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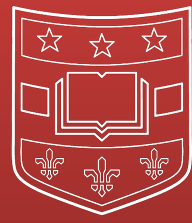
Antifungal genome mining and genetics in filamentous actinomycete bacteria isolated from local soils



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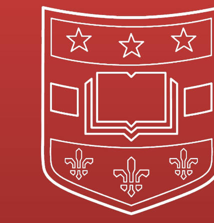
Biol 3493- Bacterial Biotechnology & Bioprospecting Spring 2017
Department of Biology, Washington University in St. Louis

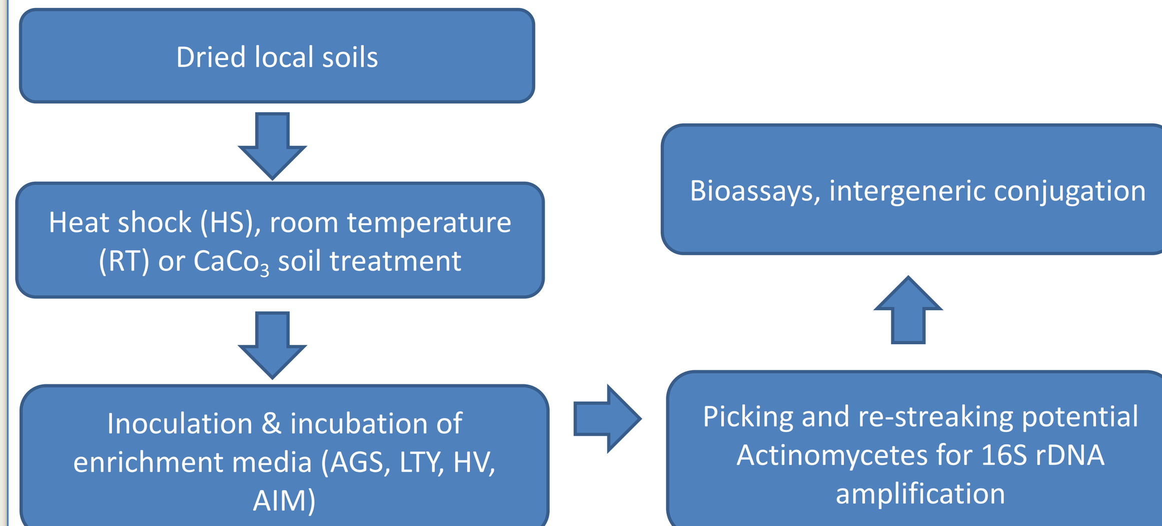


Abstract

Actinomycetes are gram positive, filamentous bacteria that produce useful antibiotics, antitumor agents, and agricultural products. A series of enrichments were undertaken to isolate actinomycetes from local soils, varying enrichment media, antibacterials, and soil treatments (including heat and $CaCO_3$). Isolates were characterized by 16S rDNA sequencing, phenotypic and morphological observations, and antibiotic production. The genetic tractability of select isolates was analyzed using a panel of integrating vectors derived from ϕC31, ϕBT1, and *OzzyJ* phage using intergeneric conjugation. Further, a semi-degenerate multiplex PCR assay to detect ϕBT1 genomic integrants was designed and tested for the first time. Finally, PCR screens were used to test if the isolates genetically encode for the production of Polycyclic Tetramate Macrolactams (PTM), a common class of antifungal natural products. We designed and tested PCR screens *in silico* that probed specific PTM biosynthetic genes in order to predict PTM chemical variability arising from gene cluster diversity. PTM production from positive isolates was assayed using coupled liquid chromatography-mass spectrometry (LC-MS). Our results indicate that we have isolated a variety of Actinomycetes, many of whom produce antifungal and antibacterial compounds, which are genetically tractable with a subset predicted to produce PTM compounds.

