Activation of Silent Biosynthetic Pathways in Cave Microorganisms for Drug Discovery

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KEYWORDS. Natural product discovery, secondary metabolite, multivariate statistical analysis

BRIEF. Cave microorganisms were exposed to a variety of stimuli for the purpose of secondary metabolic pathway activation and potential acceleration of techniques for drug discovery.

ABSTRACT. Natural products are a valuable resource for therapeutic compounds used to treat a wide variety of ailments. Cave ecosystems, with low nutrient levels and minimal interaction with the surface environment, are unique environments that host microorganisms and have been found to produce potentially valuable natural products. As in-depth genomic analysis of microorganisms has grown more common, it is clear that gene clusters that encode biosynthetic pathways for secondary metabolites are present, yet not expressed, in many microorganisms. These metabolites may be produced by microorganisms in response to environmental changes as a result of acquired genetic characteristics brought on by evolutionary pressures and adaptation to survive. Exposure to antibiotics, rare earth metals, and competing microorganisms in liquid culture was used in order to upregulate, or increase the abundance of, a particular compound(s) produced by an organism compared to the control. Liquid chromatography-mass spectrometry (LC/MS) was used to analyze and detect upregulated features in four samples from Blue Springs Cave (Sparta, TN) and Yankee Hole (Murfreesboro, TN). Compounds were selected for future isolation, characterization, and bioactivity assessment. A range of 6-41% of each sample's secondary metabolic profile was upregulated by two-fold or greater when exposed to the individual stimuli.

INTRODUCTION.

Natural products obtained from microorganisms have been valuable sources for antimicrobial and antibacterial drugs, like vancomycin in its treatment of bacterial infections [1]. Many microorganisms that have been found to produce novel compounds are actinomycetes, particularly from the genus *Streptomyces* [2]. Actinomycetes, an order of Gram-positive terrestrial and aquatic bacteria, have been heavily studied for natural product discovery.

Microorganisms may upregulate the production of secondary metabolites in response to exposure to different stimuli, like environmental alterations [3]. As a result, many microorganisms have the potential to yield a plethora of natural products; however, these compounds remain unknown because their genetic pathways are not expressed. Intentional activation of these "silent" biosynthetic pathways through exposure to different stimuli could possibly be utilized for the production and isolation of compounds used to create new drugs and help drive natural product discovery. Genomic analysis has also been used as a tool to discover and understand secondary metabolites that the organism may be able to produce [4].

Cave environments may be good sources of novel natural products because they are underexplored in their potential to host secondary metabolite-producing microorganisms [2]. The ability to survive with little light and nutrients indicates that cave microorganisms may have developed specialized survival mechanisms, with production of unique secondary metabolites being one of them [2]. Applying natural product discovery techniques to cave microorganism samples may lead to the production of valuable medicinal drugs.

Actinomycetes from Blue Springs Cave (Sparta, TN) and Yankee Hole (Murfreesboro, TN) were exposed to sub-inhibitory concentrations of antibiotics, rare earth metals, and competing organisms to upregulate secondary metabolite production and select candidates for compound isolation and characterization [5-8]. Microorganisms were screened by exposing them to different stimuli in liquid culture, extracting the produced chemical compounds, analyzing the extracts through liquid chromatography-mass spectrometry (LC/MS), and assessing the extracts' chromatogram data through principal component analysis (PCA) to choose candidates for compound isolation and characterization. Extracts were selected due to high levels of upregulation of particular features in response to exposure to the stimuli and will later be isolated, characterized, and assessed for bioactivity.

MATERIALS AND METHODS.

Seed Culture Preparation

Cave samples were identified with the initials of the researcher who extracted the sample, followed by an acronym for the cave, and an identifying number in the sequence that the sample was collected. Water extracts of cave soil samples (100 mg of soil/sample) were diluted at one-thousandth and one-ten-thousandth of the original concentration, plated on ISP Medium 2 (Difco), and allowed to grow for two weeks to isolate individual colonies. Individual colonies were picked with sterile needles and re-plated for purification. Individual glycerol stock solutions were prepared and stored at -80° C. Four purified microorganisms were chosen and inoculated into two 25 mL volumes of ISP Medium 2 to act as liquid seed cultures. Colonies were also inoculated into two 3 mL seed cultures for gDNA isolation. Seed cultures were placed in the shaking incubator at 30°C at 170 rpm; the 25 mL seed cultures were incubated for one week, while the 3 mL seed cultures were incubated for 24 hours.

