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# Isolation of microbial NRPS and PKS gene clusters for natural product isolation

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# Isolation of Microbial NRPS and PKS Gene Clusters for Natural Product Isolation

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**Abstract**

Greater than 99% of microbes living in the environment are uncultivable due to their complex nutrient and temperature requirements for growth. These microorganisms present a potential source of natural products that could be developed for biotechnological and pharmaceutical uses. Microorganisms with phosphopantetheinyl transferase (PPTase) activity are of high interest due to the role PPTase plays in activating non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) gene clusters. These gene clusters produce multi-enzymatic, multi-domain megasynthases that produce complex natural products utilized by the host organism for selective advantages. This study isolated genomic DNA from soil samples collected from the University of Richmond campus to construct metagenomic libraries with a host organism containing a BpsA reporter plasmid. These libraries were then screened for functional activity of NRPS and PKS gene clusters by virtue of PPTase activity. We then extended this study to include a library of pigmented microbes previously isolated from Chesapeake Bay sponges, *Clathria prolifera* and *Halichondria bowerbanki*. Genomic analysis of positive clones has thus far been inconclusive, however putative NRPS gene clusters have been identified in the SW202 microbial genome.

## Introduction

Interest in natural product research has increased considerably since the 1990s. They are expected to play a substantial role in drug development in coming years due to their structural diversity, relatively small dimensions, and ability to be absorbed and metabolized.[1] Higher plants, marine organisms, and microorganisms present potential untapped sources of these compounds.

The chemical compounds obtained from living organisms can be divided into two classes: primary metabolites and secondary metabolites. Primary metabolites are proteins, fatty acids, nucleotides, and sugars that are essential for life in almost all living species.[2] Secondary metabolites, commonly referred to as natural products, are extremely diversified, low-molecular weight molecules that are characteristic of a limited range of species. They often exert their

biological effects on other organisms to provide selective advantages.[3] These compounds are of particular interest because they have been shown to exhibit anti-tumor, anti-bacterial, and immunosuppressive activity.[4]

These compounds were highly sought after by pharmaceutical companies in the 1940s and '50s following the discovery of penicillin in 1928.[3] Cultivable microorganisms were targeted for their active therapeutic molecules, and during this "golden age" of microbial natural product discovery, nearly all groups of notable antibiotics were discovered.[3] This approach was quickly abandoned, however, as the continual search for new natural products led to the isolation of the same compounds over and over again. Companies were unable to isolate new compounds because greater than 99% of existing environmental microbes are uncultivable due to their complex nutrient and temperature requirements for growth. As a result, focus in the pharmaceutical industry turned towards synthetic chemistry.[3]

The relatively recent resurgence of interest in natural product isolation is a result of the shortcomings of synthetic compounds. Despite great advancements in synthetic chemistry, synthetic compounds are unable to achieve the great diversity and applicability of natural products. Thus, there is great value in isolating natural products from uncultivable organisms.

One method of doing so is to construct a metagenomic library, which is a library that allows the direct genetic analysis of genomes contained in an environmental sample.[5] This approach provides the ability to exploit the biosynthetic potential of this otherwise inaccessible resource by inserting the producer organism's DNA (eDNA; environmental DNA) into a fosmid. The fosmid is then transformed into a cultivable organism and expressed upon growth.[6] The challenge to this method is that a large, 30-120 kilobase (kb) DNA insert is needed because these compounds are produced in an assembly line-like manner by enzymes encoded in large gene clusters.[7] These clusters are called nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) gene clusters.

NRPS and PKS are multi-enzymatic, multi-domain megasynthases that produce nonribosomal peptides and polyketides, respectively.[4] NRPS utilize amino acid precursors to catalyze the

production of small biologically active peptides without the need for a nucleic acid template or ribosomal assistance.[8] PKS repeatedly add two-carbon ketide units that are typically derived from thioesters of acetate.[4] A disproportionately large number of NRPS and PKS products have successfully passed Phase III trials and been approved for clinical use, proving their enhanced ability to exhibit high molecular complexity and therapeutic activity.[6,7]

An example of a novel product produced by an NRPS is teixobactin, which is a natural product that not only qualifies as an antibiotic, but it also has properties that suggest potential for developing antibiotics that can avoid resistance development.[9] This product, which functions by inhibiting peptidoglycan synthesis in gram-positive pathogen cell walls, is produced by a two-gene NRPS cluster that contains 11 catalytic domains.[9] The proposed structure of teixobactin and its gene cluster are shown in Figure 1.

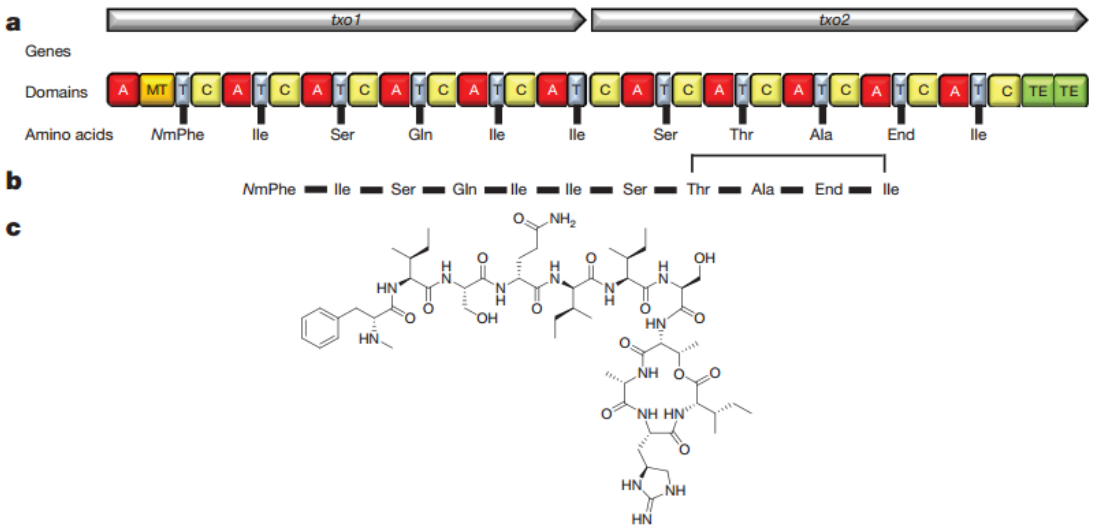


**Figure 1 | The structure of teixobactin and the predicted organization of its associated NRPS gene cluster**.[9] **a)** The two NRPS genes, named *txo1 and txo2*, the catalytic domains they encode, and the amino acids incorporated by the respective modules. Domains: A, adenylation; C, condensation; MT, methylation; T, thiolation (carrier); and TE, thioesterase (Ile-Thr ring closure). **b)** Schematic structure of teixobactin. **c)** Teixobactin structure.

Both NRPS and PKS enzymatic assembly lines are made up of discrete modules that recognize, activate, and incorporate a unit, one at a time.[8] As such, the number of modules in an NRPS or PKS typically corresponds to the number of constituents in the molecule produced, and the order

of the modules are dictated by the genome of the producer organism.[8] This organization is referred to as the collinearity rule. Within each module are a number of domains, each of which dictates the chemical moiety the module will add to a growing chain.[4] The products of these gene clusters are of particular interest due to their extremely diverse structure and function. Unlike ribosomal peptide synthesis, NRPS and PKS synthesis can utilize over 400 molecules as substrates to synthesize biologically active linear, cyclic, and branched cyclic molecules.[8]

Phosphopantetheinyl transferases (PPTases) are enzymes required for NRPS and PKS activation. PPTases catalyze the translational attachment of a 4'-phosphopantetheine (PP) cofactor to the carrier proteins of NRPS and PKS enzymes, thus facilitating their activity.[10] The 4'-PP group serves as the site of substrate and intermediate linkage, and permits the transfer of intermediates between the separate modules of these complex enzymes by activating acyl carrier proteins (ACPs), peptidyl carrier proteins (PCPs), and/or aryl carrier proteins (ArCPs) contained therein.[10] NRPS and PKS cannot function without PPTase, so the presence of this particular transferase serves as an identifier of these gene clusters in a microorganism's genome.

