides (CPPs) are composed of relatively short basic sequences of 5 to 40 amino acids and possess the ability to cross the cell membrane through a number of mechanisms. While all CPPs share membrane translocation activity the entry mechanisms may differ. The CPP membrane translocation process appears to be dependent on the cell type and the cargo that may be conjugated to the CPP (14). Recent studies suggest that most CPPs enter eukaryotic cells through endocytosis uptake mechanisms, however small arginine-rich CPPs have been shown to cross the plasma membrane in an energy independent process (15). CPPs can utilize both the clathrin-dependent and clathrin-independent endocytosis pathways to gain entrance into the cell and are able to escape from the endosomes to the cytoplasm. Translocation from the endosome appears to be facilitated by endosome acidification allowing the CPP to cross the lipid bilayer (14, 16, 17). Recently, YopM, a protein associated with *Yersinia enterocolitica* infection, was shown to have cell translocation capability. Normally,

YopM and other pathogenic proteins pass through the cell membrane via the type III secretion system (T3SS). This activity was attributed to two highly cationic amino acid regions within two alpha helices in the N-terminus and demonstrated the ability to translocate a fused protein cargo across the cellular membrane (18).

The transmembrane activity of CPPs and CPP fusion proteins represents new engineering opportunities with vast therapeutic applications. CPPs have been shown to deliver a variety of cargo including short inhibiting RNA, plasmids, proteins, liposomes, drugs and fluorescent markers, and represent a powerful strategy for drug delivery. This strategy can be used to increase cancer cell activity of these anticancer agents by facilitating their delivery into tumor cells. Furthermore, the CPP transmembrane activity showed that drug resistant cancer cells could be reverted back to a drug sensitive phenotype by conjugating CPP with Taxol and other known anticancer drugs by increasing intracellular drug concentrations (19). Also, CPPs conjugated with protein or protein domains can be used to specifically target pathways associated with a pro-cancer cell state or apoptosis pathways leading to cell death of cancer cells. While cell targeting for most CPPs is ambiguous, recent studies identified CPPs with tumor targeting capabilities (20). Finally, fluorescent labeled-CPP conjugates have been developed for surgical cancer therapy. The fluorescent-CPP with an antibody specific for the cancer cells facilitates the endosomal escape of the conjugate preserving the fluorescent signal within the cancer cell cytoplasm for easy surgical removal of only tumor tissue (21). Therefore, these unique peptides offer a variety of engineering options for the development of new anticancer agents and many other therapeutic applications.

However, many challenges remain to efficiently express and purify functional CPPs and recombinant proteins in *E. coli* systems. While CPPs can be chemically synthesized and purified from inclusion bodies, but both strategies have drawbacks related to proper protein folding and function. Also, the chemical synthesis strategy continues to cost more than traditional *E. coli* expression and purification systems expanding costs to downstream applications. In contrast, protein secretion to the media has the advantage of preserving protein structure; however secretion pathways are limited due to the cell wall barrier. Secretion of CPP fusion proteins to the periplasm may allow the translocation of the CPP fusion protein to the media or production of cell wall deficient L form strains could be generated to directly secrete CPP fusion proteins into the media. Here we describe the generation of unstable L form spheroplasts that allowed secretion to the media and subsequent purification of a CPP fusion protein with broad anticancer activity.

## 3.3. Materials and Methods

### 3.3.1. Bacterial strains, plasmids, culture conditions, and general molecular biological manipulations.

The bacterial strains and plasmids used in this study are listed in Table 6. Culture conditions for general molecular biological manipulations were performed as described (22), or according to standard protocols (23). Primers used to amplify and synthesize the corresponding DNA sequences are listed in Table 7. The $yopM_{75}$ sequence was amplified from genomic *Yesinia enterocolitica* O8 strain JB580v DNA. Sequential addition of

*ompF* and MDP DNA sequences were constructed using a two-step overlap Phusion® DNA polymerase (New England Biolabs) PCR protocol using 20 nM of each synthesis primer and target *yopM*$_{75}$ sequence for five PCR cycles. After five cycles, each amplification primer was added to a final concentration of 0.5 μM to the reaction mixture to synthesize the full length product. The PCR products and pET29a plasmid were digested with NdeI and XhoI and ligated to produce the final vector constructs with the *ompF* signal sequence and MDP sequence added to *yopM*$_{75}$ in pET29a vector (Table 6). The sequences of the modular expression cassette are listed in Table 8.

3.3.2. Protein purification and protein identification by Western blot analysis.

Wild-type *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3) containing the pET29-*ompF-yopM*$_{75}$*-MDP* construct were grown at 37˚C in LB or Pennassay L form production media containing 40 μg/ml of kanamycin and were induced with 0.5 μM IPTG (isopropyl β-D-1-thiogalactopyranoside, Sigma Aldrich) during mid-log phase. Protein samples of whole cell, cell pellet, cell cytoplasm supernatant, periplasmic proteins and media secreted protein fraction were prepared according to standard protocols (23). Protein samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting. Polyclonal immunoglobulin antibody against 6X His tag prepared from mouse was used as the probe and was incubated with *E. coli* BL21 (DE3) control and the expressed protein samples. BCIP/NitroBT (Sigma Aldrich) substrate was used to detect an anti-mouse secondary antibody conjugated with alkaline phosphatase.

3.3.3. Development of L form bacteria protein secretion system and protein quantification

*E. coli* BL21 (DE3)/pET29-*ompF-yopM*$_{75}$-*MDP* and *E. coli* BL21 (DE3)/pET29 constructs were grown at 37˚C in Pennassay media containing 40 μg/ml of kanamycin. Mid-log phase cultures were incubated with 50 μL of 5 mg/mL lysozyme for 2 hours. Cultures were then induced with 0.5 μM IPTG and incubated at 37˚C for 6 to 8 hours. *E. coli* BL21 (DE3) constructs were maintained on Pennassay 1.5% agarose media containing 40 μg/ml and 25 μg/ml ampicillin. Media fractions were separated by ammonium sulfate precipitation. The 0-40% ammonium sulfate (AS) fraction was discarded and the 40-70% AS fraction was resuspended in 500 mL of 20 μM phosphate buffer, pH 6.8, and desalted with a ZEBA spin column (Pierce). Desalted samples were resuspended in 50 mL of 20 μM phosphate buffer, pH 6.8 and filtered with a 0.2 micron filter (Millipore). Manual sample protein purification was performed using a Mono S 5/50 GL cation exchange column (GE Healthcare) on an ÄKTA FPLC (GE Healthcare) with 20 μM phosphate buffer pH 6.8 system. Samples were washed with buffer A, 20 μM phosphate buffer pH 6.8 and eluted by increasing NaCl gradient of buffer B, 20 μM phosphate buffer pH 6.8 with 1 M NaCl. Elution sample concentrations were determined using Bradford's reagent (Sigma Aldrich) and comparison to a bovine serum albumin (BSA) (Sigma Aldrich) standard curve. The elution samples were then pooled together and the concentration was determined using the Bradford reagent method and lyophilized (Labconco). 10 mg stocks were preserved in 50% glycerol and stored at -20˚C.

3.3.4 Cancer cell cultures, MTT viability and apoptosis assays

Cytotoxicity of O75M to DU-145, H232A and MDA-MB-231 cells was studied using the MTT assay. First, cells ($5 \times 10^4$) were incubated for 24 h in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine. A serial dilution (100 μM to 1 nM) from the media purified O75M 10 mg stock was generated was generated with a final glycerol concentration of 0.5%. Thereafter, the cells were washed and plated with O75M samples in triplicate in 96-well plates and cultured for 48 h. The cells were then incubated for 4 h with RPMI 1640 containing 250 μg/mL of MTT. After discarding the culture medium, 200 μL of DMSO was added to dissolve the precipitates, and the resulting solution was measured for absorbance at 570 nm with a reference wavelength of 690 nm using an Infinite200Pro plate reader (Tecan). Significant differences between samples in cytotoxicity between treatment groups were determined using a two-tailed Student's *t*-test with $p < 0.05$ being considered significant. Apoptosis analysis was performed with ApoTox-Glo Triplex Assay kit (Promega) and results analyzed for fluorescence and luminescence with the Infinite200Pro plate reader (Tecan).

