Secondary metabolite production by Streptomycetes *in situ* and *in vivo*

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Abstract

Streptomycetes are ubiquitous soil-dwelling saprophytes known to produce secondary metabolites, many of which are antibiotics. Secondary metabolism is usually a late growth phase event, brought on by different signals, e.g., nutrient starvation, environmental signals. In order to test whether *in situ* incubation changes the repertoire of expressed secondary metabolites (mainly antibiotics) compared to lab conditions, isolated *Streptomyces* sp. were embedded in AGS-Agarose medium casted in tubes and incubated in either soil or sterile petri dishes. Results show significant differences between soil and sterile petri dish incubations, based on TLC results. No antibiotic activity was found (with *E.coli* as an indicator strain). However, over 15 (conservative estimation) different spots were observed in 10 different isolates, these could be either antibiotics (to which *E.coli* is resistant) or other secondary metabolites. This research shows that *in situ* incubation have the potential of bringing forth cryptic compounds that are ecologically important as well as new antibiotics.

Introduction

Streptomycetes are Gram-positive bacteria with high G+C DNA content and a linear, relatively large (~8–10 Mb) genomes. These bacteria are ubiquitous soil saprophytes that have been studied mainly because they produce many secondary metabolites with antibiotic activity and for the interest in their life cycle. From a germinating spore, they grow vegetatively as branching filamentous hyphae. Then the mat of hyphae forms a substrate mycelium . As the mycelium develops, aerial hyphae are produced from specialized branches (Fig. 1).

Secondary metabolism usually occurs at the late growth phase. The temporal nature of secondary metabolism is genetic but expression can be influenced greatly by environmental manipulations. Therefore, secondary metabolism is often brought on by exhaustion of a nutrient, or addition of an inducer and/or by a decrease in growth rate, or by different environmental signals produced by other organisms in soil (e.g., γ-butyrolactones produced by most *Streptomyces* and by other Actinomycetes; Bibb 2005) . These events generate signals that cause a cascade of regulatory events resulting in morphological differentiation and secondary metabolite production. Formation of antibiotics is also regulated by nutrients (nitrogen, phosphorous and carbon source), metals, growth rate (Bibb 2005).

Anukool et al. (2004) have monitored streptothricin production by *Streptomyces rochei* F20 in Soil and rhizosphere compared to liquid cultures and reported evidence for *in situ* antibiotic production in sterile soil. However, expression of streptothricin biosynthetic genes (as a measure of production) wasn't detected in the nonsterile soil.

In this miniproject, an attempt to compare the production of secondary metabolites by various *Streptomyces* isolates by TLC. The hypothesis was that *in situ* incubation of *Streptomyces* sp. will induce production of secondary metabolites, and antibacterials in particular, compared to *in vitro* incubation.

Materials and Methods

Streptomyces sp. isolation and identification by phenotypic and phylogenetic analyses

A soil sample was taken from under a tree at the MBL campus (Woods hole, MA). 1 g of soil was suspended in autoclaved tap water, mixed rigorously and left to settle for an hour. Supernatant was serially diluted, plated on AGS plates (see lab manual) and incubated at 30ºC for 4 days. Twenty colonies with typical morphology (i.e. substrate and aerial mycelia) were picked and transferred to a new plate (ACM1-20). Every isolate was then examined under the microscope and the dissecting scope for evaluation of colony morphology. In addition, all isolates were cultured for 3-4 d for DNA extraction and 16S rRNA gene PCR and sequencing.