This relationship presents a target for screening of metagenomic libraries. Our lab uses a novel functional screening method that targets PPTase activity.[6] Functional screening focuses on heterologous expression of genes of interest by virtue of a readily detectable phenotype in the host organism.[6] This screening process is made possible by BpsA, which is a small and versatile single-module NRPS from *Streptomyces lavendulae*. Following activation by PPTase, BpsA autonomously generates a colored product, indigoidine, by condensing two L-glutamine residues.[6] This gene can therefore be used as a genetic reporter that enables us to recover fragments of NRPS or PKS gene clusters from eDNA libraries,"[6] as shown in Figure 2.

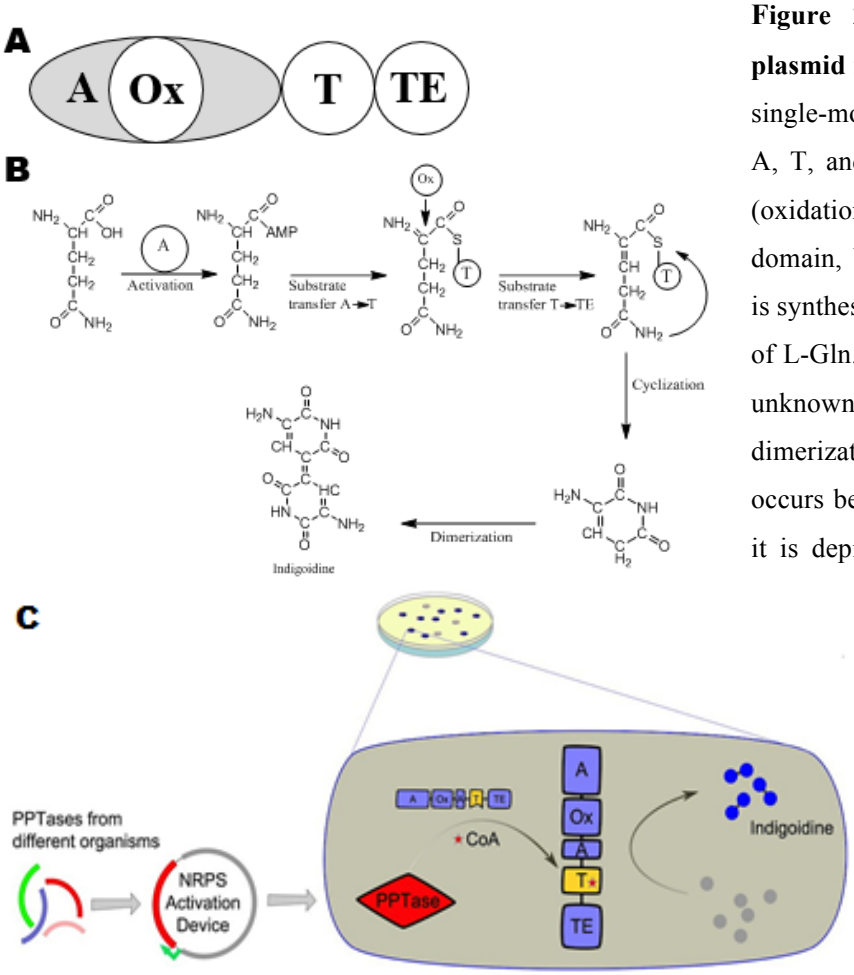


**Figure 2 | BpsA serves as reporter plasmid for PPTase activity**.[8] **a)** BpsA is a single-module NRPS enzyme with a single A, T, and TE-domain with an Ox-domain (oxidation domain) integrated into the A-domain, **b)** The blue pigment, indigoidine, is synthesized by BpsA from two molecules of L-Gln. The functions of the domains are unknown, as well as the mechanism of dimerization. It is unknown if the oxidation occurs before or after cyclization, although it is depicted here as taking place before dimerization, **c)** schematic representation of the functional screen using BpsA as a reporter plasmid.

Our work has focused on identifying and isolating NRPS and PKS gene clusters from soil and sponge associated microbial samples to identify novel natural products (i.e. new secondary metabolites). We have extracted eDNA directly from microbes isolated from soil and environmental samples, propagated it in a cultivable host containing the BpsA reporter plasmid, and subjected the clones to functional screening. Positive clones are being studied to determine their nucleic acid sequence and bioinformatics analysis is being used to identify gene clusters in a microbial genome.

## Experimental[a]

*Soil Collection*

Soil was collected from various locations on the University of Richmond campus. Soil from approximately 6-12 cm below the surface was sieved through 2 mm to remove large rocks and sediment. DNA was extracted immediately following sample collection.

*HMW DNA Extraction from Soil*

The protocol for HMW eDNA extraction was adopted from several existing protocols to meet the conditions of our soil.[11,12,13,14,15,16,17,18,19] An indirect cell lysis procedure was used with the aid of acid-washed polyvinylpolypyrrolidone (PVPP) and 20% SDS during homogenization to reduce humic acid contamination and release bacteria from soil aggregates.

Soil [50 g, dry weight] was homogenized in a Waring blender at medium speed in 0.1 M phosphate buffer [pH 5.5], acid-washed PVPP, and 20% SDS for three 1-minute intervals with intermittent cooling in an ice bath for one minute. The homogenate was diluted with 50 ml sterile water and centrifuged at 1,000 x g for 15 minutes at 4 °C to remove fungal biomass and soil debris. The supernatant was collected and the soil pellet was subjected to two more rounds of homogenization and centrifugation. The resulting supernatants were pooled and then centrifuged at 12,000 x g for 20 minutes at 4 °C to collect the bacterial fraction.

The bacterial pellets were combined and resuspended in 2% (w/v) sodium hexametaphosphate [pH 8.5 adjusted with $Na_2CO_3$] before being subjected to centrifugation at 10,000 x g for 15 minutes at 4 °C to eliminate free or extracellular DNA that may have been present in the sample. The resulting pellet was resuspended in 40 ml ice-chilled dispersion solution [2% sodium hexametaphosphate pH 8.0 containing 0.2% (w/v) sodium deoxycholate and 25 mg/mL PEG 8,000] by using a sterile spatula or vigorous pipetting. The suspension was then homogenized for one minute at low speed. The resulting fine suspension was incubated in the incubator shaker at 4 °C at 100 rpm overnight.

[a] The protocol for each procedure is listed in the Appendix at the end of this paper.

Following incubation, the suspension was pipetted into a clear, round-bottom oakridge centrifuge tube and 1.3 g/mL autoclaved nycodenz solution was pipetted to underlay the cell suspension. Dispersion solution was added to fill each centrifuge tube. The tubes were incubated at 4 °C for 30 minutes to allow larger particles to settle to the bottom of the tube prior to centrifugation. The samples were then centrifuged at 10,000 x g at 4 °C for 20 minutes in a swing-out rotor. A diffuse band containing bacterial cells was resolved at the nycodenz-aqueous interface.

The cell layer was pipetted off the nycodenz cushion and collected in a sterile tube. The bacterial fraction was washed by suspending it in TE buffer and gently inverting the tube 4-6 times. The cells were collected by centrifugation at 15,000 x g at 4 °C for 15 minutes and suspended in TE buffer to yield the bacterial suspension for lysis.

*Cell Lysis*

The bacterial cells were lysed within agarose plugs to avoid shearing the eDNA. Through this process, HMW eDNA remained in the agarose plug while cell debris and contaminants freely diffused during lysis and washing steps.

A 0.5 mL aliquot of the bacterial suspension was mixed with 1.0% LMP agarose in 0.5x TBE and cast into 100 µl plug molds. The molds were then placed on ice for 10 minutes to allow the agarose to solidify. The cell-containing agarose plug was incubated in lysis buffer [10 mM Tris pH 8.0, 50 mM NaCl, 0.1 M EDTA, 1% sarkosyl, 0.2% sodium deoxycholate, 1 mg/mL lysozyme] at 37 °C for 1 hour. The plug was then transferred to ESP buffer [1% sarkosyl, 1 mg/mL proteinase K in 0.5 M EDTA, pH 8] and incubated at 55 °C for 16 hours. The solution was then decanted and replaced with fresh ESP buffer, and the plug was incubated at 55 °C for an additional hour. The plug was stored in 50 mM EDTA [pH 8.0] at 4 °C until needed for electroelution.