## 3.4. Results and Discussion

### 3.4.1. Cell penetrating peptide synthesis and secretion model design.

Development of a functional *E. coli* CPP expression system allows rapid CPP-fusion protein production for a broad CPP based anticancer agent production program. The CPP expression system was designed to utilize both ligation and DNA synthesis

approaches to develop functional CPP and CPP fusion protein products. Previous identification of transmembrane activity in the N-terminal α-helices of YopM, a T3SS protein associated with *Salmonella enterocolitica* infection, provided a CPP from a bacterium that was capable of expression in *E. coli* BL21(DE3), albeit in inclusion bodies (18). Primers designed to amplify the first 73 amino acids of *yopM*, associated with the two N-terminal α-helices with two extra C-terminal amino acids used as a linker for addition of a protein cargo allow the CPP to be cloned into pET29a. Addition of *ompF* signal sequence to export the CPP/CPP cargo peptide via cellular secretion pathways was designed to use a primer overlap PCR strategy with the 5' end of the *yopM* sequence. Also, a DNA synthetic approach was used to add the mitochondrial disrupting peptide (MDP) sequence through overlapping primers complementing the 3' end of the $yopM_{75}$ sequence. The MDP sequence was previously reported to have cancer cytotoxicity inducing apoptosis (24). The final *ompF-*$yopM_{75}$*-MDP* construct will be cloned into pET-29a to produce the expression vector pET-29-*ompF-*$yopM_{75}$*-MDP* (Fig. 12A; Fig. 12B). Utilizing the NdeI and XhoI restriction enzymes allows expression of the CPP fusion protein with a C-terminal 6x His-tag. The addition of the 6x His-tag CPP can be used both in purification and identification of the CPP-fusion protein by Ni-NTA column and Western blot, respectively. The addition of the *ompF* signal sequence directs the nascent CPP to the Type I secretion pathway for export into the periplasm and ultimately into the media for purification (25). Export of CPP proteins into the periplasm allows bi-direction transmembrane activity of the CPP to partially or completely escape into the media for purification. Furthermore, this model accounts for alternate methods for the release of the CPP construct into the media. Possible strategies for the cellular

CPP release include the addition of cell wall permeabilization agents like glycerol or formation of L-form *E. coli* BL21(DE3) spheroplast or protoplast expression strains to facilitate or increase CPP secretion into the media (26).

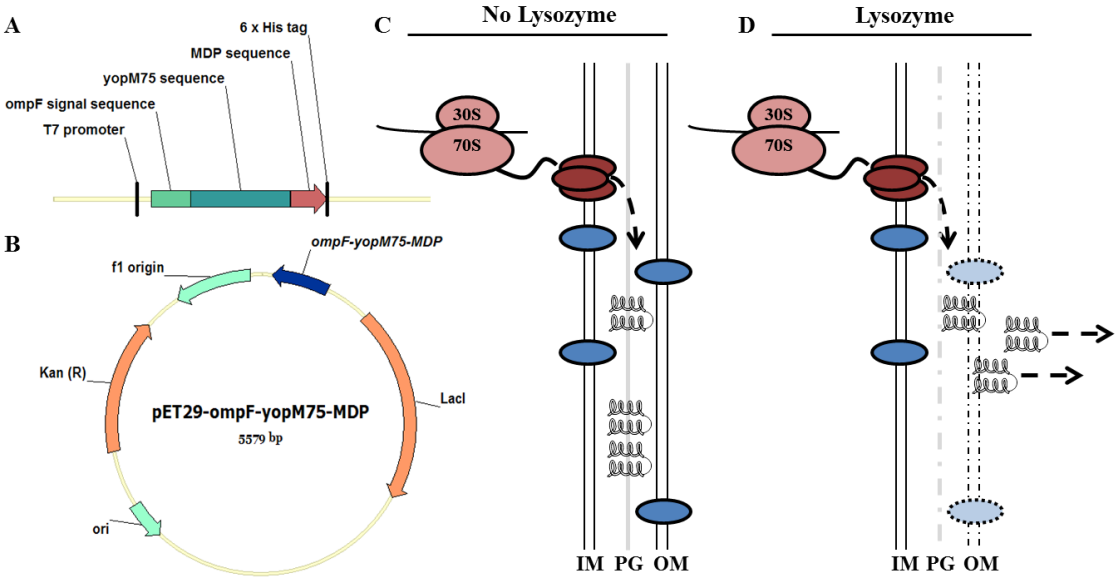


**Figure 12.** Model of CPP secretion system. (**A**) Expression cassette modular design with an inducible promoter, *ompF* signal sequence, CPP ($yopM_{75}$) and MDP (mitochondrial disrupting peptide). (**B**) Plasmid pET29-*ompF*-$yopM_{75}$-*MDP* construct with kanamycin resistance. (**C**) OmpF signal sequence directed Type I secretion pathway export of CPP to periplasm and release into the media. (**D**) Increased CPP export into the media can be achieved by agents that increase outer membrane permeability or degrade the cell wall. (IM, inner membrane; PG, peptidoglycan; OM, outer membrane)

3.4.2. Identification, cloning and expression of potential cell penetrating peptides.

Construction of a CPP with anticancer activity was based on the model design. First, $yopM_{75}$ was amplified by PCR, digested with NdeI/XhoI and ligated into the NdeI and XhoI sites of pET-29a. The pET-29a-$yopM_{75}$ construct was used to transform *E. coli*

DH5α and were verified by sequencing. Next, the *ompF* signal sequence and mitochondrial disrupting peptide sequence (*MDP*) were added using an overlap primer extension PCR method to synthesize the *ompF-yopM*$_{75}$*-MDP* construct was digested with NdeI/XhoI and ligated into NdeI/XhoI sites of pET29a (Table 7). Sequence verified pET29a- *ompF-yopM*$_{75}$*-MDP* constructs were used to transform the expression strain, *E. coli* BL21 (DE3). Protein expression experiments with *E. coli* BL21 (DE3)/pET29a-*ompF-yopM*$_{75}$*-MDP* and control strain *E. coli* BL21 (DE3)/pET29a were performed in 50 mL cultures and induced with IPTG during mid-logarithmic growth. Whole cell fractions containing the media, cytoplasm and membrane fractions were analyzed by SDS-PAGE gels to determine protein expression. The whole cell fraction showed the presence of a 13 kDa band visible in the IPTG induced sample (Fig. 13). After cell lysis, a similar band was found in the cell pellet fraction consisting of insoluble membrane protein and protein inclusion bodies showing the CPP-fusion protein was expressed but packaged into inclusion bodies (Fig. 13). This result also showed the OmpF-YopM$_{75}$-MDP protein was expressed, but the signal sequence was not cleaved in the whole cell or the cell pellet inclusion body fraction accounting for the increase in molecular weight. Next, the periplasmic fraction was examined for protein secretion and in contrast to the cell pellet fraction, the periplasmic fraction showed an 11 kDa band related to the YopM$_{75}$-MDP protein secreted into the periplasm (Fig. 13). This result demonstrated the functional activity of the OmpF signal sequence directing protein secretion to the periplasm and the cleavage of the signal sequence upon entering the periplasm. However, examination of the media for the presence of the YopM$_{75}$-MDP did not show the release of the protein through the outer membrane into the media. Previous studies demonstrated the

localization and accumulation of OmpF signal proteins in the periplasm and counter reports of extracellular secretion. (25) This result supports the notion that the formation of inclusion bodies was due to the accumulation of protein in the periplasm and did not release the protein into the extracellular media due to periplasm crowding. However, protein expression from a strong promoter like the T7 promoter may offset the balance between secretion protein synthesis and secretion pathway periplasm export. Also, the increase inclusion body formation may be a cellular reaction to the cytoplasmic accumulation of OmpF-YopM$_{75}$-MDP with inherent toxic properties or induced toxicity from accumulation.