Environmental Isolation

METHODS



RESULTS

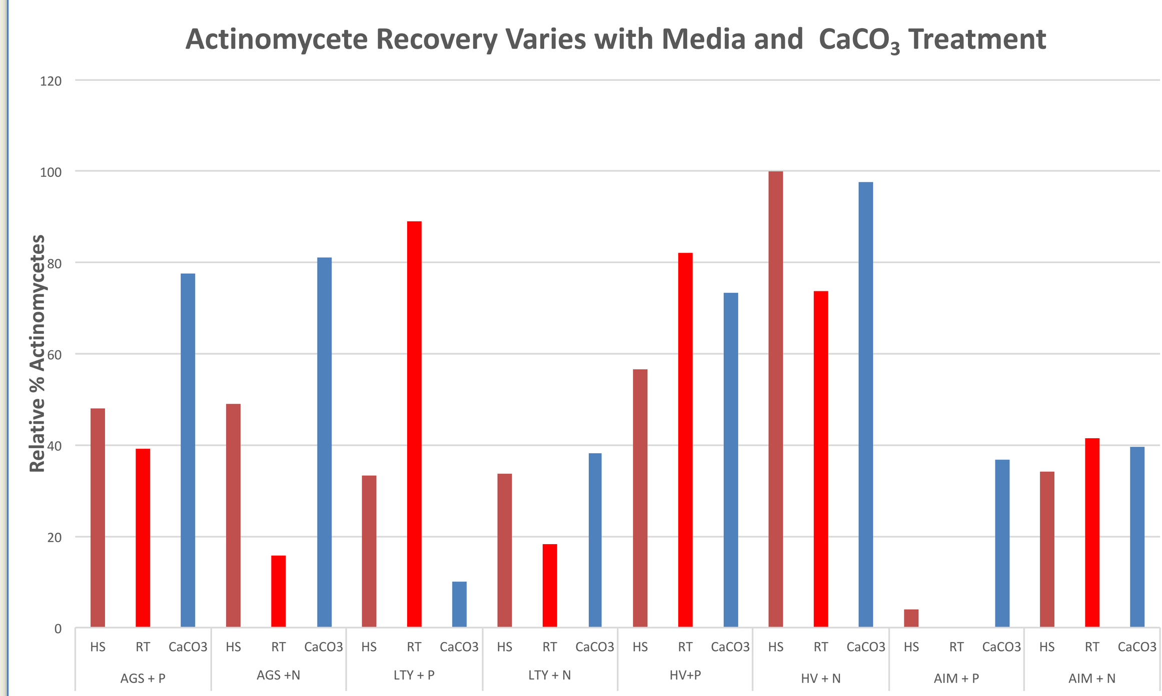


Figure 1: Analysis of selective treatment efficacy for isolation of Actinomycetes: media type, heat shock, calcium carbonate treatment and antibiotic treatment.

- Soil treated with $CaCO_3$ had higher relative % Actinomycetes than non-treated soil
- No difference in relative % Actinomycetes between heat shocked versus room temperature soil samples
- No difference in relative % Actinomycetes between polymyxin B (PMB) and nalidixic acid (NaI) treatment
- HV, on average, had the highest relative % Actinomycetes of all media types

Environmental Isolation

	Bioassay results against Bacterial and Fungal Indicator Organisms (ZOI in mM)			
Actinomycete Strain	*E. coli*	*B. subtilis*	*S. cerevisiae*	*C. albicans*
SP17KL001	12	20	7	6
SP17KL002	0	10	9	9
SP17KL007	10	17	10	0
SP17KL011	6	0	0	0
SP17KL012	0	0	0	0
SP17KL013	0	12	0	0
SP17KL014	0	0	0	0
SP17KL015	0	10	0	0
SP17KL016	10	0	0	0
SP17KL018	5	8	0	0
SP17KL019	10	6	0	0
SP17KL020	0	12	0	0
SP17KL023	0	14	0	0
SP17KL024	0	0	0	0
SP17KL031	0	0	8	0
SP17KL032	0	10	0	0
SP17KL033	0	0	0	0
SP17JA01	0	8	9	0
SP17JA03	10	16	0	0
SP17JA05	0	10	0	0
SP17JA07	0	13	0	0
SP17JA08	0	13	9	0
SP17JA09	0	9	0	0
SP17JA010	15	0	8	0
SP17JA012	0	0	11	0
SP17JA014	0	12	7	8
SP17JA016	0	8	0	0
SP17BM01	0	6.5	0	0
SP17BM03	0	10	7	7
SP17BM04	0	13	0	0
SP17BM06	9	10	0	0
SP17BM10	0	7	0	0
SP17BM11	6	7	6	9
SP17BM13	0	0	0	0
SP17BM14	8	14	14	10
SP17BM17	14	10	8	0
SP17BM18	0	10	19	14