*Electroelution of Agarose Plugs*

Electroelution was used to recover the HMW eDNA from the agarose plugs. Pretreated dialysis tubing [90 °C in a 1 mM EDTA / 2% $NaHCO_3$ solution, stored at 4 °C in 50% ethanol] was rinsed with sterile $H_20$ followed by sterile 1X TAE buffer. The plugs were equilibrated in 1X

TAE buffer at 4 °C before being placed into the dialysis tubing filled with sterile 1X TAE. Electroelution was carried out in a gel electrophoresis chamber with 1X TAE buffer in a field strength of 4-5 V/cm and after 2 hours the polarity was reversed for exactly one minute to dissociate eDNA that may have impacted on the side of the membrane. The eDNA was removed from the tubing using a wide-bore pipette tip and stored at 4 °C.

*Isolation of Sponge-Associated Microbe DNA*

Pigmented microbes were isolated from Chesapeake Bay sponges, *Clathria prolifera* and *Halichondria bowerbanki.*[b] Individual colonies were isolated and grown on marine media. The resulting cells from one colony, arbitrarily named SW202, were suspended in isolation buffer [0.15 M Tris, 0.1 M EDTA, pH 8.0] and lysed using 100 mg/mL lysozyme and 25 mg/mL RNase. After incubating at 27 °C for one hour, 20% SDS was added and the sample was mixed by inverting the tube. The cells were then incubated at 65 °C until the solution cleared. Phenol:chloroform:isoamylalcohol was added and the sample was centrifuged at 14,000 rpm at 4 °C for 5 minutes. The eDNA-containing aqueous layer was collected by using a wide-bore pipette tip and the eDNA was precipitated with ethanol before being suspended in TE buffer for storage.

[b] Sponge samples were collected by Dr. Malcolm Hill's Laboratory.

*Metagenomic Library Construction*

eDNA from both the soil and sponge-associated microbe samples was used to construct a metagenomic library. The libraries were constructed using the end repair, ligation reaction, and phage packaging steps of the Epicentre CopyControl™ Fosmid Library Construction Kit with the pCC2FOS2 vector, shown in Figure 3, with the following modifications: the "EcoBlue1" E. coli strain containing the BpsA reporter plasmid was used as the plating strain; cells were plated on ZYP-5052 Glutamine plates [10 g/L N-Z amines, 5 g/L yeast extract, 0.5% glycerol, 0.05% D-glucose, 0.2% α-glucose, 100 mM L-glutamine, 25 mM $(NH_4)_2SO_4$, 50 mM $KH_2PO_4$, 50 mM $Na_2HPO_4$ and 1.5 % (w/v) agar] with 12.5 µg/mL chloramphenicol and 50 µg/mL spectinomycin.

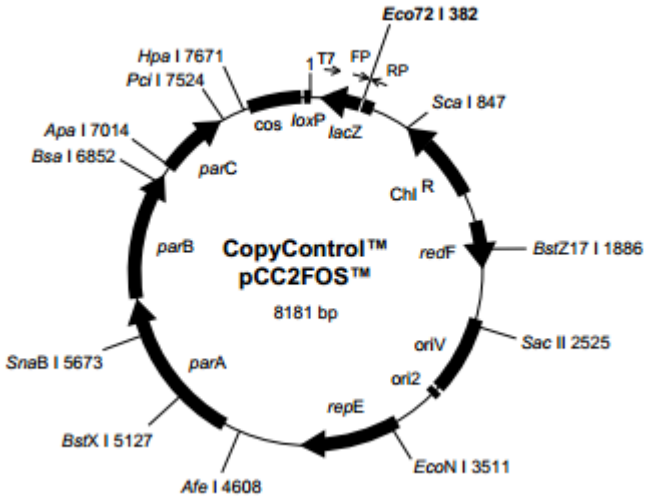


**Figure 3 | CopyControl™ pCC2FOS fosmid used to create metagenomic library for soil and SW202 eDNA.** eDNA was inserted at the Eco72 I site, which begins at base pair number 382; FP forward primer, RP reverse primer.

It was calculated that 766 cfu/mL would be needed for 99% coverage of the genome with an insert size of 30-50 kb:

$$N=\frac{\ln(1-P)}{\ln(1-f)}=\frac{\ln(1-0.99)}{\ln\left(1-\frac{3.0\times10^{4}}{5.0\times10^{6}}\right)}=766 \text{ cfu/mL}$$

P is the desired probability; f is the proportion of the genome contained in a single clone; and N is the required number of fosmid clones; cfu is the number of colony forming units.

Plates were incubated at 37 °C overnight and then incubated at room temperature for up to 8 days to allow for indigoidine production.

Positive clones, identified by the production of indigoidine and thus their blue color, were isolated and grown on ZYP-5052 plates. This served as a positive control to verify that the colonies exhibited PPTase activity. Once verified, they were grown in LB media, which lacks the high glucose content of ZYP-5052 media, as a negative control. The liquid cell culture was stored in a glucose solution at -80 °C.

*Genomic Sequencing and Annotation*

SW202 genomic eDNA was sent to the University of Maryland Genetics Institute to be sequenced using NextGen PacBio sequencing. The resulting genome was annotated using both Rapid Annotation using Subsystem Technology (RAST)[c] and Antibiotics & Secondary Metabolite Analysis SHell 2.0 (AntiSMASH 2.0)[d] programs.

*Fosmid Insert Sequencing*

Fosmids were isolated from positive SW202 clones using the GeneJET™ Plasmid Miniprep Kit from Thermo Scientific. Samples were then sent to Operon for SimpleSeq sequencing using the pCC1FOS™ forward primer, whose sequence was provided by Epicentre. The resulting sequences[e] will be compared to the SW202 genomic sequence using a BLAST search to determine the sequence of the 30-50 kb insert.

**Results and Discussion**

Several aspects of the HMW eDNA extraction from soil protocol led to the successful incorporation of eDNA into a metagenomic library. First, the use of an indirect lysis protocol, which isolated the cells from soil debris, eukaryotic cells, and extracellular DNA prior to cell

[c] From: Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O.
*BMC Genomics,* 2008

[d] From: antiSMASH 2.0 — a versatile platform for genome mining of secondary metabolite producers.
Kai Blin, Marnix H. Medema, Daniyal Kazempour, Michael A. Fischbach, Rainer Breitling, Eriko Takano, & Tilmann Weber. *Nucleic Acids Research* (2013) doi: 10.1093/nar/gkt449.

[e] We have not yet determined the sequence for insert DNA.

lysis, produced a sample with much fewer contaminants than would be produced with a direct lysis protocol, in which the whole sample undergoes lysis and the DNA is isolated from the crude sample. The PVPP added to the homogenization step formed a complex with humic acids in the soil, and acid-washing the PVPP optimized this interaction. This helped prevent humic acids from inhibiting metabolic activity downstream. Humic acid contamination was also reduced by the use of PEG, which reduces the coprecipitation of humic acid, and a basic pH for the homogenization solution. NaCl caused the soil to precipitate with the cell debris and proteins when isolating bacterial cells. The addition of SDS enhanced the breakdown of polymer bridges that contribute to soil aggregates, which typically trap bacterial cells that would have been lost during the centrifugation steps. Washing the bacterial cells with sodium hexametaphosphate prior to lysis helped remove free DNA from the bacterial fraction, insuring that the eDNA incorporated into the metagenomic library was from the target organisms. Lastly, the nycodenz density gradient helped purify the bacterial fraction further by removing remaining contaminants.

Lysis within the agarose plug produced HMW eDNA fragments. Electroelution from the agarose plug maintained the size of the fragments without the risk of denaturing the DNA by heating the plug or treating it with GELase. The resulting DNA was used with the CopyControl™ Fosmid Library Production Kit without the need for further purification steps.