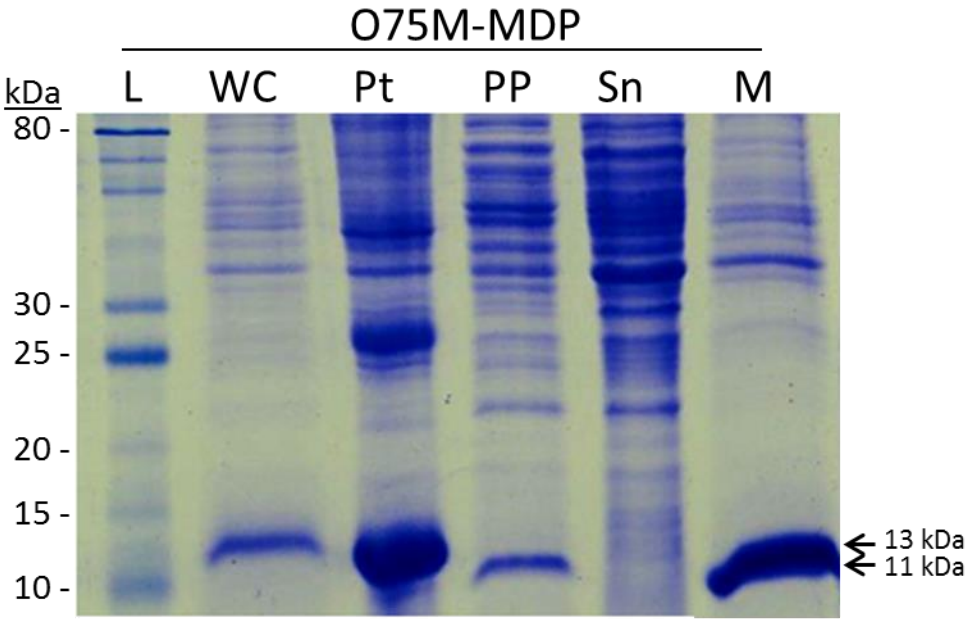


**Figure 13.** YopM$_{75}$-MDP expression and secretion to the media. The initial expression analysis of the whole cell (WC) fraction showed YopM$_{75}$-MDP at 13 kDa. Further analysis showed YopM$_{75}$-MDP formed inclusion bodies isolated in the cell pellet (Pt) also at 13 kDa correlating with the YopM$_{75}$-MDP still possessing the OmpF, signal

sequence. In contrast, soluble $YopM_{75}$-MDP was not detected in the cytoplasm supernatant (Sn) fraction. Examination of the periplasm showed secretion and accumulation of $YopM_{75}$-MDP at 11 kDa, related to the excision of OmpF during secretion. Initial $YopM_{75}$-MDP was not detected from the media, however when cells were treated with lysozyme the 11 kDa band was present and corresponded to the release of $YopM_{75}$-MDP into the media.

3.4.3. Development of L form *E. coli*, secretion of peptides to the media and purification.

The secretion of proteins into the media has been extensively researched and often requires the addition of membrane permeabilization molecules or cell wall disruption agents. Previous protein export attempts report using molecules like glycerol increase the diffusion rate of proteins through the cell wall, but the diffusion rates to the growth media remained slow (26). Therefore, cell wall disrupting agents like penicillin or lysozyme appear to be the best suited agents for high yield protein secretion systems. The addition of these agents leads to the degradation of the peptidoglycan layer and either partial or complete removal of the outer membrane in the production of L-form bacterial spheroplasts or protoplasts, respectively. In order to construct active protein expression L-form *E. coli* BL21(DE3)/ pET29a-*ompF-yopM*$_{75}$*-MDP* cells, 50 mL Penassay broth media cultures were grown to mid-logarithmic growth and 50 µL of 5 mg/mL lysozyme stock was added and incubated for 2 hours prior to induction with final IPTG concentration of 0.5µM. Lysozyme treated and non-treated cultures were streaked on Pennassay L-form agar media containing kanamycin and ampicillin to compare culture morphology. Kanamycin was used for selection of strains carrying the pET29 construct,

while a serial dilution of ampicillin was used to test for L-form phenotype demonstrating increased resistance to peptidoglycan targeting antibiotics like ampicillin. The remaining culture was analyzed for protein secretion into the media.

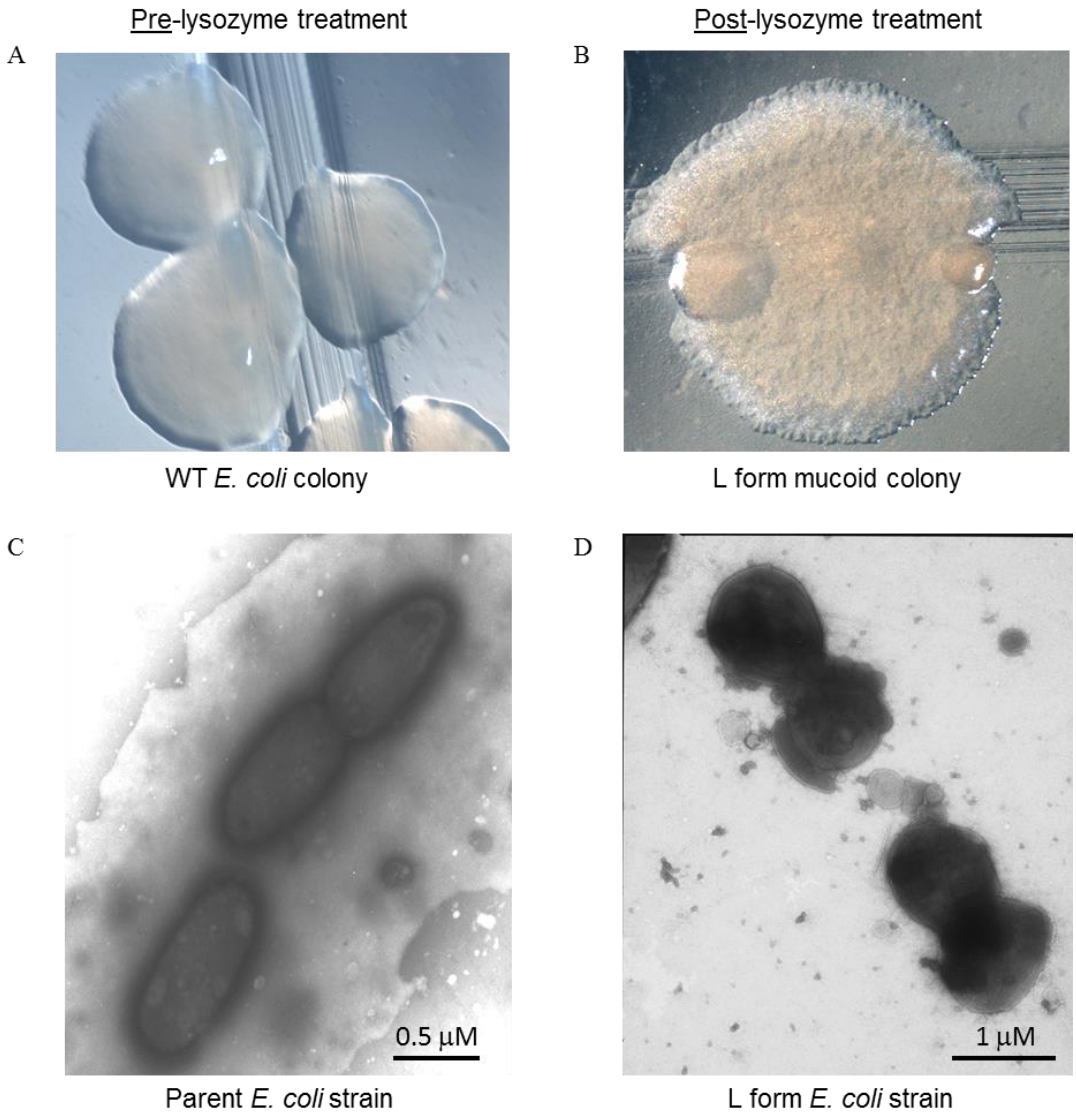


**Figure 14.** Formation of L form BL21 (DE3) *E. coli*. Parental *E. coli* BL21 (DE3) colonies demonstrate tan colonies with distinct boundries (**A**), while lysozyme treated *E. coli* BL21 (DE3) formed white/milky mucoid colonies depicting L form phenotype (**B**). Morphology analysis by TEM at 17,000X showed parental *E. coli* BL21 (DE3) cells as

smooth rod shaped bacteria (**C**). In contrast, L form *E. coli* BL21 (DE3) showed a round, cocoid bacteria with frayed outermembrane and the presence of membrane blebs (**D**).