DNA extraction was done using the UltraClean Microbial DNA isolation kit (MoBio, Carlsbad, CA) according to the manufacturer's specifications. DNA was then diluted 1:100 and used as a template for 16S rRNA gene amplification, using universal eubacterial probes (8F, 1492R; Lane DJ 1991). 16S rRNA genes were amplified in a 25-µl reaction mixture consisting of 12.5 µl of 2 X Promega master mix, 15pmol or each primer, 1 µl of template DNA. Amplification conditions for the PCR included an initial denaturation step of 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 46°C for 30 sec, and 72°C for 1.5 min and a final extension step of 72°C for 5 min. Reaction products were checked for size and purity on 1% agarose gel. PCR products were cleaned with ExoSAP-IT (USB Corp., USA). DNA sequencing was performed by the chain termination method using an ABI Prism. Sequence analysis was done using RDP10 (Cole et al, 2009) as well as with BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi). Phylogenetic trees were constructed with RDP's Tree Builder and visualized using FigTree (ref).

Isolate embedding in agarose plugs and incubation

Isolates were inoculated in 3 ml of NB and incubated at 30ºC for 72 h, on a rotary shaker at 250 rpm. Cultures were then centrifuged (7000 x g, 2') washed and resuspended in FW-base. 100 µl of the washed cultures were then added to 5ml of melted AGC-Agarose medium (45-50 ºC; AGS-Agarose medium was made according to the AGS protocol with 1% agarose instead of agar). The melted medium was then poured into sterile cylinders and left to harden. Each agarose plug was then cut to five 1-2 cm plugs, and those were either embedded in soil (S, 2 plugs; collected at the same collection site as for isolation) or put in an empty petri dish (P, 3 plugs).

The two soil plugs and two of the plate plugs were incubated in a humid chamber with constant umidity, while the last plate plug was incubated outsite the chamber. All incubation were at 30ºC, for 72 h.

Extraction of secondary metabolites and thin layer chromatography (TLC)

After incubation, plugs were washed in sterile deionized water and homogenized using a pestle, in eppendorf tubes. Each homogenate was added with 250 µl ethyl-acetate, vortexed and incubated for 12 h at room temperature. After the incubation, tubes were centrifuged (10,000 x g, 2 min) and the supernatant was transferred to a new tube. Ca. 5 µl of each extract were blotted on a Silica-Gel 60 F264 (Merck) used for TLC. TLC plates were developed with choloroform:methanol (4:1) and visualized by UV light. Retention factors (R_f) were calculated by the following equation $R_f = \frac{Distance\ Compound\ Traveled}{Distance\ Solvent\ Traveled}$. In parallel, Rifamicin and chloramphenycol were used as positive controls.

Antibiotic diffusion disc assay

Three ml of LB-Soft agar (2% LB w/v, 0.5% Agar w/v) added with 100 µl of an overnight culture of *Escherichia coli* were overlaid on an LB plate (2% LB w/v, 1.8% Agar w/v) and left to harden. Filter paper disks were saturated with ethyl acetate extracts, air-dried and placed onto the plates. Plates were incubated for 24 h at 35°C.

Oxygen profile of *Streptomyces* embedded in agarose

The oxygen profiles of two isolates, embedded in agarose, as well as a sterile control were measured using an oxygen microelectrode (Unisense, Denmark).

Results

Twenty morphologically different *Streptomyces* sp. were isolated from soil and used for subsequent TLC analysis. These isolates all had the typical Streptomycete colony and cell morphologies (Fig. 1) and all sequenced 16S rRNA genes (10 isolates) were Actinomycetes, nine of which were *Streptomyces* sp. These isolates were identified as closely related (>99%) to *Streptomyces amakusaensis*, *Streptomyces aureus*, *Streptomyces sanglieri* , *Streptomyces griseus*, *Streptomyces litmocidini*, *Streptomyces galilaeus* , *Streptomyces inaequalis* and *Rhodococcus fascians* (Fig. 2). All but one of these bacteria (*R. fascians*, a phytopathogen) are known to produce antibiotics.