The results from the functional screen of both metagenomic libraries are summarized in Table 1. The production of indigoidine suggests the presence of a whole or partial NRPS or PKS gene cluster in the insert DNA. An example of a positive clone on a ZYP-5052 plate is shown in figure 4.

| | **SW202** | **Soil** |
|---|---|---|
| **Total Number of Clones** | 869 | 1,432 |
| **Number of Positive Clones** | 5 | 2 |

**Table 1 |** Summary of colony production of soil and SW202 metagenomic libraries.

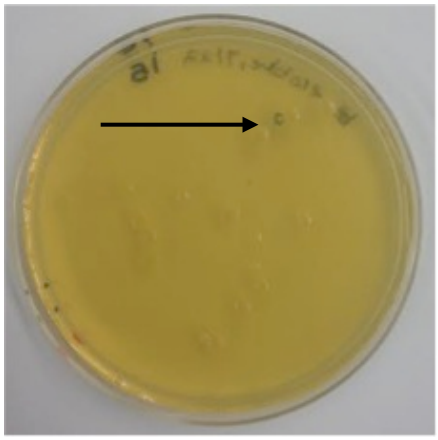

**Figure 4 | Positive clone on a ZYP-5052 plate.** Positive clones were identified by the production of indigoidine, which turned the colony blue. The arrow is pointing to SW202C, which is one of the five positive clones identified from the SW202-associated microbe.

The results of the control experiments are shown in Figure 5.

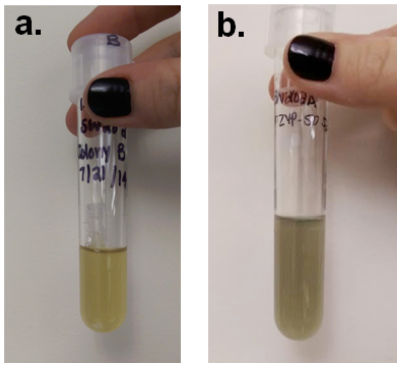


**Figure 5 | Control experiments verified the presence of PPTase in the positive colonies.** Cells were grown in liquid media to confirm the assay. **a.** positive clones were grown in LB Media as a negative control **b.** positive clones were grown in ZYP-5250 media.

Positive clones grown in LB media for longer than 16 hours showed the possibility of turning blue. Because the LB media contains small amounts of glutamine, we hypothesized that this coloration is a result of low-level indigoidine production, as opposed to pigment production by the microorganism.

The results from the PacBio sequencing are summarized in **Table 2**.

| | |
|---|---|
| **# contigs (>= 0 bp)** | 19 |
| **# contigs (>= 1000 bp)** | 17 |
| **Total length (>= 0 bp)** | 5,389,822 |
| **Total length (>= 1000 bp)** | 5,389,822 |
| **Largest contig** | 924,773 |
| **GC (%)** | 43.55 |
| **# N's per 100 kbp** | 0.00 |

**Table 2 | Sequencing results from NextGen PacBio Sequencing.** All statistics are based on contigs of size >= 500 bp, unless otherwise noted. The total size of the genome is approximately 5.4 million base pairs. Data was provided by the University of Maryland Genetics Institute.

Annotation of the SW202 genome provided additional information about the organism and its potential of containing NRPS and PKS gene clusters. The results from the RAST annotation are shown in Figure 6. RAST identified 8 secondary metabolite subsystems in the SW202 genome. None of these subsystems are directly linked to known NRPS or PKS gene clusters.

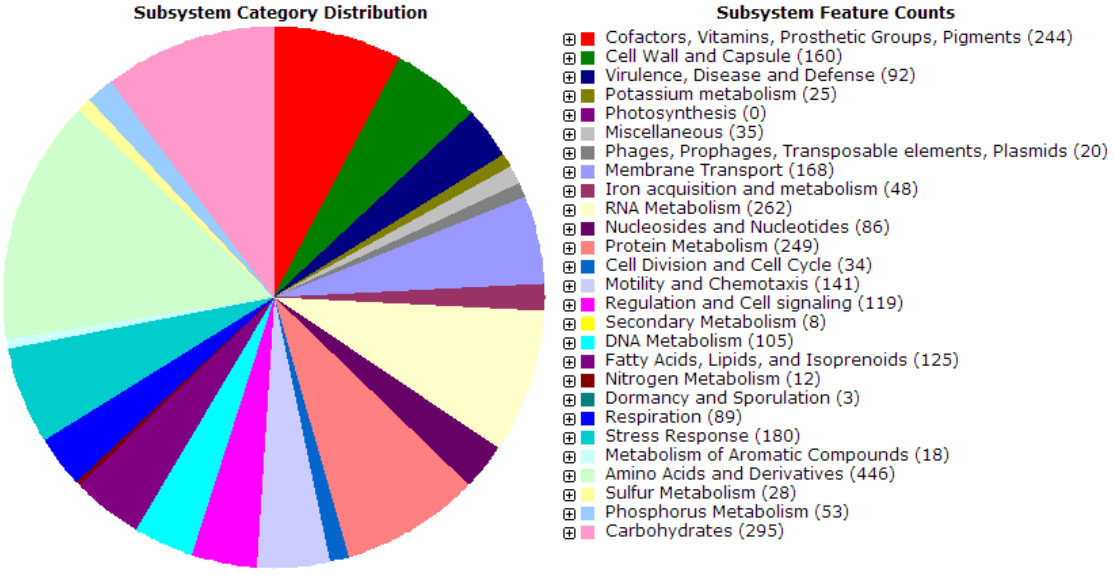


**Figure 6 | RAST annotation of SW202 genomic DNA**. The curation of genomic data (i.e. annotation) is done via the curation of subsystems by an expert annotator across many genomes rather than by a gene-by-gene basis. Frequently, the subsystems represent a collection of functional roles that make up a metabolic pathway, a complex, or a class of proteins.[20]

The annotation results from AntiSMASH 2.0 are shown in Figure 7.

| Cluster | Type (AntiSMASH Identification) | Contig |
|---|---|---|
| Cluster 1 | Bacteriocin | 2 |
| Cluster 2 | NRPS | 3 |
| Cluster 3 | NRPS-lantipeptide | 7 |
| Cluster 4 | NRPS-t1PKS | 4 |
| Cluster 5 | NRPS | 4 |
| Cluster 6 | NRPS | 5 |
| Cluster 7 | Bacteriocin | 1 |
| Cluster 8 | NRPS-t3PKS-bacteriocin | 1 |
| Cluster 9 | Bacteriocin | 6 |

**Figure 7 | AntiSMASH 2.0 annotation uses profile hidden Markov models (profile HMM) to identify secondary metabolite gene clusters.**[21] These models turn multiple sequence alignment into a position-specific scoring system that can be used to search databases for remotely homologous sequences.[22] The clusters are color coded by predicted secondary metabolite type.

AntiSMASH 2.0 annotation identified 9 gene clusters in SW202 associated with secondary metabolites. Of the 9 gene clusters identified, four (clusters 2, 4, 5, and 6) are novel clusters. Cluster 4 is particularly interesting because it is predicted to be a hybrid NRPS and type I PKS gene cluster. Type I PKS differ from type II PKS in that they contain multiple enzymes with unique active site domains that control the sequential addition of two-carbon units, whereas type II PKS are minimal systems made up of only three proteins.[23] Further investigation into cluster four is shown in Figure 8.

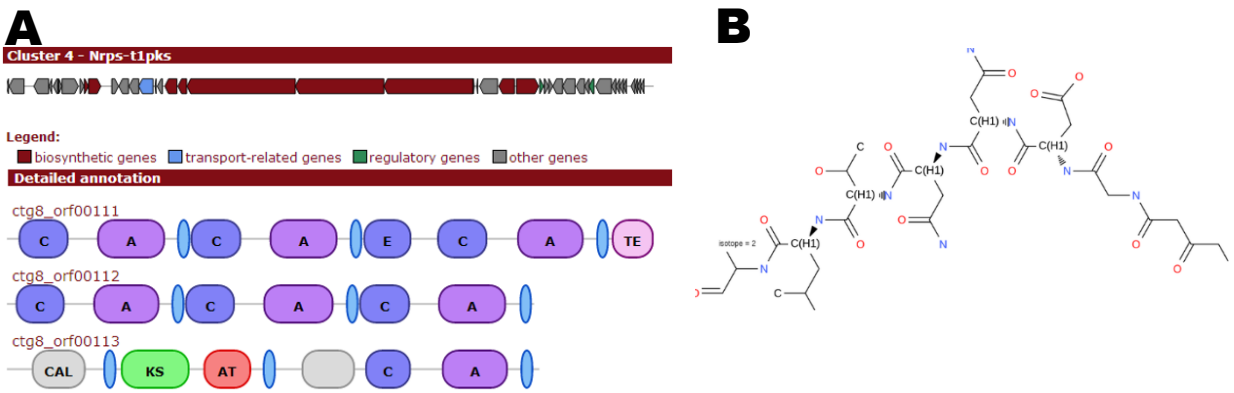


**Figure 8 | Investigation into cluster 4 identified by AntiSMASH 2.0 annotation**. **a)** The predicted gene structure for cluster 4 is shown above, and the corresponding domains are shown by open reading frame (orf) below. Orf 111 resembles the typical NRPS domain sequence **b)** The core scaffold of the resulting structure is roughly predicted based on assumed PKS/NRPS colinearity. Modification reactions are not considered.