Table 1: Bioassay results of environmental isolate strains to bacterial and fungal indicators.

- 12/37 strains had bioactivity against *E. coli* (gram negative)
- 27/37 strains had bioactivity against *B. subtilis* (gram positive)
- 14/37 strains had bioactivity against *S. cerevisiae* (fungi)
- 7/37 strains had bioactivity against *C. albicans* (fungi)

Conclusion: Environmental actinomycetes inhibited Gram + *B subtilis* bacteria most frequently, followed by Gram – *E. coli*, with fewer strains inhibiting either of the yeast strains. Are antifungal producers rarer in nature?

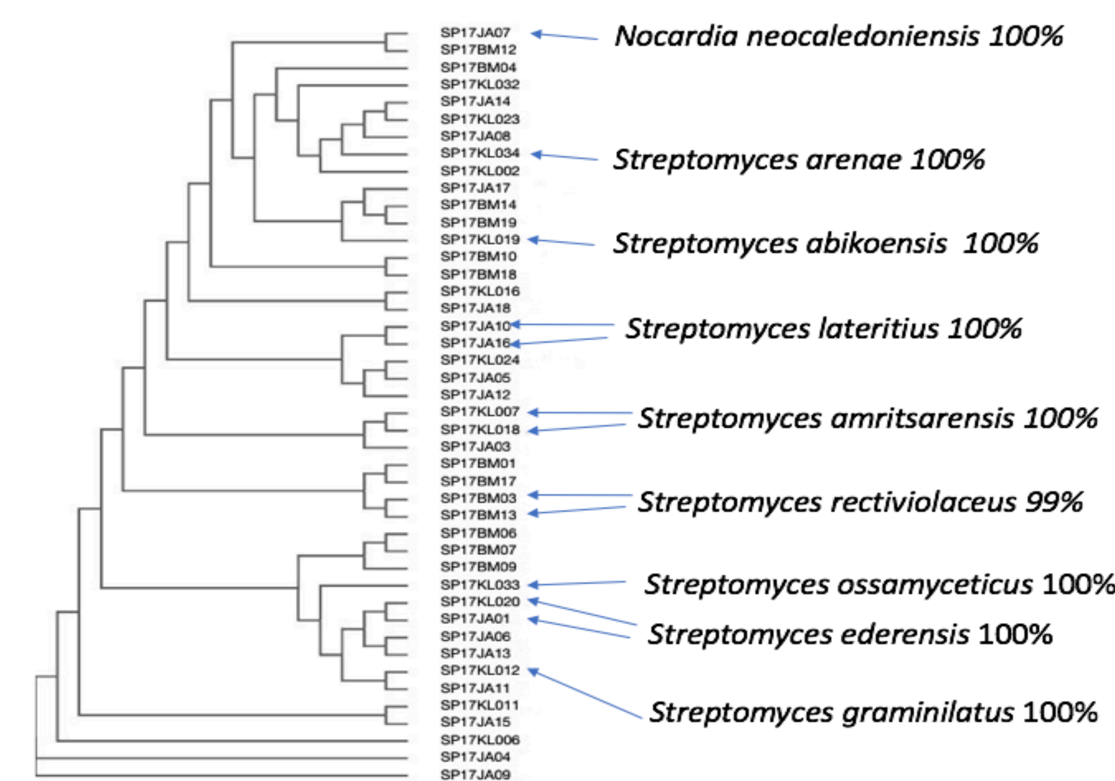
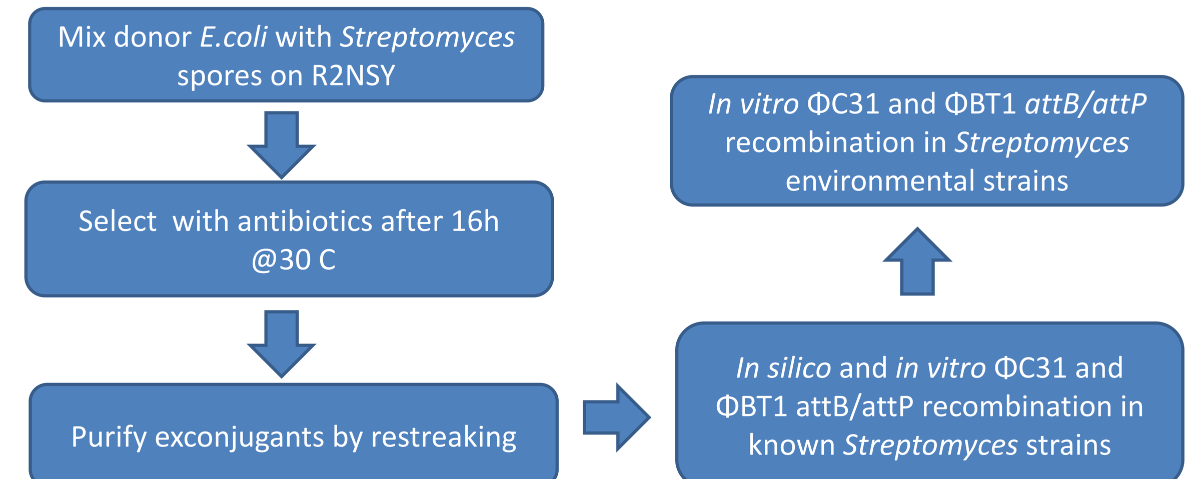