Examination of the cell culture morphology showed a difference between lysozyme treated and non-lysozyme treated cells. Lysozyme treated cell formed large white and tan mucoid colonies with undefined boundaries, while the untreated cells formed tan oval colonies with defined boundaries (Fig 14A; Fig. 14B). Next, the cell phenotype was analyzed by TEM to identify the presence of L-form spheroplasts or protoplasts. TEM micrographs of lysozyme treated sample showed the presence of round cells, with partial attachment of the outer membrane and membrane blebbing (Fig. 14D). In comparison, the untreated cells were rod shaped with a smooth outer membrane (Fig. 14C). These results, showed the formation of L-form spheroplasts from *E. coli* BL21 (DE3)/ pET29a-*ompF-yopM*$_{75}$*-MDP* cells with similar colony morphology and cellular phenotypes to previously described L form bacteria (13).

Comparison of media fractions between lysozyme treated cultures and non-lysozyme treated cultures showed the presence of an 11 kDa protein in the lysozyme treated cultures. This protein correlates with the protein identified in the periplasmic fraction of similar molecular weight (Fig. 13). This protein was purified by anion exchange chromatography with a yield of 32 mg/L and produced an elution protein at 11 kDa correlating to both the periplasmic and media fractions (Fig. 15). This protein was confirmed by antibody detection of the C-terminal His tag using Western blot analysis, which verified the presence of YopM$_{75}$-MDP in the media post lysozyme treatment. Next, the elution samples were pooled and dialyzed to reduce contaminating salt

concentrations. The pooled sample was concentrated by lyophilization and resuspended in 50% glycerol for storage. The purification of $YopM_{75}$-MDP represents a fast and efficient strategy for the secretion and purification of CPP fusion proteins by utilizing the unique cell wall deficiencies of L form *E. coli* BL21 (DE3) cells. The rapid generation of CPPs and CPP fusion proteins can be used to produce therapeutic concentration and test for novel activities.

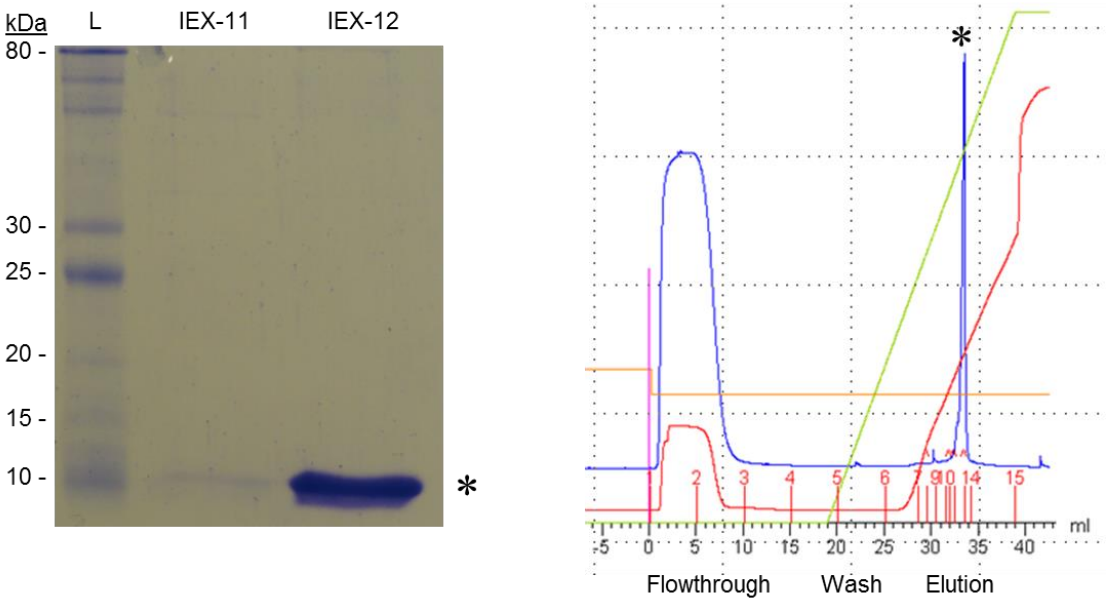


**Figure 15.** Purification of $YopM_{75}$-MDP from the media. The prepared media fractions were separated and purified by anion exchange chromatography yielding a single band at 11 kDa corresponding to $YopM_{75}$-MDP (*).

3.4.4. Antiproliferative activity of CPP.

Cancer cell viability screening demonstrated $YopM_{75}$-MDP antiproliferative activity toward lung, breast and prostate cancer cell lines. Purified YopM75-MDP was used for MTT cell viability assays and determined the antiproliferative activity $IC_{50}$ value

in the low µM range for each cancer cell type. The $IC_{50}$ values were calculated to be 2.47 ± 0.2µM, 3.27 ± 0.2µM and 6.99 ± 0.12µM for DU-145 prostate cancer cells, H232A lung cancer cells and MDA MB-231 breast cancer cells, respectively (Fig. 16). The DU-145 prostate cancer cell viability assay result was further supported by the Apo-Tox-Glo result. The $IC_{50}$ was calculated to be 3.99 ± 0.2µM and was similar to the MTT assay result. The toxicity assay showed similar fluorescence to the control demonstrating the cells were not rapidly lysing. While, caspase 3/7 activity increased with increasing concentrations of $YopM_{75}$-MDP demonstrating the induction of apoptosis through the induction of the caspase cascade (Fig. 17). These results show similar cytotoxicity toward cancer cell lines, while reducing toxicity toward *E. coli* (24). The MDP sequence bears high sequence similarity to synthetic antimicrobial peptides that exhibited cytotoxicity toward *E. coli* and 3T3 mouse embryonic fibroblasts (27). The addition of a CPP that originated in *Yersinia enterocolitica*, a relative of *E. coli*, would suggest that antimicrobial activity may be reduced toward bacteria through the fusion of $YopM_{75}$. Furthermore, the activity of $YopM_{75}$-MDP provides further evidence of the vast potential of CPP fusion proteins. The purification of $YopM_{75}$-MDP from the media demonstrated the ability to generate novel *E. coli* L form expression systems with lysozyme treatment to release OmpF based signal proteins. Finally, the formation of *E. coli* L form protein secretion cells advances our goal toward generating CPP expressing anticancer bio-agents capable of intra-tumor growth and anticancer protein expression.

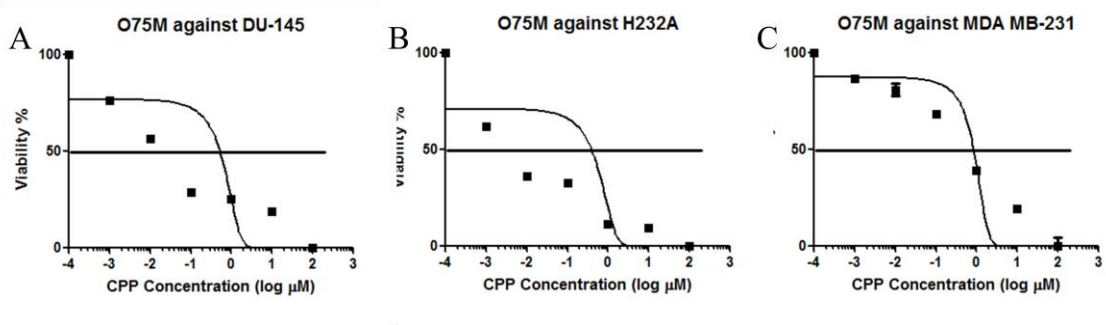


**Figure 16.** Cancer cell antiproliferation assays. YopM$_{75}$-MDP was tested for activity against three cancer cell lines. (**A**) prostate cancer cell line DU-145, (**B**) breast cancer cell line H232A, (**C**) lung cancer cell line MDA MB-231.