An NCBI BLAST search of open reading frame 111 (depicted as ctg8_orf00111 in Figure 7a) produced the results shown in Figure 9.

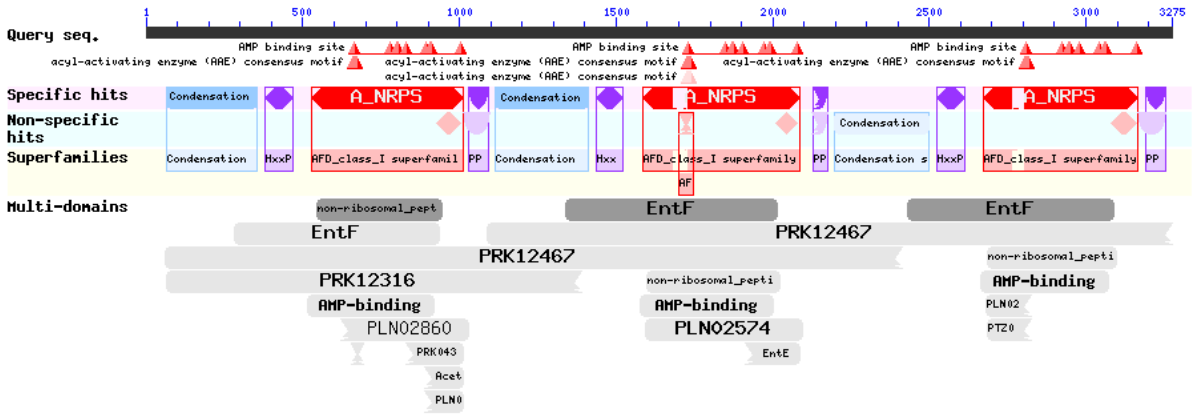


**Figure 9 | Conserved domains from ctg8_orf111 show properties typical of NRPS gene cluster**. A NCBI BLAST returns results that verify the presence of an NRPS gene cluster in the AntiSMASH 2.0 cluster 4. Enterobactin synthase component F (EntF) is a protein involved in enterobactin synthesis. Enterobactin modification of antibiotics have shown to enhance antibacterial activity against pathogenic E. coli strains under certain conditions. EntF has also shown to covalently bind with PPTase thus identifying as a part of the biological process of NRPS activation. The A_NRPS represents an amino acid adenylation domain, which is responsible for the specific recognition of amino acids and this type of domain is typically found in multi-domain NRPS. The following condensation domain is also

characteristic of NRPS. Together, these data suggest that SW202 has the potential to produce a novel antibiotic by means of an NRPS.

This data can be used to identify targets for natural product isolation. By identifying a target product, positive clones can be screened for a DNA sequence known to be contained within a desired NRPS or PKS gene cluster. This clone can then be grown to preferentially produce a specific product (or part of the product).

Sequencing of the fosmid insert of two positive clones, arbitrarily named SW202A and SW202B, has thus far been inconclusive. One returned sequence was positively identified by a BLAST search as part of the BpsA reporter plasmid, so it will be necessary to further purify the fosmid sample before sequencing. A clean sequence may be compared to the genomic sequence of SW202 in order to determine the sequence of the 30-50 kb insert and, in conjunction with AntiSMASH 2.0 annotation, predict the NRPS or PKS product.

We also hope to isolate the fosmids containing soil eDNA for sequencing. The soil sample, however, contains many different microorganisms, so we are unable to sequence the genome to compare with the insert sequence as was done with SW202.

**Conclusions**

Secondary metabolites produced by NRPS or PKS gene clusters present a promising target for drug discovery. eDNA samples harvested from soil and sponge microbes were successfully incorporated into metagenomic libraries that could be screened for PPTase activity via a BpsA reporter plasmid. Positive clones suggest the presence of NRPS and/or PKS gene clusters in the collected eDNA, and genomic analysis of the SW202 eDNA suggest their presence as well. Clusters identified by genomic analysis serve as potential targets for isolation. Sequencing the eDNA insert of positive clones will provide further insight into the specific cluster or natural product produced by our target organisms.

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[2] Hanson J.R. (2003) The classes of natural products and their isolation. From: Natural Products: The Secondary Metabolites. 17, P004-P004. DOI:10.1039/9781847551535-FP004

[3] Aitken, E. (n.d) The importance of natural products in drug discovery and development: a history in short and future perspectives. From Victoria University of Wellington.

[4] Ansari M.Z, et al. (2004) NRPS-PKS: a knowledge-based resource for analysis of NRPS/PKS megasynthases. *Nucl. Acids Res.* 32 (suppl 2): W405-W413. doi: 10.1093/nar/gkh359

[5] Thomas, T., Gilbert, J., and Meyer, F. (2012) Metagenomics – a guide from sampling to data analysis. *Microbial Informatics and Experimentation*. 2:3.

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# Appendix

## Table of Contents:

# HMW DNA Isolation from Soil

1. Collect soil from the University of Richmond campus. Sieve through 2 mm mesh.
2. Suspend 50 g (dry weight) soil in **300 ml cold 0.1 M phosphate buffer (pH 5.5)** and **10 g acid-washed PVPP**.
3. Homogenize in Waring blender at medium speed for three 1-minute intervals with cooling in an ice bath for 1 minute between intervals.
4. Add a 2-ml portion of **20% SDS** and blend for an additional 5 seconds. *The SDS breaks polymer bridges to help release bacteria from soil aggregates.*
5. Place sample on ice for 5 minutes to allow foaming to settle.
6. Transfer ~150 ml of homogenate to 250-ml centrifuge bottles and dilute with 50 ml **cold sterile water**.
7. Centrifuge at 1,000 x g for 15 minutes at 4 ºC to remove fungal biomass and soil debris. *Humic material covers the range of buoyant densities of bacterial cells so it will not be completely separated in gradient.*
8. Carefully transfer supernatant to a fresh 250-ml centrifuge bottle. Be careful not to disrupt pellet while collecting supernatant.
9. Subject the soil pellet to two more rounds of homogenization (with 100 ml **cold sterile water**) and centrifugation. Pool supernatants and store at 4 ºC until further processing.
10. Centrifuge pooled supernatants at 12,000 x g for 20 minutes at 4 ºC to collect bacterial fraction.
11. Combine bacterial pellets and resuspend in 200 mL cold **2% (w/v) sodium hexametaphosphate, adjusted to pH 8.5 with 0.2% Na2CO3**. Homogenize for one minute at low speed and then centrifuge at 10,000 x g for 20 minutes at 4 ºC. *This step eliminates any free/extracellular DNA that may be present in the sample.*
12. If necessary, this pellet may be stored at -20 ºC in isopropanol (not highly recommended).