Sequencing and Genetic Identification

gDNA isolation, PCR, and 16S rRNA sequencing were performed to identify the organisms. gDNA purification was performed using the Wizard Genomic DNA Purification Kit (Promega). However, TE25S Buffer was used instead of EDTA, and 120 µL of hydrated lysozyme was used for the necessary lytic enzymes.

Polymerase chain reactions (PCR) were performed on each of the tubes to amplify the 16S rRNA for sequencing and genetic identification of each sample. Each PCR tube contained 35 µL sterile water, 5 µL 10X PCR Buffer, 1 µL of the respective gDNA, 1 µL each primer (27F and 1525R), 1.5 µL dNTP (10 mM), 3 µL DMSO, and 1 µL polymerase. The sequence for the forward primer (27F) was AGAGTTTGATCCTGGCTCAG, and the sequence for the reverse primer (1525R) was AAGGAGGTGATCCAGCCGCA. PCR was run using the following thermocycler conditions: 95°C for 4 minutes, followed by 35 cycles of denaturation for thirty seconds at 95°C, annealing for one minute at 52°C, and extension for one minute at 72°C, final extension for 10 minutes at 72°C and held at 4°C overnight. Gel electrophoresis was run at 100 V for 45 minutes. To purify the PCR product, the QIAquick PCR Purification Kit Protocol was utilized (Qiagen). Tubes containing genetic material were stored in the freezer at -20°C until sent for sequencing. Finally, the genetic material was sequenced and the most closely related organisms were determined for each sample.

Fermentation Cultures and Extraction

Fermentation cultures were prepared in order to activate silent biosynthetic pathways that may yield potentially valuable secondary metabolites. After the 25 mL seed cultures were incubated at 30°C at 170 rpm for one week, they were removed from the incubator. Seven different fermentation cultures for each bacterial sample were prepared by adding 1 mL of the sample's seed culture to seven

different flasks containing 25 mL of ISP Medium 2. Each of these different fermentation cultures exposed the bacteria sample to six different stimuli and one, containing ISP Medium 2 and the sample, was prepared as a control. Each of the following compounds or competing organisms was added to one of the fermentation cultures per organism: 25 µL of 120µM streptomycin, 25 µL of 120µM rifampicin, 17.6 µL of 1900µM $LaCl_3$, 25.9 µL of 200µM $ScCl_3$, 200 µL of *T. pulmonis* in liquid culture (isolated from Snail Shell Cave), or 200 µL of *R. wratislaviensis* in liquid culture (from NRRL Culture Collection) [6, 9]. The liquid bacteria cultures of *T. pulmonis* and *R. wratislaviensis* were added to the fermentation cultures 24 hours after the start of fermentation to avoid overgrowth of competing organisms. These were incubated at 30°C at 170 rpm for one week.

The fermentation cultures underwent extraction to capture the secondary metabolites produced by the organism. First, the cultures were removed from the incubator, 25 mL of methanol was added to each flask, and the cultures were incubated under the same conditions for one hour. The cultures were transferred to conical tubes, centrifuged at 3,750 rpm for 10 minutes (additionally centrifuged, if necessary, until transparent), and the supernatant was transferred to a new conical tube. Afterward, 8 mL of the extract was then transferred into a test tube and dried overnight; the residue of each extract was stored at 4°C. The following day, the dried extracts were massed and a mixture of 50% methanol/50% water was added to each test tube (at a ratio of 5 µL/mg of dried extract) to rehydrate the sample. Extracts were vortexed, allowed to sit for two hours, and then vortexed again.