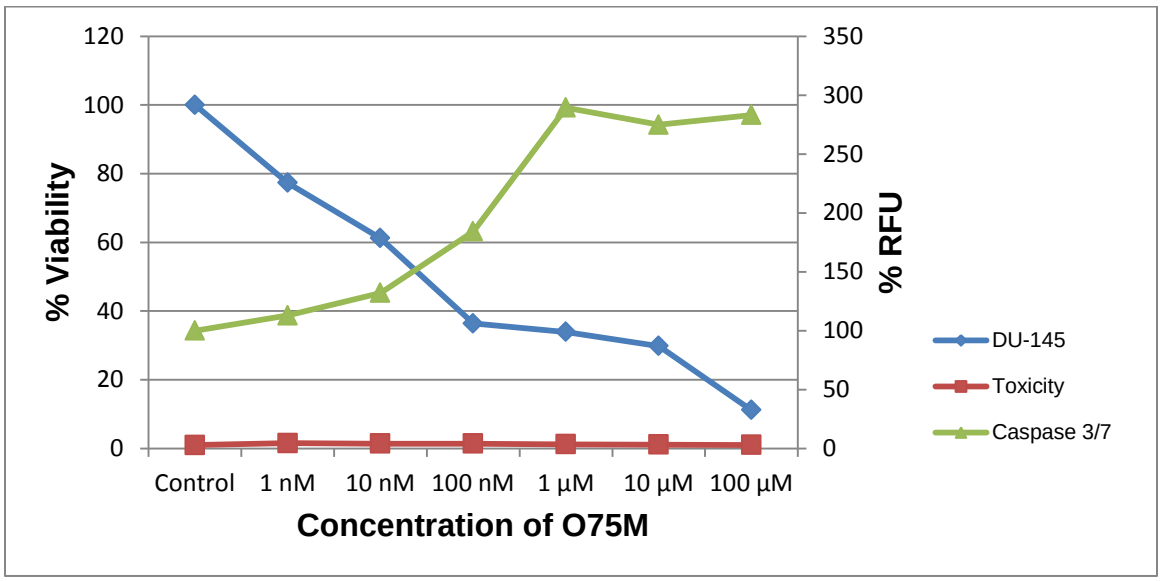


**Figure 17.** Apoptosis pathway induction assay. YopM$_{75}$-MDP showed induced caspase activity in prostate cancer cell line DU-145. Also, YopM$_{75}$-MDP did not show increased toxicity, while demonstrating similar cell viability results to the MTT analysis.

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**Table 6.** Bacterial strains and plasmids used in this study

| Strains or plasmids | Description[a] | Source or reference |
|---|---|---|
| *Yesinia enterocolitica* O8 strain JB580v | Wild type strain | |
| *Escherichia coli* | | |
| DH5α | General cloning host | Lab stock |
| BL21(DE3) | Host strain for heterologous gene expression | Novagen |
| SW01 | BL21(DE3) harboring pET29 | This work |
| SW02 | BL21(DE3) harboring pET29-ompF-*yop75*-MDP | This work |
| Plasmids | | |
| pET29 | Kan$^r$, general cloning vector | Novagen |
| pET29-*yop75* | Kan$^r$, *yop75* CCP cloned into MCS | This study |
| pET29-ompF-*yop75* | Kan$^r$, *yop75* with *ompF* signal sequence | This study |
| pET29-ompF-*yop75*-MDP | Kan$^r$, *yop75* with *ompF* signal sequence and MDP gene sequence | This study |

[a] Kan$^r$, kanamycin resistance; MCS, multiple cloning site; MDP, mitochondrial disrupting peptide.

[b]strain was provided as a gift by Dr. Kristen Walker, University North Carolina (UNC) School of Medicine.

**Table 7.** Primers used for construction of CPPs and gene expression

| Name (primers sorted by gene) | Sequence (5' → 3')[a] | Gene Name |
|---|---|---|
| FP-yop75-NdeI<br>RP-yop75-BamHI-XhoI | CTAGCACATATGTTTATAACTCCAAGAAATGTATC<br>CGCTCGAG GGATCCGGCTTGTCGGTCCAGGC | *yop75* |
| Amplification primer<br>FP-OmpF-SRP OV-NdeI<br><br>Synthesis primers<br>FP-OmpF-SRP OV2<br><br>RP-OmpF-SRP OV | CTAGCACATATGATGATGAAGCGCAATATTCTG<br><br>GCTCTGTTAGTAGCAGGTACTGCAAACGCTTTTAT<br>AACTCCAAGAAATGTATC<br>TGCAGTACCTGCTACTAACAGAGCAGGGACGATC<br>ACTGCCAGAATATTGCGCTTCATCAT | *ompF* signal sequence |
| Amplification primer<br>RP-MDP-CPP-XhoI<br><br>Synthesis primer<br>RP-CPP-MDP-OV | CCCTCGAGTTTCGCCAGTTTCGCCAG<br><br>TTTCGCCAGTTTCGCCAGTTTTTTCGCCAGTTTCGC<br>CAGTTT GGCTTGTCGGTCCAGGC | *MDP* |

[a]Sequences in capital letters are designed either to provide restriction sites (underlined) for directional cloning, or to provide extra base(s) in front of restriction sites for efficient restriction enzyme digestion, or to provide overlapping sequences for CPP expression cassette assembly during multiplex PCR.

**Table 8**. DNA and Protein sequences

| Name of Sequence | Sequence (5' → 3')[a] |
|---|---|
| *ompF* signal sequence | ATGATGAAGCGCAATATTCTGGCAGTGATCGTCCCTGCTCTGTTA GTAGCAGGTACTGCAAACGCT |
| OmpF signal sequence | MMKRNILAVIVPALLVAGTANA |
| *yopM*$_{75}$ | ATGTTTATAACTCCAAGAAATGTATCTAATACTTTTTTGCAAGAAC CATTACGTCATTCTTCTGATTTAACTGAGATGCCGGTTGAAGCAG AAAATGTTAAATCTAAGACTGAATATTATAATGCATGGGCGGTAT GGGAACGAAATGCCCCTCCGGGGAATGGTGAACAGAGGGAAATG GCGGTTTCAAGGTTACGCGATTGCCTGGACCGACAAGCCGGATCC TCTAGAGTCGACCTGCAGGCATGCAAGCTTGCGGCCGCACTCGAG CACCACCACCACCACCACTGAGATCCGGCTGCTAACTCGAC |
| YopM$_{75}$ | MFITPRNVSNTFLQEPLRHSSDLTEMPVEAENVKSKTEYYNAWAVW ERNAPPGNGEQREMAVSRLRDCLDRQALE |
| *MDP* | AAACTGGCGAAACTGGCGAAAAAACTGGCGAAACTGGCGAAA |
| MDP | KLAKLAKKLAKLAK |
| *ompF-yopM*$_{75}$*-MDP* | ATGATGAAGCGCAATATTCTGGCAGTGATCGTCCCTGCTCTGTTA GTAGCAGGTACTGCAAACGCTATGTTTATAACTCCAAGAAATGTA TCTAATACTTTTTTGCAAGAACCATTACGTCATTCTTCTGATTTAA CTGAGATGCCGGTTGAAGCAGAAAATGTTAAATCTAAGACTGAAT ATTATAATGCATGGGCGGTATGGGAACGAAATGCCCCTCCGGGG AATGGTGAACAGAGGGAAATGGCGGTTTCAAGGTTACGCGATTG CCTGGACCGACAAGCCAAACTGGCGAAACTGGCGAAAAAACTGG |

| | |
|---|---|
| | CGAAACTGGCGAAA |
| OmpF-YopM$_{75}$-MDP | MMKRNILAVIVPALLVAGTANAMFITPRNVSNTFLQEPLRHSSDLTE<br>MPVEAENVKSKTEYYNAWAVWERNAPPGNGEQR<br>EMAVSRLRDCLDRQAKLAKLAKKLAKLAK |

CHAPTER 4

# SIGNIFICANCE AND FUTURE DIRECTIONS

## Engineering of CPP anticancer bio-agents

## 4.1. Significance of Research

The development of new anticancer agents with the ultimate goal of preventing and curing cancer has been the goal of researchers for many years. Current anticancer strategies employ a number of agents including natural products or peptide anticancer agents that target cancerous or pro-cancerous cell states. Cancer drug targets include many distinct cancer pathways such as metabolic, apoptosis, genetic and epigenetic that distinguish individual cancer cell lines.