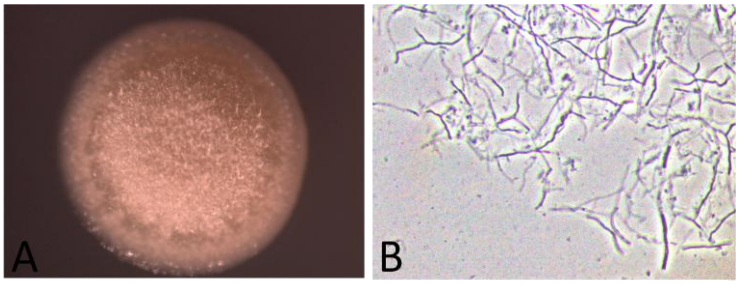

Figure 1. Colony (A) and cell (B) morphology of ACM-5

Figure 2. Weighbor Joining Phylogenetic tree of isolated *Streptomyces* sp. and closely related type strains.

Of the 20 isolates, 10 were used for ethyl acetate extraction and TLC (i.e., only ten of the isolates grew to high enough density to be used). The plates were evaluated and R_f was calculated for each spot on the TLC plates (Table 1, Fig. 3).

Table 1. R_f values for ethyl acetate extracts from *Streptomyces*-inoculated agarose plugs

Isolate	Soil A	Soil B	Plate	Plate	Plate (out)
ACM3	0.83	0.79	0.76	0.77	0.85
	0.96	0.92	0.81	0.83	0.89
ACM5	0.77	0.74	0.65	0.66	0.61
		0.81	0.72	0.73	0.73
					0.77
ACM6	0.83	0.83	0.84	0.84	0.00
	0.86	0.86	0.91	0.91	
	0.89	0.89	0.95	0.95	
	0.94	0.94			
ACM8	0.38	0.25	0.23	0.23	0.64
	0.80	0.44	0.64	0.64	0.80
	0.91	0.60	0.80	0.80	0.89
	0.96	0.82	0.89	0.89	
		0.89			
ACM9	0.39	0.39	0.62	0.62	0.66
	0.76	0.76	0.68	0.68	0.74
ACM11	0.83	0.79	0.80	0.80	0.80
	0.88	0.82	0.84	0.84	0.84
	0.91	0.85	0.98	0.98	0.98
	0.96	0.93			
		0.98			
ACM14	0.00	0.00	0.83	0.83	0.00
			0.92	0.92	
ACM16	0.77	0.77	0.74	0.74	0.79
			0.78	0.78	0.84
ACM19	0.90	0.88	0.80	0.80	0.80
			0.84	0.84	0.84
ACM20	0.68	0.68	0.67	0.69	0.74
			0.76	0.75	0.81
Mixture (10 isolates)	0.84	0.84	0.79	0.79	0.00
	0.86	0.86	0.81	0.81	
	0.93	0.93	0.85	0.85	
			0.91	0.91	
Cont - Rif	0.41				
Cont - Chl	0.87				