Figure 2: 16S rDNA phylogenetic tree of environmental isolates with nearest identities to *Streptomyces* homologs.

Intergeneric Conjugation

METHODS



Streptomyces Strains:	E. coli Strains:
l-S. lividans 66	301-JV36 + pJVD52.1 (Apr^R)
r-S. roseosporus	303-JV36 + pSET152 (Apr^R, ΦC31)
v-S. viridochromeogenes	372-S17-1 + pJVD52.1 (Apr^R)
c-S. coelicolor	373-S17-1 + pSET152 (Apr^R, ΦC31)
	418-JV36 + pJMD5 ($Thio^R$, ΦBT1)
	649-JV36 + pAdBO2 (Apr^R, OzzyJ)

Table 2: Conjugal donors and *Streptomyces* recipients used in control conjugations.

Intergeneric Conjugation

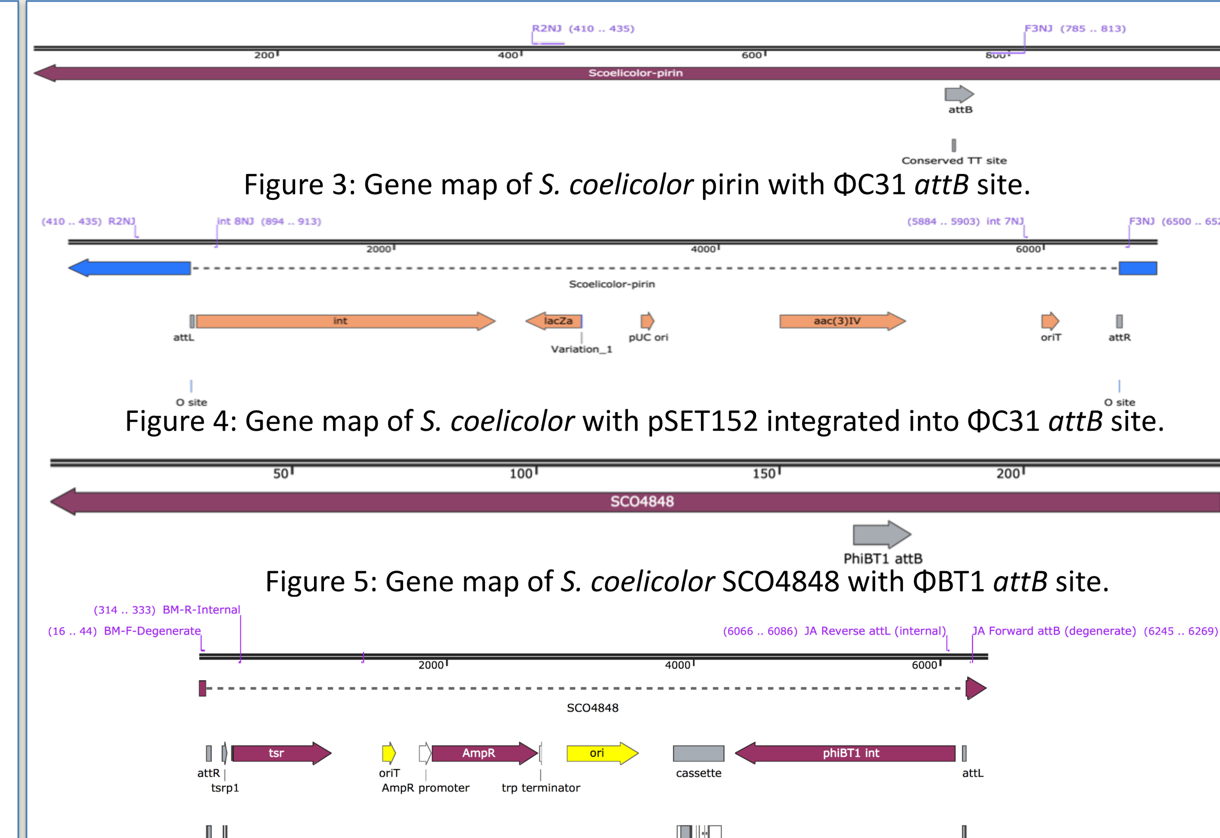


Figure 3: Gene map of *S. coelicolor* pirin with ΦC31 *attB* site.

Figure 4: Gene map of *S. coelicolor* with pSET152 integrated into ΦC31 *attB* site.

Figure 5: Gene map of *S. coelicolor* SCO4848 with ΦBT1 *attB* site.

Figure 6: Gene map of *S. coelicolor* SCO4848 with pJMD5 integrated into ΦBT1 *attB* site