13. Resuspend cell pellet in 40 ml ice-chilled **dispersion solution** by removing pellet from sides of the tube with a sterile spatula or vigorous pipetting. Homogenize in blender for one minute at low power.
    i) 2% sodium hexametaphosphate pH 8 containing 0.2% (w/v) sodium deoxycholate and 25 mg/ml PEG (MW 8,000).
14. Incubate fine suspension in a 250-ml bottle at 4 ºC for at least two hours with gentle mixing by placing bottle on rotating platform at 100 rpm. *This solution may be incubated overnight without apparent decrease in cell or DNA yield (Liles).*
15. Pipette 20 ml of cell suspension into two round-bottom, clear 40 ml centrifuge tubes and underlay the cell suspension with 7 ml of a **Nycodenz** solution using a Pasteur pipette. Add sufficient dispersion solution to fill each centrifuge tube.
    i) 1.3 g/ml, autoclaved and cooled to 4 ºC.
16. Incubate the tubes at 4 ºC for 30 minutes to allow larger particles to settle to the bottom of the suspension prior to centrifugation
17. Centrifuge tubes at 10,000 x g at 4 ºC for 20 minutes in a swing-out rotor. A diffuse band containing bacterial cells will be resolved at the Nycodenz-aqueous interface, although some cells may remain in the supernatant.
18. Pipette the cell layer off of Nycodenz cushion and collect in sterile 30 mL tube.
19. Wash the bacterial pellet twice by suspending in 200 ml **TE buffer.** May use clean paintbrush to carefully resuspend bacterial cells.
    i) 33 mM tris (pH 8.0) and 1 mM EDTA.
20. Collect by centrifugation and suspend in 100 ml TE buffer to yield bacterial suspension for lysis.

# HMW DNA Isolation from Soil: Preparation

**Acid-Washed Polyvinylpolypyrolidone (PVPP)**

1. Suspend 15 g insoluble PVPP in 200 ml **3.0 M HCl** for 12-16 hours at room temperature. (5 pm – 9 am = 16 hrs)
    a. Dilute concentrated (usually 12 M) HCl under the hood. Be sure to wear protective clothing (i.e lab coat, eyewear, gloves, etc.). While stirring, pour liquid HCl into bottle containing ~600 mL $dH_20$. Once all the HCl has been added, add the remaining $dH_20$ and let it stir for a minute or two.
2. Filter through #4 filter paper. The PVPP will be very thick. Allow it to filter long enough to get as much liquid out as possible. Also be sure to get as much of the PVPP from the beaker as possible – you don't want to leave behind a lot of product.
3. Suspend in 200 ml of **20 mM potassium phosphate (pH 7.4)** and mix by stirring for 1-2 hours.
    a. Add 4.56 g into one liter (or 3.648 g into 800 ml) of $dH_2O$. Let it stir until all the solid has dissolved. Once completely dissolved, check the pH. If necessary, adjust the pH to 7.4 using NaOH or HCl.
4. Check the pH of the suspension. Repeat the suspension/washing process with 20 mM potassium phosphate (pH 7.4) until the suspension reaches pH 7.0.
5. Once suspension reaches desired pH, filter through #4 filter paper and air dry over night.

**0.1 M Phosphate Buffer (pH 5.5), 500 ml**

To 500 ml $dH_2O$, add:

1. 6.615 g monosodium phosphate
2. 0.554 g disodium phosphate.

Check the pH and adjust if necessary.

**20% SDS, 10 ml**

To 10 ml of $dH_2O$ 15 mL tube, add:

1. 2.0 g SDS

Screw on the cap and invert a few times to mix. Leave it upright to allow the bubbles to subside. May be filtered, but autoclaving is not necessary.

**2% (w/v) Sodium Hexametaphosphate (pH 8.5, adjusted with $Na_2CO_3$), 500 ml**

To 500 ml $dH_2O$, add:

1. 10 g sodium hexametaphosphate
2. 2 g $Na_2CO_3$

Heating the solution while stirring will help the sodium hexametaphosphate go into solution. Check the pH. It should be ~8.0. If too low, slowly add solid $Na_2CO_3$ until it reaches the desired pH.

**Dispersion Solution, 200 ml**

To 200 ml $dH_2O$, add:

1. 4.0 g sodium hexametaphosphate
2. 0.4 g sodium deoxycholate

**Nycodenz**

To 38 ml $dH_2O$, add:

1. 50 g Nycodenz

Solid will not go into solution until it is autoclaved. Store at room temperature away from light.

**TE Buffer**

To 500 ml $dH_2O$, add:

1. 2.0 g Tris base
2. 0.1861 g EDTA

Adjust to pH 8.

# Making Agarose Plugs and Lysing Cells Within

1. Mix an aliquot of 0.5 ml cells with 1.0% LMP agarose in 0.5x TBE.
2. Cast mixture into plug molds of 100 μl. *If necessary, store plugs at 4 ºC in 50 mM EDTA, ph 8.0.*
3. Extrude Cell-containing agarose plug into **lysis buffer** (10 ml per 1 cc plug). Incubate at 37 ºC for 1 hour.
    i) 10 mM Tris, pH 8.0
    ii) 50 mM NaCl
    iii) 0.1 EDTA
    iv) 1% Sarkosyl
    v) 0.2 % Sodium deoxycholate
    vi) 1 mg lysozyme per ml
4. Transfer plug to 40 ml **ESP Buffer (pH 8)**. Incubate at 55 ºC for 16 hours.
    i) 1% Sarkosyl and 1 mg proteinase K per ml in 0.5 M EDTA, pH 8
5. Decant solution and replace with fresh ESP Buffer. Incubate at 55 ºC for an additional hour.
6. Place plugs in 50 mM EDTA and store at 4 ºC.

# Making Agarose Plugs and Lysing Cells Within: Preparation

**5x TBE (to be diluted for 0.5x TBE)**

To 1 liter of $dH_2O$, add:

1. 54 g Tris base
2. 27.5 g Boric Acid
3. 20 ml 0.5 M EDTA, pH 8.0

Dilute 1:10 for 0.5 TBE.

**1.0% LMP agarose in 0.5x TBE**

To 30 ml $dH_2O$, add:

1. 0.3 g Agarose

Microwave for 30 seconds. Check to see of all the agarose has dissolved. If not, microwave for another 10 seconds. Repeat if necessary.

**Lysis Buffer, 100 ml**

To 100 ml $dH_2O$, add:

1. 0.1211 g Tris
2. 3.722 g EDTA

Check the pH of this solution. Adjust to pH 8.0 with NaOH. Then, add:

3. 0.2922 g NaCl
4. 1.0 g Sarkosyl
5. 0.2% Sodium deoxycholate
6. 0.1 g lysozyme (found in freezer)

**ESP Buffer (pH 8.0), 100 ml**

To 100 mL $dH_2O$, add:

1. 1.0 g Sarkosyl
2. 0.1 g proteinase K
3. 15.8 g EDTA

Adjust the pH to 8.0. Contents will not completely dissolve until the pH ~8.

**50 mM EDTA, 100 ml**

To 100 ml, add:

1. 1.861 g EDTA

# Electroelution to Purify DNA from Gel Plug[a]

Pretreating Dialysis Tubing:

- Heat membranes (8-10 cm strips) at 90 $^{o}$C in **1 mM EDTA/ 2% $NaHCO_3$** for 10 minutes, boil in $H_2O$ for 10 minutes, and rinse several times in $H_2O$.
- Store at 4 $^{o}$C in 50% ethanol
- Immediately prior to use, rinse thoroughly with sterile $H_2O$ and then in sterile 1x TAE buffer

Electroelution:

1. Prepare fractions to contain ~300 mg gel (about 10 x 6 x 5 mm)
2. Equilibrate fractions in 50 ml sterile (filter sterilized) 1x TAE buffer at 4 $^{o}$C with occasional mixing for 30 minutes
3. Place gel slice lengthwise into the dialysis bag that is sealed at one end. Add 300-400 μl sterile 1x TAE
4. Carefully remove air from the bag and steal the other end with a dialysis clip, and trim excess dialysis membrane
5. Position the gel slice longitudinally to one side of the dialysis bag, i.e., parallel to one of the creased edges of the bag. Fill gel electrophoresis chamber with sterile 1x TAE and allow it to equilibriate to 4 $^{o}$C (in cold room).
6. Completely submerge dialysis bag such that the length of the gel slice is parallel to the electrodes and the side containing the gel slice is closest to the negative pole.
7. Carry out electroelution using a field strength of ~4-5 V/cm and after 2 hours the polarity is reversed for exactly one minute to disassociate the DNA that has impacted on the side of the membrane.
8. Carefully remove assembly from the buffer chamber, blot it dry, undo one of the dialysis clips, and gently remove the DNA using a wide-bore pipette tip.