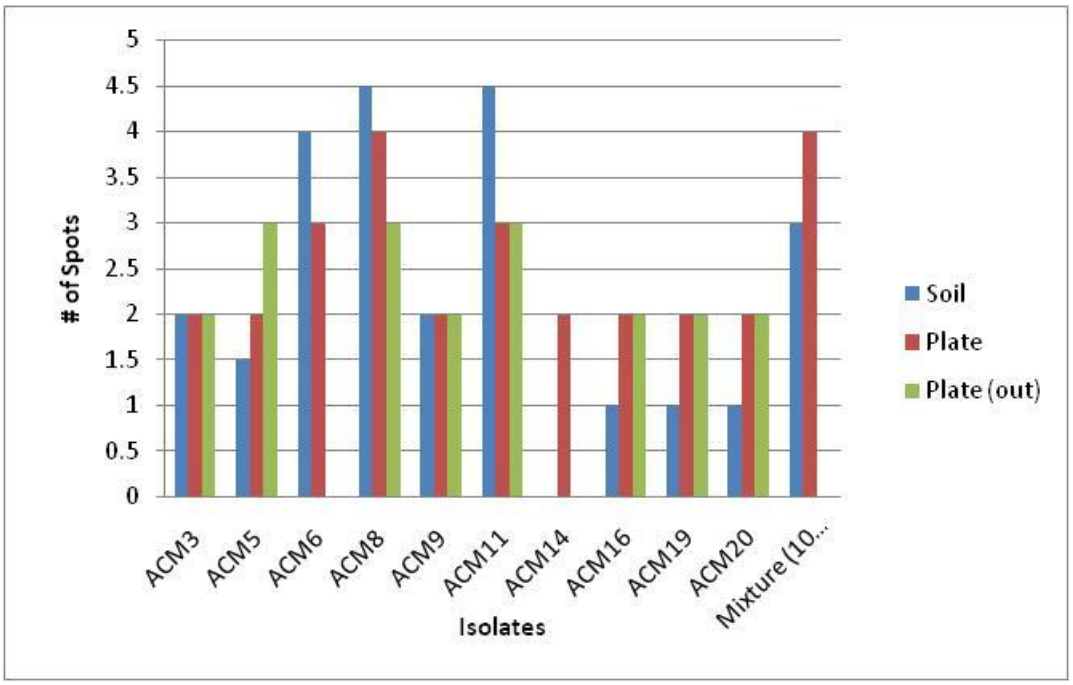

Figure 3. Number of peaks observed for *Streptomyces* isolates under different conditions. For soil and plate samples n=2, plate (out) n=1.

The spot patterns on TLC plates show qualitative as well as quantitative differences – in most cases, plate replicates had the same pattern, whereas patterns often varied between soil replicates. In addition, patterns of soil incubated isolates were different from those incubated in a petri dish.

These differences in spot patterns suggested possible differences in antibiotic production. In order to test this, disc diffusion assays with *E.coli* as the indicator strain were set up (Fig. 4). Surprisingly, not one of the extracts tested showed antibiotic activity.

Figure 4. Disc diffusion assays of isolates. A – incubation in soil, C – incubation in sterile plate and E- Incubation in sterile plate, outside humid chamber.

Discussion

Production of secondary metabolites is a key process in *Streptomyces* ecology as these metabolites serve different roles, from siderophores to signaling molecules and antibiotics. The production of secondary metabolites is dependent on various environmental factors and signals, some of which are produced by the organism itself (i.e., A—factor) and some are probably produced by other members of the soil community. Up until now, over 7000 compounds, produced by *Streptomyces* (Berdy, 2005), have been identified. The identification of cryptic biosynthetic pathways may be done by genomic analyses or by empirically changing the signals the bacterium is exposed to and try to induce production while keeping the ecological context in mind. *In situ* incubation of *Streptomyces* has the potential of understanding the ecology of these bacteria, in their natural environment, by tapping into the crosstalk between them and the other soil community members. In this project, I attempted to induce production of these molecules in general, and specifically antibacterials, by incubating *Streptomyces* in their natural environment, while maintaining the ability to probe secreted compounds.

The profile of secondary metabolites secreted by *Streptomyces* incubated *in* situ is significantly different from that of bacteria incubated in a petri dish. A difference that is apparent even when using insensitive methods like TLC to profile the secondary metabolites (Table 1). Even though it was hypothesized that *in situ* incubation will induce production of antibiotics, which was not the case (Fig. 4). However, this may be due to the use of a single indicator strain (E. coli) and not a suite of different, ecologically relevant, bacteria and fungi. In addition, the spots that were observed might have been different signaling molecules that facilitate the crosstalk between the Streptomycete and its environment. These observed profiles are dependent on the extraction method as well as the development solution. In this work only a single set of extraction and development was used, making detection limited. Use of multiple extraction methods, along with a sensitive measuring method (e.g., LC-MS) and possibly isolation of these compounds should allow us to evaluate the entire potential of secondary metabolite production in that specific environment.

This method of *in situ* incubation can be used to try and answer many questions regarding ecology of soil dwelling bacteria (and other environments as well). By allowing one to confine the immediate environment of a bacterium, while keeping it exposed to the various signals in its

environment, should allow eavesdropping the variety of signals and responses in the natural environment.

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