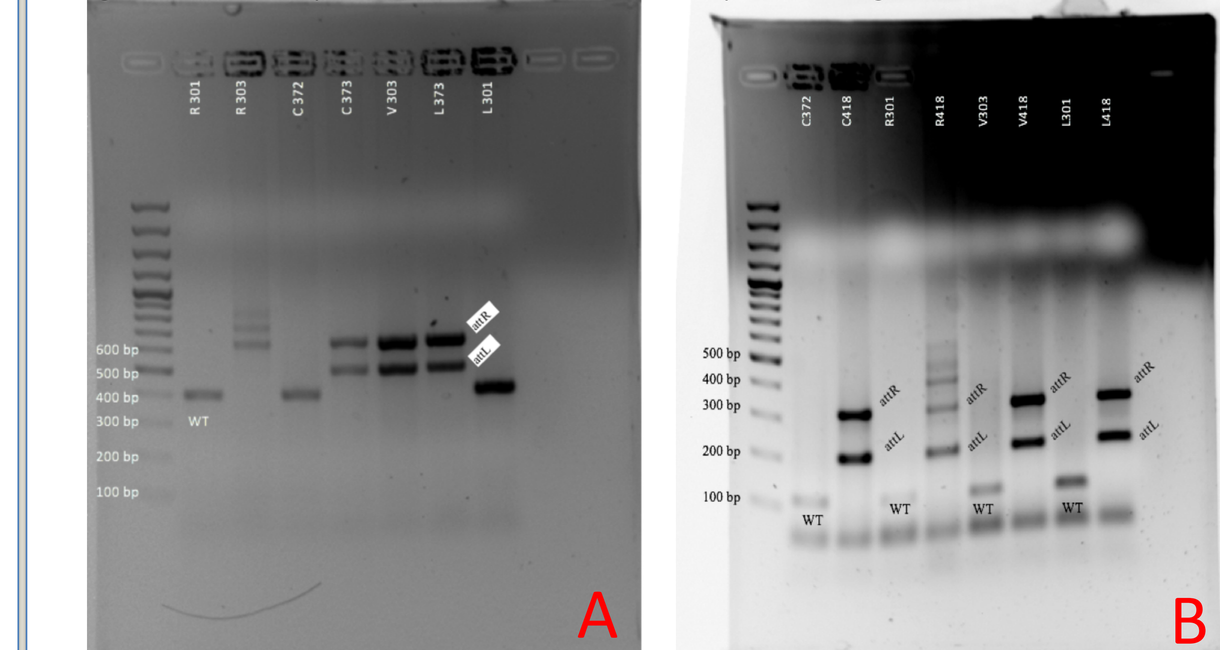


Figure 7: Gel electrophoresis of (A) ΦC31 and (B) ΦBT1 conjugation into known *Streptomyces*