Many epigenetic targets have demonstrated their potential as a cancer therapy and continuing research in HDAC inhibition is focused on isoform-specific agents like FK228 that display better pharmacological properties that pan-HDAC inhibitors (1). Since the approval of FK228 by the FDA for cutaneous T-cell lymphoma more depsipeptide compounds like the thailandepsins and spiruchostatins have been identified with different HDAC selectivity and the potential to target different cancer types (2, 3). These depsipeptides share similar genetic organization and biosynthetic pathways and offer an opportunity for engineering hybrid depsipeptides with novel HDAC inhibitory function.

Further research of the FK228 biosynthetic pathway showed the in *trans* recruitment of both a Ppant transferase and an AT domain during the production of FK228. The stand alone AT domain and Ppant transferase in *C. violaceum* were found to be constitutive genes suggesting that these proteins may be involved in essential cell functions. The Sfp-type Ppant transferases found within the gene clusters are often dispensable for the associated polyketide or NRPS biosynthesis. However, our research suggests that Sfp-type Ppant transferases are essential to *C. violaceum* and the true

physiological role(s) for stand-alone Ppant transferase remains to be established. Previous research demonstrated and increased physiological role where the broad spectrum activity of Sfp-type Ppant transferases substituted Ppant transfer activity for fatty acid biosynthesis in *B. subtilis* and *Pseudomonas aeruginosa* in the absence of ACPS-type Ppant transferases (4, 5).

The "stand alone" AT domain in *C. violaceum* demonstrated the first evidence of "cross talk" between modular NRPS-PKS systems and fatty acid biosynthesis pathways. The bridge between fatty acid biosynthesis and polyketide synthesis has been addressed previously in *Streptomyces* species containing many type II polyketide synthases which are analogous to the type II fatty acid biosynthetic pathway composed of discrete proteins. Early findings showed *S. coelicolor* contained three different ACP-like proteins, rather than a single ACP, that were not shared among PKS and fatty acid biosynthesis pathways providing one level of pathway specificity (6). Furthermore, FabD, the malonyl-CoA:AcpP acyl-transferase found in fatty acid biosynthesis was shown to be active on both AcpP and type II PKS ACPs (7). FabD is often found within the fatty acid biosynthetic operon (*E. coli*) and a homologue from the fatty acid biosynthesis operon in *S. glaucescens* was shown to transfer malonate to both tetracenomycin (Tcm) ACP and fatty acid ACP. Alternatively, pathway specificity may be determined by the carrier domains and the specific docking domain protein-protein interactions allow for the proper biosynthesis of fatty acid biosynthesis, PKS and NRPS pathways. Future research on the docking domains of ACPs will address questions associated with the similarity to fatty acid ACPs to elucidate the specificity and additional functions of these domains. The expanded physiological roles of Ppant

transferases and acyl transferases suggests that they may represent new targets for antimicrobial treatment of bacteria that have these enzymes associated with essential pathways like fatty acid biosynthesis.

The future of CPP research will continue to develop new applications further enhancing the broad range of CPP activities. Our research shows the engineering potential of CPPs for the development of new therapeutic agents. However, in the future we would expand this project to engineer and express CPPs and CPP-fusion peptides in *E. coli* that can target specific cells, differentiate healthy from unhealthy cells and interact with specific intracellular pathways. This new method for releasing the outer cell wall through L form bacteria production allowed CPP-MDP secretion to the media for rapid purification and may be expanded to purify new CPP-fusion constructs to test for anticancer activity. The secretion of CDP-MDP into the media was critical for future research goals toward generating an anticancer CPP tumor targeting bio-agent. Future experiments may require the development of a stable L form bacteria through genetic mutation(s) to prevent reversion back to the parental phenotype in the construction of these new CPP based bio-agents. Also, the expression of the CPP may need to be adapted to the tumor microenvironment depending on the final host selected for growth and CPP expression within the tumor.

## 4.2. Targeting the tumor microenvironment

Cancer is a major public health problem facing mankind and yet the development of effective cancer treatments with no or few side effects remains one of the most difficult scientific challenges of our time. Cancer occurs in many types (such as breast

cancer, skin cancer, or leukemia – cancer heterogeneity) and the effectiveness of conventional treatments (surgical removal, radiotherapy and chemotherapy) varies (8). Consequently, there have been intensive efforts to develop alternative cancer therapies including bone marrow transplantation, immunotherapy, gene therapy, hormone therapy, and inhibition of angiogenesis (formation of blood vessels) or specific protein targets (mostly kinases) in key signaling pathways, all with mixed success (9). A fundamental requirement for any cancer therapy is to differentially suppress the proliferation of cancer cells or selectively eliminate cancer cells, while minimizing toxicity toward normal cells to an acceptable level.

Most human cancers are solid tumors. Solid tumors generally have an architecture in which the inner core maintains a very low oxygen microenvironment (known as "hypoxia"). Hypoxia occurs when tumor mass outgrows angiogenesis 10, 11). The hypoxic microenvironment of solid tumors often contains areas of necrosis that are resistant to both radiotherapy and chemotherapy; resistance to chemotherapy is, in part, due to the poor drug bioavailability as a result of the distance between inner tumor mass and peripheral blood vessels. Solid tumors with a hypoxic/necrotic core are also predisposed to increased tumor spreading (known as "metastasis") (12). Paradoxically, the pathophysiologic characteristics of hypoxia in solid tumors also provide a unique opportunity for the development of tumor-selective treatments, including prodrugs activated by hypoxia, hypoxia-specific gene therapy, targeting the hypoxia-inducible transcription factor, and also the use of anaerobic bacteria as tumorcidal bio-agents (12). The early observations of cancer regression following bacterial infections in patients has inspired active pursuits of nonpathogenic anaerobic bacteria as anticancer bio-agents over

the last 60 years (13-15). In fact, the human body between external skin and inner digestive lumen is normally well oxygenated except for the hypoxic/necrotic cores within solid tumors where some anaerobic bacteria administered systemically through the blood circulation, or injected locally into solid tumors, can infiltrate and proliferate. As a consequence, tumor mass in the bacteria-colonized regions is selectively destroyed (known as "oncolysis") and nutrients released are consumed by the bacterial population.

The first generation of anticancer bio-agents were natural isolates of facultative or obligate anaerobic bacteria, belonging to the genus of *Bifidobacterium*, *Clostridium*, or *Salmonella*. Experiments performed on animal models or cancer patients demonstrated that administered bacterial spores or vegetative cells germinate and/or multiply in the hypoxic/necrotic regions of solid tumors, and that therefore limited oncolysis in these tumor regions can occur. However, a common problem hindering this approach is that anaerobic bacteria die when they reach to the outer layers of solid tumors where oxygen levels elevate. Some bacterial strains also produced endotoxins that resulted in excessive cytotoxicity toward normal cells, which inspired the creation of attenuated strains with proven safety records (16-18). Thus, the first generation of anticancer bio-agents could only destroy the inner parts of solid tumors but could not completely eradicate them; tumors would re-grow from the remaining viable outer rings.