Eluted DNA can be used immediately for fosmid library construction.

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# Isolation of Sponge-Associated Microbe DNA

1. Sample should already be resuspended in 3 ml of Isolation Buffer (0.15 M Tris, 0.1 M EDTA, pH 8.0). If it is not, use the big pipette to resuspend cell pellet in 3 ml of Isolation Buffer in a sterile 15 ml tube.
2. Add 50 µl lysozyme (100 mg/ml). Add 8 µl RNase (25 mg/ml). Mix by gently inverting. Incubate at 37°C for at least 1 hour (set another heat block to 65°C in the meantime).
3. Add 500 µl of 20% SDS. Mix by gently inverting. Transfer to three 1.5 ml microcentrifuge tubes.
4. IMMEDIATELY incubate at 65°C for at least 5 minutes or until it clears. Transfer to a 50 ml centrifuge tube by gentle pouring.
5. **In the hood**, add 6 ml phenol:chloroform:isoamylalcohol. Vortex short burst (**still in the hood**).
6. Centrifuge 14,000 rpm at 4°C for 5 minutes.

   Expect three phases: aqueous (top), phenol (bottom), and a goopy, rubbery interface. The DNA is in the top aqueous layer. The interface and phenol layers were previously thought to have very high DNA concentration, but these were false positives due to phenol absorbance at 270 nm.
7. Remove the aqueous (top) layer using a wide-bore pipette tip, transferring to a clean 50 ml centrifuge tube. Pour the phenol layer and interface into a phenol waste container.
8. Add as much ice cold 95% ethanol as possible to ppt the DNA. Mark the centrifuge bottle with a small X where you expect the DNA to pellet.
9. Centrifuge at 10,000 rpm at 4°C for 10 minutes to pellet the DNA. Pour off the supernatant into the non-halogen waste container. Leave the tube upside-down on a paper towel for ~5-10 minutes to remove any remaining ethanol.
10. Add 800 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA) and secure in a tilted position so that the TE covers the place you marked (you want the TE to cover the pelleted DNA). Allow resuspension overnight at room temperature.

# CopyControl™ Fosmid Library Production Kit with pCC1FOS Vector: Protocol

Check to make sure that all of the kit contents are available and prepare each of the additional reagents before beginning the procedure

**A. Shearing the Insert DNA**

Kit component used in this step: **Fosmid Control DNA**

Prepare a 1% agarose gel. Load 100ng of the Fosmid Control DNA into one lane and 10 µl of the DNA sample. Run the gel at 30-35V overnight.
**Do not include ethidium bromide in the gel or running buffer

Stain the gel with ethidium bromide after the run is complete and visualize the gel. If the genomic DNA sample migrated slower than the control DNA, then it must be sheared 20 times with a needle. If it migrated about the same, then you may continue to the next step. If it migrated faster than the control DNA, then the insert is too small and DNA must be re-isolated.

**B. End-Repair of the Insert DNA**

Kit components used in this step: **End-Repair Enzyme Mix, 10X Buffer, dNTPs, ATP**

This step generates blunt-ended, 5'-phosphorylated DNA. The end-repair reaction can be scaled as dictated by the amount of DNA available.

1. Thaw and thoroughly mix all of the reagents listed below before dispensing; **place on ice**. Combine the following on ice:

    x µl sterile water
    8 µl 10x End-Repair Buffer
    8 µl 2.5 mM dNTP Mix

8 µl 10 mM ATP

up to 20 ng sheared insert DNA (approx. 0.5 µg/µl)

4 µl End-Repair Enzyme Mix

_____

80 µl total reaction volume

2. Incubate at room temperature for 45 minutes

3. Add SDS to a final concentration of 0.1% and heat for 10 minutes at 70 °C

4. Add 2 volumes of ethanol and mix. Spin in a microcentrifuge at top speed for 20 minutes to pellet the DNA and carefully pour off the ethanol. Air-dry for 10 minutes. Resuspend in a minimum volume of 1X TE Buffer and proceed with ligation and packaging.

**E. Ligation Reaction**

Kit components used in this step: **Fast-Link 10X Ligation Buffer, Fast-Link DNA Ligase, ATP, CopyControl pCC1FOS Cloning-Ready Vector.**

1. Refer to appendix A to determine the approximate number of clones needed for the library. A single ligation reaction will produce $10^3$-$10^6$ clones, depending on the quality of the insert DNA. Based on this information, calculate the number of ligation reactions you will need to perform.

2. Combine the following reagents in the order listed and mix thoroughly after each addition.

A 10:1 molar ratio of CopyControl pCC1FOS Vector to insert DNA is optimal.
0.5 µg CopyControl pCC1FOS Vector = ~0.09 pmol vector
0.25 µg of ~40-Kb insert DNA = ~0.009 pmol insert DNA

x µl sterile water

1 µl 10X Fast-Link Ligation Buffer

1 µl 10 mM ATP

1 µl CopyControl pCC1FOS Vector (0.5 µg/µl)

x µl Concentrated insert DNA (0.25 µg of ~40-kb DNA)

1 µl Fast-Link DNA Ligase

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10 µl Total Reaction Volume

3. Incubate at room temperature for 4 hours.

**Note:** *Overnight ligation reactions at 16 °C may be performed but should not be necessary. Transfer reaction to 70 °C for 10 minutes to inactivate the Fast-Link DNA Ligase. Proceed to Part F or, if desired, the reactions can now be frozen and stored overnight at -20 °C.*

**F. Packaging the CopyControl Fosmid Clones**

Kit components used in this step: **MaxPlax Lambda Packaging Extracts, EPI300-T1$^{R}$ Plating Strain**

1. On the day of packaging reactions, inoculate 50 ml LB broth + 10 mM $MgSO_4$ _ 0.2% Maltose with 0.5 ml of the EPI300-T1$^{R}$ overnight culture. Shake the flask at 37 °C to an $A_{600}$ of 0.8-1.0 (~2 hours). Store the cells at 4 °C until needed (Part G). The cells may be stored up to 72 hours at 4 °C if necessary.

2. Thaw, on ice, one tube of MaxPlax Lambda Packaging Extracts for each ligation reaction performed in part E.

3. When the extracts are thawed, **immediately** transfer 25 µl (one-half) to a second 1.5-ml microfuge tube and place on ice. Return the remaining 25 µl of the MaxPlax Packaging Extract to a -70 °C freezer for use in step 7

**Note:** *Do not expose the MaxPlax Packaging Extracts to dry ice or other $CO_2$ source.*

4. Add all 10 µl of the ligation reaction from part E to the 25 µl of the thawed extracts being held on ice.

5. Mix by pipetting the solution several times. Avoid the introduction of air bubbles. Briefly centrifuge to bring all of the liquid to the bottom of the tube.

6. Incubate the packaging reactions at 30 °C for 2 hours.

7. After the 2 hour packaging reaction is complete, add the remaining 25 µl of MaxPlax Lambda Packaging Extract from step 3 to each tube.

8. Incubate the reactions for an additional 2 hours at 30 °C.

9. At the end of the second incubation, add Phage Dilution Buffer (PDB) to **1 ml final volume** in each tube and mix gently. Add 25 µl chloroform to each and store at 4 °C. A viscous precipitate may form after the addition of chloroform. This precipitate will not interfere with the library production. Determine the titer of the phage particles (packaged fosmid clones) in part G, and then plate the fosmid library in part H. Or, store the phage particles as described in Appendix D.

**Note:** *In the construction of metagenomic fosmid libraries from environmental water or soil microbes, the amount of PDB to be added to the packaged phage may require some adjustment depending on the starting amount of DNA. If the DNA used in ligation is lower than the protocol recommends, then the addition of 0.5 ml of PDB may be needed.*

**G. Titering the Packaged CopyControl Fosmid Clones**

Kit components used in this step: **EPI300-T1$^R$ Plating Strain from Part F, step 1.**

Before plating your library, it is recommended that you determine the titer of the phage particles (packaged CopyControl Fosmid clones). This will aid in determining the number of plates and dilutions required to obtain a library that meets your needs.