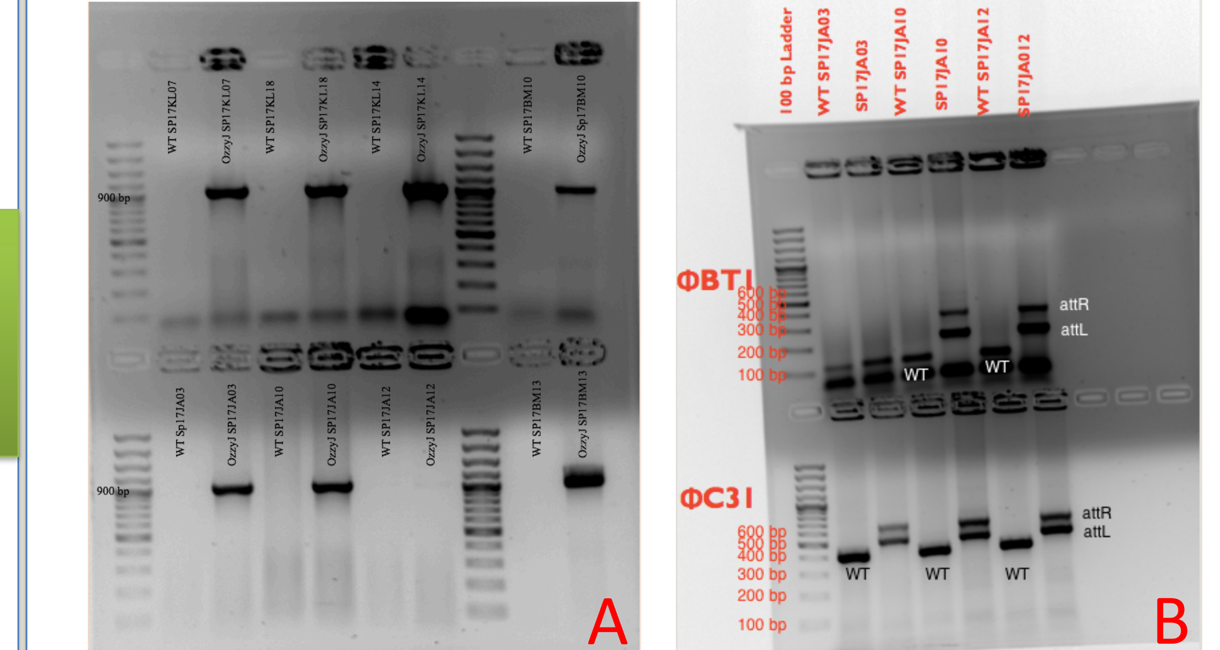


Figure 8: Gel electrophoresis of (A) *OzzyJ* and (B) ΦBT1/ ΦC31 conjugation into environmental isolates.