The second generation of anticancer bio-agents were genetically engineered anaerobic bacteria that produce prodrug-activating enzymes or tumor necrosis factors (13, 19). Prodrugs are injected intravenously and are activated by the enzymes at the sites of bacterial spore germination and vegetative growth inside solid tumors. This generation of anticancer bio-agents achieved greater oncolytic effects and some of them have actually

entered clinical trials, but the results have still not been sufficient to warrant practical cancer therapy. The effectiveness of prodrug injections is hampered by poor prodrug bioavailability due to the distance between peripheral blood vessels and the hypoxic core; an extra dose of tumor necrosis factor only slightly enhanced the necrosis in the already necrotic regions of solid tumors. Further improvement by combining anticancer bio-agents with vascular-targeting chemicals resulted in still only modest increases of oncolytic activity (19). Nevertheless, the benefits of exploiting tumor hypoxia for cancer therapy have yet to be fully realized (12).

4.3. Selection of anaerobic expression host

The production and secretion of CPPs will generate a new anticancer treatment combining the tumor targeting and anticancer activities of the bacteria with the anticancer activity of the CPPs. The continuing anticancer CPPs development in *E. coli* BL21(DE3) can lead to the engineering of many specific anticancer agents that are capable of targeting multiple pathways to treat cancer. *E. coli* BL21(DE3) can be used to engineer the anaerobic expression cassette, the integration of the expression into bacterial chromosome and CPP secretion into the media. *E. coli* BL21(DE3) has been used successfully to express and secrete the CPP fusion proteins into the media. A secondary benefit of engineering *E. coli* strains is the potential for large scale media production of CPPs, because most current CPPs are chemically synthesized or derived from peptide digestion. The expression cassettes constructed in *E. coli* strains can be easily modified by exchanging promoters and signal sequences targeting protein secretion pathways that work in different recipient anaerobic bacterial hosts such as *Bifidobacterium longum* and

attenuated *Salmonella typhimurium*. Both *B. longum* and *S. typhimurium* have higher (G+C) contents than that (50.8%) of *E. coli,* therefore codon usage would be evaluated and adjusted to accommodate these differences. Although *B. longum* and attenuated *S. typhimurium* have some shortcomings (Table 10), currently they are the best practical hosts for this research. *Clostridium novyi-NT* and *Clostridium sporogenes* are Gram-positive, spore-forming, strictly obligate anaerobic bacteria and have demonstrated strong oncolytic effects with a high degree of safety. Although they would be adaptable hosts for the purpose, they are recalcitrant to genetic manipulation making them more difficult to use for this research (20). Transfer of the anaerobic expression cassette and integration into the genome of *Clostridia* strains represents a formidable task and may require new methods for efficient genetic transformation. Also, since CPPs have shown greater antibacterial activity toward some Gram-positive bacteria than Gram-negative, both *Clostridia*and *Bifidobacterium* may present additional challenges to CPP secretion system engineering (21,22). Therefore, strains of *Clostridia* and *Bifidobacterium* are considered as secondary candidates for this research.

**Table 10.**. Parts of data were adopted from (13-15); *E. coli* strains are chosen as priming hosts for pilot experiments.

| Genus/Species[a] | Available?[b] | Genetically Engineerable?[c] (Comment/Reference) | (G+C) Content[d] | Advantages[e] | Disadvantages[e] | Priority[f] |
|---|---|---|---|---|---|---|
| *E. coli* BL21(DE3) or variant strains | Commercial sources | Yes (Genetic workhorse) (21) | 50.8 | - Easy genetics<br>- Collateral benefit<br>- Good for pilot experiments | - Alone not strong oncolytic<br>- Non-spore former | Pilot/1st |
| *B. adolescentis* | - | Yes (22) | 59.2 | - Non-toxic<br>- Intravenous or oral administration<br>- Modestly high (G+C)% content | - Alone not strong oncolytic<br>- Non-spore former<br>- Difficult to store and handle<br>- Susceptible to non-permissive conditions | |
| *B. bifidum* | - | Unknown (22) | | | | |
| *B. infantis* | - | Yes (22) | ~59-61 | | | |
| *B. longum* | Private, Public | Yes (23,24) | 60.1 | | | 1st |
| *C. acetobutyricum* (*C. beijerinckii*) | - | Yes (25) | 30.9 | - Spore former<br>- Spores are easy to produce, stable and economical to use<br>- Intravenous administration<br>- Demonstrated strong oncolytic ability<br>- Demonstrated safety of nonpathogenic strains<br>- Attenuated strains available | - Some strains are pathogenic<br>- Strain variation exists<br>- Very low (G+C)% content<br>- Difficult genetics | |
| *C. novyi-NT* and derived strains (attenuated) | Private | No (16,20) | 28.9 | | | 2nd |
| *C. sordellii* | - | Unknown (16) | ~28-30 | | | |
| *C. sporogenes* (*C. butyricum*) (*C. oncolyticum*) | Private, Public | Yes but very difficult (26, 27) | ~28-30 | | | 2nd |
| *S. choleraesuis* | - | Yes (28) | 52.1 | - Intrinsic antitumor activity<br>- Can target both large and small tumors<br>- Attenuated strains available | - Wild-type strains are pathogenic<br>- Safety is a concern<br>- May induce weak innate immune response<br>- Alone not strong oncolytic | |
| *S. typhimurium* VNP20009 (attenuated) | Private, Public | Yes (29, 30) | 52.2 | | | 1st |

[a] *E. coli*, *Escherichia coli* – Gram-negative, facultative, suitable for pilot experiments; *B, Bifidobacterium* – Gram-positive, obligate anaerobe; *C, Clostridium* - Gram-positive, strictly obligate anaerobe; *S, Salmonella* - Gram-negative, facultative anaerobe. Several strains of *C. acetobutyricum* were renamed as *C. beijerinckii*. Several strains of *C. oncolyticum* used to be named *C. butyricum*, and later renamed as *C. sporogenes*.

[b] Private, private sources through (intended) collaborations; Public, public sources such as strain stock centers; -, not concerned.

[c] Genetic engineerability refers to the availability of appropriate vectors and proven methods of bacterial transformation.

[d] The (G+C) contents, wherever available, are from microbial genome databases. ~, Estimated. A close (G+C) content assumes similar codon usages.

[e] Advantages/disadvantages collectively refer to the genus.

[f] Subject to personal judgment.