LC/MS and Analysis

Extracts were analyzed using LC/MS and PCA and were compared to the control extract to assess the upregulation of secondary metabolism caused by stressing the bacteria with different stimuli. For LC/MS analysis, 20 µL of extract was injected to be analyzed. LC/MS was run for fifty-five minutes, including a 30-minute gradient and 25-minute wash in the following two buffers: Buffer A was composed of 950 mL sterile water, 50 mL acetonitrile, and 10mM ammonium acetate, while Buffer B was composed of 950 mL acetonitrile, 50 mL sterile water, and 10mM ammonium acetate. Samples were run in duplicate in order to ensure the validity of the data received. LC/MS chromatograms for each sample set were visually assessed to observe upregulated peaks by comparing and noting differences in runs containing one of the stimuli to the control. The raw data was first processed using XCMS software (Scripps Center for Metabolomics) and was statistically analyzed and compared using PCA. PCA, a method used to statistically compare samples through use of visual plots to show degree of relation among different data points, was used to determine and compare how each sample reacted to different stimuli through comparison of upregulated features. Particular features from the dataset that were found to be highly upregulated and to have ideal mass-to-charge ratio (m/z) values were chosen as potential candidates for future isolation and characterization.

RESULTS.

Principal Component Analysis (PCA)

Microorganisms were exposed to stimuli in liquid culture in order to activate silent biosynthetic pathways and analyze the upregulation of different features from the LC/MS chromatogram data. PCA was used to statistically compare how each sample reacted to different stimuli by the generation of visual plots. The color code for the different stimuli is used throughout this section and is as follows: purple is R. wratislaviensis, orange is T. pulmonis, red is rifampicin, dark blue is ScCl3, grey is LaCl3, light blue is streptomycin, and yellow corresponds to the control. Figure 1A shows a loadings plot, which compares the degree of similarity among all of the cultures containing a stimulus to the sample set; cultures that are closer to one another on the graph are statistically more similar. Figure 1B is an S-plot that statistically compares the similarity between the R. wratislaviensis culture and the control for each feature. Features that are farther from the center are said to be more statistically dissimilar from the others. Figure 1C compares relative intensity values among stimuli in each sample for selected upregulated mass-charge ratio values to determine the stimuli in which these features were most highly upregulated.

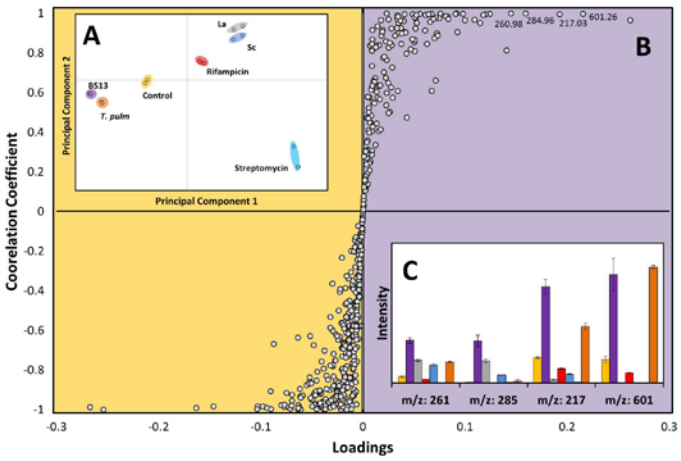

Figure 1. The HMBlue0331 organism was selected for the creation of an individual graph due to a higher number of ideal selected features for analysis. A) This figure is a loadings plot for the HMBlue0331 sample, which shows the correlations among the upregulated features from each of extracts that were exposed to different stimuli. B) This figure is an S-Plot comparing the control (left) to the R. wratislaviensis co-culture (right) and highlights interesting m/z values. C) This bar graph shows the compared intensity values for each culture condition for the four different selected features. The bars are color-coded to match their respective bubbles in the PCA plot in Fig. 1A.

Figure 2A shows a PCA plot for the KDYank031 organism, which used the same properties as Fig. 1A to compare cultures containing different stimuli and to find and prioritize ideal features based on their degree of upregulation from the control. Figures 2B and 2C show similar plots for the BBBlue012 and KDYank042 organisms. In Figure 2D, a loadings plot is shown which compares statistical similarity among all features for that organism; features that are closer together are more similar. When compared to Fig. 2A, specific regions of the graph were colored to show specific features that were more highly upregulated when exposed to certain stimuli, and selected features were distinguished by their m/z values. This type of graph was generated for the BBBlue012 and KDYank042 organisms in Figures 2E and 2F. Figure 2G, 2H, and 2I, which are similar to Figure 1C, show the comparison of the relative intensity values for each of the different color-coded stimuli in the selected features to determine the stimuli in which the feature was most highly upregulated for the respective organism.