- ΦC31 and ΦBT1 PCR multiplex assay for intergeneric conjugation into known and environmental strains. Exconjugants verified through agarose gel electrophoresis, detecting of wt, *attL* and *attR* bands predicted from *in silico* model
- *OzzyJ* PCR assay for intergeneric conjugation into environmental strains verified through amplification of the Apr^R (*aac(3)IV*) locus

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- We would like to acknowledge our mentor Dr. Joshua A.V. Blodgett and DBBS teaching assistants Yunci Qi and Dinesh Gupta.
- Presented by a group (Students of Biol3493 SP17)

Genome Mining

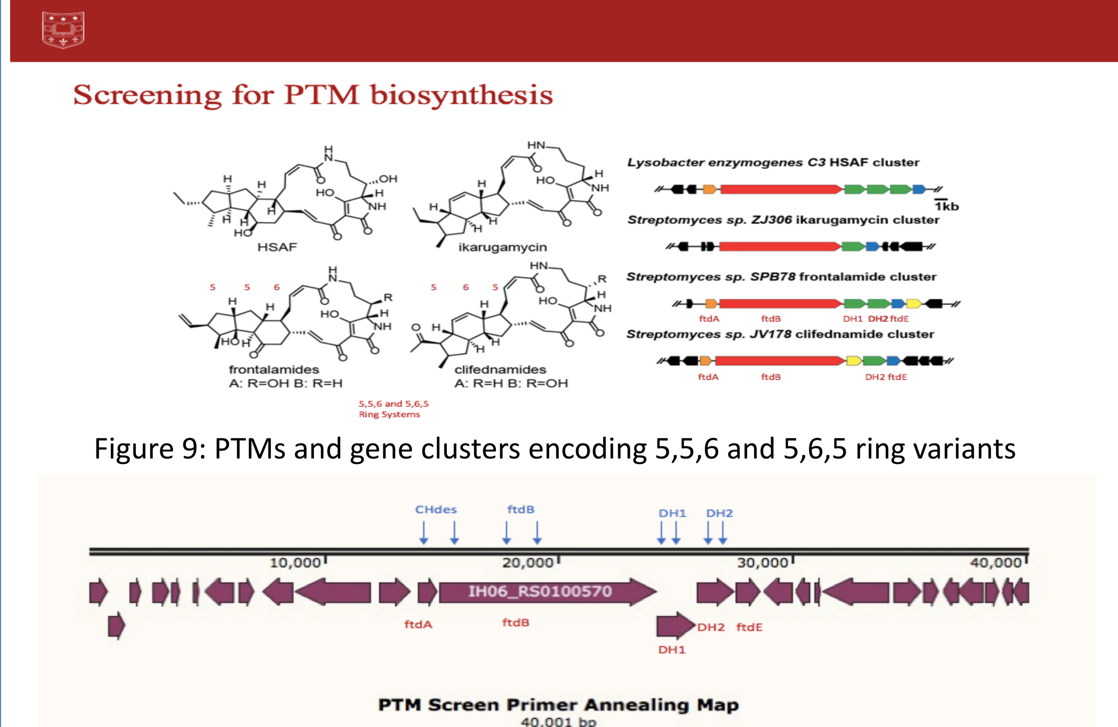
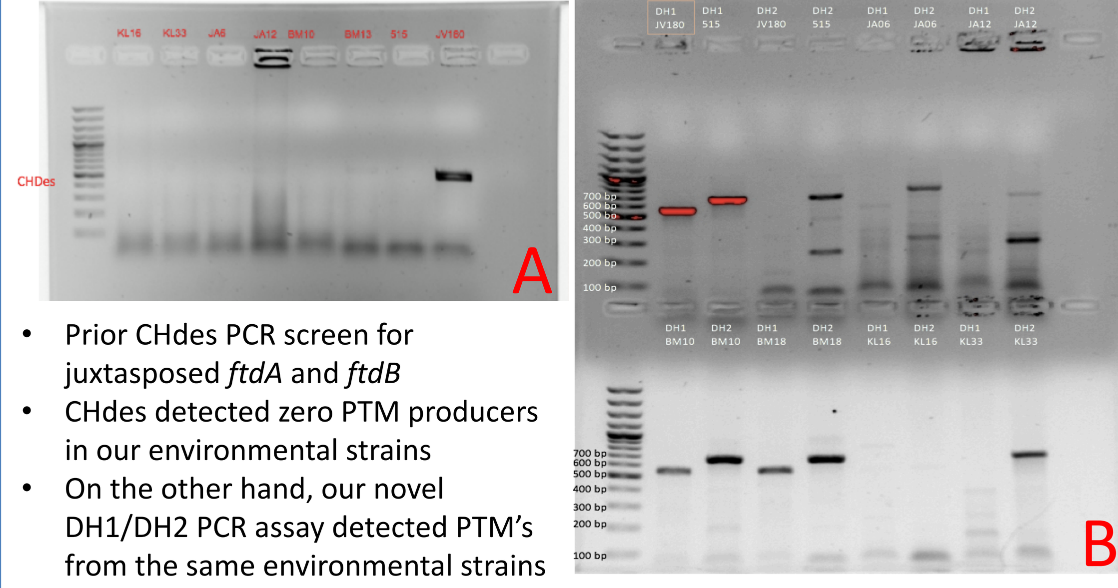