1. Make serial dilutions of the 1 ml packaged phage particles from part F, step 9 into Phage Dilution Buffer (PDB) in sterile microfuge tubes.

A) 1:$10^1$ Dilute 10 μl of packaged phage into 90 μl PDB

B) 1:$10^2$ Dilute 100 μl of the 1:$10^1$ dilution into 900 μl PDB

C)1:$10^3$ Dilute 100 μl of the 1:$10^2$ dilution into the 900 μl PDB

2. Add 10 μl of each above dilution, and 10 μl of the undiluted phage, individually, to 100 μl of the prepared EPI300-T1$^R$ host cells from Part F, step 1 above (you should have 4 tubes total). Incubate each tube for 1 hour at 37 °C.

3. Spread the infected EPI300-T1$^R$ cells on an LB plate + 12.5 μg/ml cloramphenicol and incubate at 37 °C overnight to select for CopyControl Fosmid clones.

4. Count colonies and calculate the titer of the packaged phage particles from Part F, Step 9. To determine the titer, use the formula on page 15 of the epicentre protocol.

**H. Plating and Selecting the CopyControl Fosmid Library**

Based on the titer of the packaged CopyControl Fosmid clones and the estimated number of clones required (see appendix A), calculate the volume of the packaged fosmid clones that will be needed to prepare the CopyControl Fosmid library.

1. Based on the titer of the phage particles determined in part G, dilute the phage particles from part F, step 9 with PDB to obtain the desired number of clones and clone density on the plate. Proceed to the next step or store the diluted phage particles as described in Appendix D: Infect the bacterial cells and resuspend in the appropriate volume of liquid media based on the expected titer. Then, transfer the resuspension to a sterile tube and add sterile glycerol to a final concentration of 20%. Mix the solution and store in aliquots at -70°C.

2. Mix the diluted phage particles from part H, step 1 with EPI300-T1$^{R}$ cells prepared in part F, step 1 in the ratio of 100 μl of cells for every 10 μl of diluted phage particles.

3. Incubate the tubes at 37 °C for one hour.

4. Spread the infected bacteria on an LB plate + 12.5 μg/ml chloramphenicol and incubate at 37 °C overnight to select for the CopyControl Fosmid clones.

5. We recommend plating as much of the library as possible. Storage of the packaged phage for more than 72 hours at 4 °C will result in a severe loss of phage viability and the plating efficiency will be severely compromised. We recommend storing the phage as an amplified library (see Appendix D, method C as described in Part H, step 1) for best results.

# CopyControl™ Fosmid Library Production Kit: Reagent Preparation

**LB Broth + 10 mM $MgSO_4$ + 0.2% Maltose** (50 ml)

2XYT Broth may be used in lieu of LB Broth.

To 50 ml of broth, add:

- 0.0602 g $MgSO_4$
- 0.1 g Maltose

Stir with stir bar until fully dissolved, then autoclave.

**LB Plates + 12.5 µl/ml Cloramphenicol** (200 ml)

Prepare in a 500 ml flask.

To 200 ml ultrapure water, add:

- 2 g tryptone
- 1 g yeast extract
- 2 g NaCl
- 3 g agar

May also use LB mix if available with the addition of 3 g agar.

Stir with stir bar to bring into solution (it may not be fully dissolved), then autoclave.

Once the solution cools to ~ 55 °C (can touch the glass without a glove), add:

- 73.5 µl cloramphenicol (34 mg/ml)
-

Swirl the flask and pour ~10 plates. Let them cool on bench overnight before storing in the fridge. Label the plates appropriately.

**70% Ethanol** (10 ml)

To a 15 ml culture tube (with the white cap), add:

- 3 ml ultrapure water
- 7 ml ethanol

**3M Sodium Acetate (pH 7.0)** (10 ml)

To 10 ml ultrapure water, add:

- 2.461 g Sodium Acetate

Stir on stir plate for a few minutes to fully dissolve the components. Then pH the solution using HCl

**Phage Dilution Buffer –10 mM Tris-HCl (pH 8.3), 100 mM NaCl, 10 mM $MgCl_2$** (100 ml)

To 100 ml ultrapure water, add:

- 0.1211 g Tris
- 0.584 g NaCl
- 0.2033 g $MgCl_2 \bullet 6\ H_2O$

Stir on stir plate for a few minutes to fully dissolve the components. Then pH the solution using HCl

**TE Buffer –10 mM Tris-HCl (pH 7.5), 1 mM EDTA** (100 ml)

To 100 ml ultrapure water, add:

- 0.1211 g Tris
- 0.0372 g EDTA

Stir on stir plate for a few minutes to fully dissolve the components. Then pH the solution using HCl

**1% Agarose Gel** (30 ml)

To 30 ml TAE, add:

- 0.3 g agarose

Microwave ~ 40 seconds to heat and dissolve agar into TAE (watch to make sure it doesn't boil over). Let solution cool until it is comfortable to touch to skin. Once cooled, pour into gel mold, add comb for wells, and allow it to fully cool and solidify.

# Making 2YP-5052 Glutamine Agar for EcoBlue Cells

Combine in 400 mL ultrapure $H_2O$

- Agar – 7.5 g
- Yeast Extract – 2.5 g
- N-Z Amines – 5 g

Autoclave this solution

Combine in 100 mL ultrapure $H_2O$

- Glutamine – 3.29 g
- Glycerol – 2.5 mL
- D-glucose – 0.25 g
- α-Lactose – 1.0 g
- Sodium phosphate dibasic – 3.549 g
- Ammonium sulfate dibasic – 1.652 g
- Potassium phosphate monobasic – 3.402 g

Filter-sterilize this solution and add it to the 400 mL autoclaved solution to make a final volume of 500 mL

Once the solution has cooled enough to touch the flask to your skin, add 500 µL of 100 mg/ml spectinomycin. Pour plates and store at 4° C.

*Do NOT autoclave glutamine, as it will degrade.*

# Optimization of Gel Electrophoresis for HMW DNA

The following conditions were found to be optimal for running HMW DNA in an agarose gel:

- 0.4% agarose in TAE buffer, 50 ml
- Run gel at ~3 v/cm for 4.5-5 hours at 4 °C

# Disk Diffusion Assay for Testing Potential Antimicrobial Agents

Preparing the Plate:

1. Make 20 ml agar plates for each bacterium strain. Plates must be identical in agar amount.
2. Let the plates dry overnight. Store at 4 °C

Inoculating the plates with bacteria:

1. Label the plates as necessary. Up to 9 disks (3 x 3) may be tested on each plate. Each plate should contain a control disk that will be inoculated with sterile water.
2. Using a sterile spreader, spread 100 $\mu$l of liquid bacteria culture (optical density ~1 at 600 nm) on their designated plates. Ensure that the bacteria are very well spread out.
3. Let the plates dry until no liquid remains on the top of the plates. This will take about 15-20 minutes in an incubator at 37 °C or up to 30 minutes at room temperature.

Spotting the potential antimicrobial agent:

1. Acquire one empty sterile petri dish, metal forceps, and sterile paper disks (BD cat #2310396 mm blank paper discs), a flame source, and the plates.
2. Open the sterile petri dish and pour the sterile paper disks inside. Sterilize the forceps in the flame and allow them to cool while remaining sterile. Keep the flame source lit.
3. Once cooled, carefully pick up one of the paper disks with the sterile forceps and carefully place it on the plate. Re-sterilize the tip of the forceps in the flame.
4. Immediately after placing the disk, spot 10 $\mu$l of the potential antimicrobial agent in the paper disk. You must do this immediately after putting the disk on the plate so the disk does not absorb liquid from the media. Be careful not to bump the plate from this point forward or else the zone of inhibition will not be circular.
5. Repeat the process for each potential antimicrobial agent.
6. Place the plates in the incubator overnight.

Measuring the zone if inhibition

1. If the antimicrobial agent is inhibitory for the strain of bacteria that you are studying, there will be a circle around the disk where the bacteria did not grow. The diameter of this zone of inhibition must be measured. Measure across the zone of inhibition three times and average these values. Also record whether the edge of the zone was well defined.
2. Repeat the experiment on three different days with enough measurements to do statistical analysis.