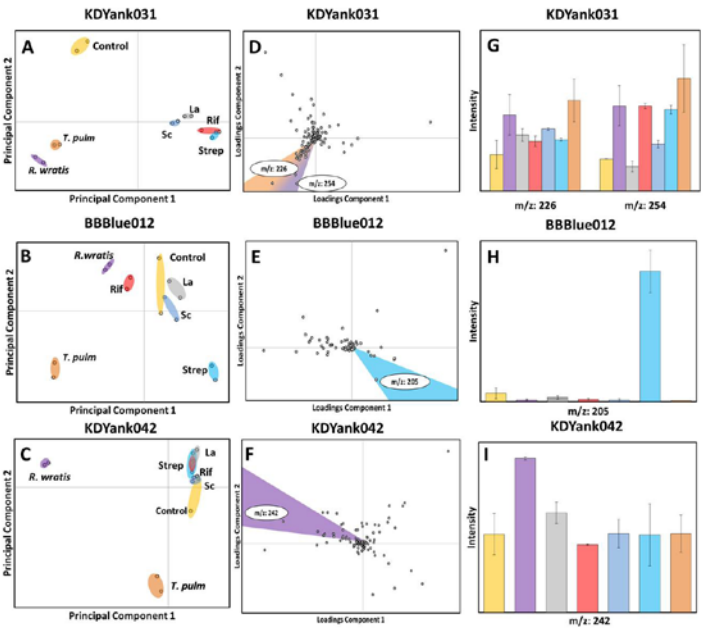

Figure 2. (A-C) These figures show PCA plots comparing each fermentation culture condition for the KDYank031, BBBlue012, and KDYank042 samples. D-F)

These figures show loadings plots for each sample with highlighted regions (color-coded for respective stimuli color) and mass-charge ratio (m/z) values for selected features. G-I) These color-coded bar graphs compare averaged LC/MS intensity values, with error bars representing standard deviation, for each condition for the respective highlighted features in Figs. 2D-2F.

Secondary Metabolic Profile Upregulation

Figure 3 shows the percentage of the features upregulated by two-fold or greater for each stimulus to analyze the overall effect of each stimulus in activating the microorgansims' silent biosynthetic pathways. When exposed to individual stimuli, 6-41% of each organism's secondary metabolic profile was upregulated by two-fold or greater. When the total set of upregulated features for each organism was analyzed, a range of 25-54% of each organism's total secondary metabolic profile was upregulated by two-fold or greater when the response to each stimulus was compared.

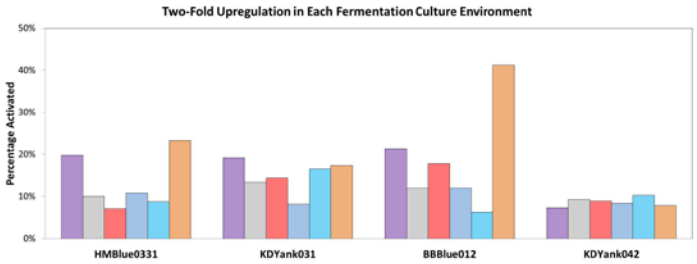

Figure 3. This figure compares the percentage of the respective organism's secondary metabolic profile that was upregulated by two-fold or greater for each stimulus to the control for each of the four samples. The colors of each of the bars for each sample correspond to the colors for each stimulus in Fig. 3A.

16S rRNA Genetic Identification

Table 1 shows the closest genetic relative for each organism through 16S rRNA sequence comparison to known organisms in the EZTaxon database (EZBioCloud). HMBlue0031 was determined to be most closely related to the actinomycete species Micromonospora coxensis, which is notable as actinomycetes have been known producers of valuable natural products.

Table 1. This table reveals the closest known relative to the sample organisms when the 16S rRNA sequences were compared using the EZTaxon database. The table shows the length of each 16S fragment, the similarity of the sample organism to the identified closest relative, and the completeness of the sequenced 16S fragment.