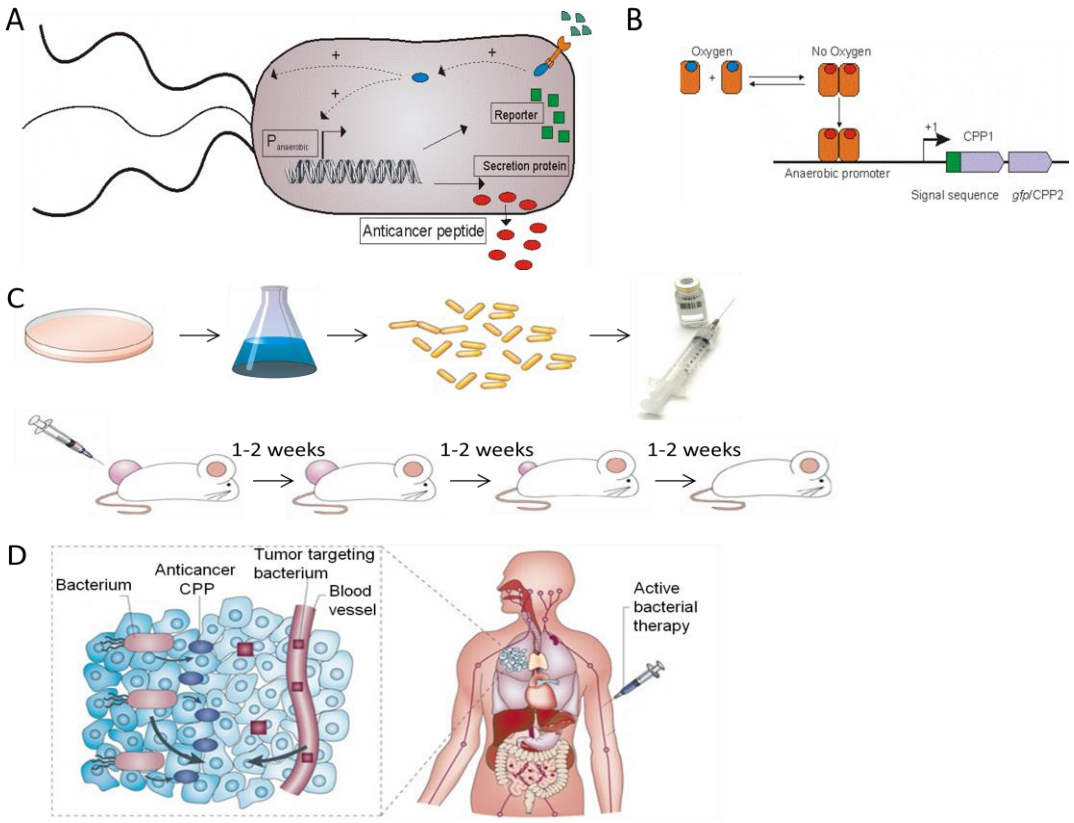


**Figure 18.** Anticancer bio-agent experimental design image**.** A) Engineering of anticancer tumor targeting bacterium shows induction of gene expression in an anaerobic environment leading to secretion of anticancer cell penetrating peptide (CPP) and production of a reporter protein (Gfp). B) The expression cassette module is activated under hypoxic condition leading to gene expression of CPP and reporter genes. C) Production and formulation of bacterial spores or bacteria for testing in mouse cancer model. D) Future systemic application of tumor targeting CPP expressing anticancer bio-agents. Part D was adapted with permission from (31).

## 4.4. CPP fusion peptide expression cassette design

Efficient production of CPPs in engineered bacteria requires all the necessary genes to be arranged properly under the proper regulatory circuits. Specifically, designing

the expression cassette with four exchangeable modules will allow rapid amendments to each individual component of the CPP expression system. The first module will contain an interchangeable promoter from the given host genome so that the expression of the CPP gene is controlled by environmental influences simulating a hypoxic tumor microenvironment (Fig. 18B). Since the genome sequences of all prioritized host bacteria but *C. sporogenes* are available, promoters activated under hypoxic growth conditions can be identified and verified experimentally. The second module will contain a signal sequence that directs the peptide to the corresponding protein secretion pathway. The third module will contain the CPP with transmembrane activity that can carry an anticancer protein or protein domain such as MDP, directed toward apoptosis or cancer pathways (Fig. 18A). Some additional CPPs that possess inherent domain targeting ability that have anticancer activity could be used to increase the number of anticancer peptides expressed by the host (Table 10). The CPPs may be modified to contain a small peptide or protein fusion product with known function to block protein/protein interactions and inhibit cell proliferation or induce apoptosis. The fourth module allows for co-expression of a fusion protein, another CPP, or a fluorescent marker gene (*gfp*) from the same promoter. However, the fourth module may be optional. In order to facilitate expression cassette transfer, integration and mutant selection in a receiving host, a marker gene (conferring antibiotic resistance) driven by a constitutive promoter would be placed upstream of the expression cassette. Finally, the integration of the expression cassette into the host genome will be facilitated by transposon insertion to complete the construction of the anticancer CPP expressing bio-agent (Fig. 18C, Fig. 18D).

**Table 10.** Cell penetrating peptides that display promising anticancer activity**.**

| CPP | Protein Source | Types of Cancer | Mode of Action | Bactericidal activity |
|---|---|---|---|---|
| **PNC-27**[1] | Hdm-2 binding protein | Breast, colon, osteosarcoma | Necrosis and p53 binding apoptosis (32) | Unknown |
| **PNC-28**[1] | Hdm-2 binding protein | Breast, colon, bone, pancreatic | Apoptosis via p53 (33) | Unknown |
| **CytC**$^{77\text{-}101}$ | Cytochrome C | Untested | Apoptosis pathway via mitochondria (34) | Gram + > Gram- |
| **PEGA-pVEG** | Vascular homing peptide | Breast | Anti-angiogenesis (35) | Gram + > Gram- |
| **CB1a** | cecropins | Stomach, lung, leaukemia | Membrane disruption (36) | Poor activity |
| **MAP**[2] | Artificial model amphipathic peptide | Breast, leukemia, melanoma | Membrane disruption (37) | Gram + > Gram- |
| **Transportin**[2] | Galanin and mastoparan fragments | Breast, leukemia, melanoma | Membrane disruption (37) | Gram + > Gram- |

[1]PNC-27 and PNC-28 were synthesized with penetratin CPP attached to carboxyl terminus of Hdm-2 binding protein that binds p53

[2]Map and Transportin show hemolytic toxicity

## 4.5. Anti-tumor activity analysis in mouse tumor models

Formulated anticancer bio-agents will be tested first on mouse models carrying human tumor xenografts by intravenous (systematic) injection (38-40). Anaerobic bacteria have demonstrated the ability to circulate safely through bloodstream, infiltrate and selectively (germinate if spores) grow inside solid tumors in the hypoxic/necrotic regions. As a pure physiological consequence, tumor mass will be destroyed and the released nutrients will be consumed by proliferating bacteria. Secondly, the bacterial growth under hypoxic conditions will activate gene expression from the anaerobic promoter and the anticancer cell penetrating peptide will be secreted outside of the cell. There is no need for injection of other anticancer prodrugs as anticancer CPPs are produced de novo inside solid tumors. Although obligate and facultative anaerobic bacteria will cease to grow and eventually die as they progress to the outer rings of solid tumors where the oxygen levels elevate or evoke an immune response, CPPs will continue to diffuse and selectively kill proliferating cancer cells. Therefore, a strong synergy between the physical consumption of cancer mass by engineered anaerobic bacteria and the anticancer activities of CPPs could have the potential to eradicate many kinds of solid tumors regardless of cancer genotype. Promising test results on mouse models would warrant future tests on large animal models and cancer patients according to preclinical and clinical trials.

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# CURRICULUM VITAE

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"A Synthetic Biology Approach to Developing New Anticancer Agents"

The aims of my doctoral dissertation research are to determine the complete biosynthetic pathway of an important anticancer drug FK228 and to engineer heterologous expression systems to explore the new natural products and cell penetrating peptide based anticancer agents. Through my research we precisely reconstituted the FK228 biosynthetic gene cluster in *E. coli* cells, identified two house-keeping genes (*fabD1* and *fabD2*) belonging to the fatty acid synthesis loci and a discrete Sfp-type phosphopantetheinyl transferase gene (*sfp*) that are necessary for FK228 biosynthesis in *C. violaceum*. This discovery presents an opportunity for engineering of FK228 analogs and was utilized in the development of both natural product and cell penetrating peptide expression systems. Utilizing a combination of expression plasmids I reconstructed the biosynthetic pathway of the potent anticancer compounds tryprostatin A and tryprostatin B. In addition, we developed an anticancer protein secretion system based on cell penetrating peptides to deliver anticancer peptide-based "cargo" into tumor cells. The development of natural product and cell penetrating peptide heterologous expression systems serves as a platform to implement and expand the production of future anticancer agents and validates our current effort toward engineering novel tumor-targeting bio-agents with natural product or cell penetrating peptide expression capability.

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**Wesener, S.R**., V. Potharla and Y.-Q. Cheng (2011) Reconstitution of FK228 biosynthetic gene cluster reveals cross talk between modular polyketide and fatty acid biosynthesis. *Applied and Environmental Microbiology* 77:1501-1507.

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## Presentations

Shah, G.R., S.R. Wesener, Y.-Q. Cheng*. Poster presentation: Engineering Novel Anticancer Bio-Agents. American Association for Cancer Research (AACR). Chicago, USA. Mar 31-Apr 4, 2012.

Wang, C., V. Potharla, **S.R. Wesener**, L. Doughty and Y.-Q. Cheng*. Poster presentation: Platform for Discovery and Combinatorial Biosynthesis of New Histone Deacetylase Inhibitors as Anticancer Agents. Cancer Epigenetics Conference, organized by American Association for Cancer Research (AACR). San Jaun, Peurto Rico. Jan 20-23, 2010.

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