Figure 9: PTMs and gene clusters encoding 5,5,6 and 5,6,5 ring variants

Figure 10: PTM primer annealing locations for CHdes, *ftdB*, DH1, DH2 screens



- Prior CHdes PCR screen for juxtaposed *ftdA* and *ftdB*
- CHdes detected zero PTM producers in our environmental strains
- On the other hand, our novel DH1/DH2 PCR assay detected PTM's from the same environmental strains

Figure 11: Gel electrophoresis of PTM CHdes screen (A) vs. DH1/DH2 screen (B) of environmental strains.

- Discovery of KL33 as a new dehydroxy-clifednamide PTM producer
- 5, 5, 6 ring system PTMs require both DH1 and DH2 genes, while 5, 6, 5 ring system PTMs require only DH2
- Two lines of evidence suggests KL33 lacks *ftdA*: LC-MS data and negative CHdes PCR screen

Figure 12: Overlapping UV and mass spectrometry peaks at 7.2 min retention time indicates KL33 produces de-hydroxylated clifednamide as compared with a JV178 control. KL33 lacks a PTM signal at 6.5 min characteristic of hydroxy- clifednamide.

Conclusion

- We optimized conditions for the enrichment culture of Actinomycetes, and 16S rDNA sequencing provided genus level identification of our environmental isolates. Most were *Streptomyces*, but *Kitasatospora*, *Kribbella* and *Nocardia* were also isolated
- We were successful in integrating phage vectors (ΦC31, ΦBT1, and novel phage *OzzyJ*) into known *Streptomyces* strains and environmental *Streptomyces* isolates using intergeneric conjugation
- Through our analysis of PTM gene clusters, we found most PTM producing strains among our isolates lack an *ftdA* gene. In addition, we developed a more effective PCR screen for detecting and characterizing PTM clusters in soil isolates using the DH1 and DH2 genes compared to previous screens that detect juxtaposed *ftdA* and *ftdB* PTM genes