Organism Name	Length (bp)	Closest Relative	Similarity (%)	Completeness (%)
HMBlue0331	1416	Micromonospora coxensis	99.22	98.2
KDYank031	1447	Bascillus drentensis	99.01	98.1
BBBlue012	1264	Bradyrhizobium betae	92.89	89.5
KDYank042	703	Bascillus drentensis	82.53	47.8

DISCUSSION.

This study sought to utilize exposure of cave microorganisms to different stimuli to enhance secondary metabolite upregulation for natural product discovery. Extracts were selected from the data as candidates for compound isolation and characterization based on high upregulation of features detected from PCA and raw chromatogram analysis, which potentially allows for prediction of characteristics of the secondary metabolites like molecular weight.

PCA analysis of the HMBlue0331 sample organism highlighted four unique, distinct features that were selected as leads for future isolation, characterization, and assessment of bioactivity (Fig. 1). As this organism also produced dark brown extracts compared to the yellow-orange color of the liquid medium, the sample's extracts were visually distinguished and gained greater interest compared to others in the data set. As the T. pulmonis and R. wratislaviensis co-culture extracts for this sample showed high upregulation for the selected features and the visually interesting dark color, these extracts were primarily selected for initial attempts at isolation.

Furthermore, PCA analysis of the KDYank031, BBBlue012, and KDYank042 sample microorganisms highlighted additional features that were selected for future assessment (Fig. 2). It can be noted that in Figures 2G-2I, the T. pulmonis, R. wratislaviensis, and streptomycin-treated extracts showed high degrees of upregulation when compared to the control. This, combined with the similar trend that can be observed for the HMBlue0331 sample organism, provides stronger evidence that these stimuli could be highly useful for upregulation of secondary metabolite production and natural product discovery, reinforcing ideas in previous literature [5-7].

When the overall effect of the stimuli upon the sample's secondary metabolic profile was assessed, 6-41% of each organism's secondary metabolic profile was upregulated by two-fold or greater when exposed to each individual stimuli (Fig. 3). In total, 25-54% of each organism's total secondary metabolic profile was upregulated by two-fold or greater when responses to each stimulus were compared. It can be therefore concluded that exposure to these stimuli can activate previously silent biosynthetic pathways and increase secondary metabolite production, causing the generation or upregulation of novel compounds that could be potentially utilized for the production of medicinal drugs.

The HMBlue0331 organism is most closely related to Micromonospora coxensis (Table 1), the only identified actinomycete from the sample set. Actinomycetes have been known to produce secondary metabolites used in the production of medicinal drugs [4]. Furthermore, the Micromonospora genus has been known to produce antibiotic compounds, such as telomycin [10]. This strengthens the possibility that this organism could produce novel natural products with valuable medicinal purposes.

Actinomycetes have numerous secondary metabolite gene clusters, which are often silent [11]. With the demonstrated ability of these methods to upregulate the secondary metabolic profile of an actinomycete and other microorganisms, these methods for secondary biosynthetic pathway activation pose strong potential in their use for natural product discovery. Different methods for activating silent gene clusters have been developed and successfully utilized in natural product discovery, with several presented in this study. As PCA serves as a tool for comprehensive analysis of secondary metabolomes derived from microorganisms extracts, it has the advantage of selecting the leads for isolation based on their uniqueness, rather than abundance as it has been historically performed.

Future studies will attempt to isolate and characterize the selected compounds through size-exclusion chromatography, high-performance liquid chromatography, and mass spectrometry. Isolated compounds will be assessed for bioactivity through thin-layer chromatography. Additional attempts at isolation and assessment of other samples from caves such as Snail Shell Cave (Rockvale, TN) and Ellison's Cave (Walker County, GA), which were not assessed using LC/MS due to time constraints, will occur. Therefore, these extract will undergo the same analysis methods, to select candidates for isolation and characterization and assessment of bioactivity.

Based on the current results, these methods may accelerate natural product discovery and help to find novel compounds that could be used for the production of valuable medicinal drugs. Furthermore, these results strengthen the idea that cave ecosystems are apt environments for discovery of microorganisms that may yield secondary metabolites due to their extreme environments. These compounds could be useful in the alleviation of ailments and could therefore be highly valued by the medical industry.

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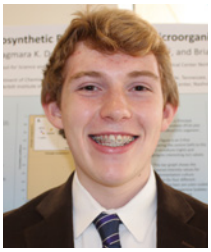

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