Genetically induced production of secondary metabolites in *Bacillus megaterium*

Majie C. Foster, Jay L. Brewster and P. Matthew Joyner
Natural Science Division, Pepperdine University, Malibu, California, USA

Abstract

Historically, the most important source of new antibiotic drug leads has been small organic compounds made by bacteria. Many antibiotics have been developed into pharmaceutical agents from these molecules (often called secondary metabolites) produced by soil bacteria. *Bacillus* species are soil bacteria known for producing various antimicrobials including gramicidin, bacitracin, surfactin, and others. *Bacillus megaterium* is a widely used model gram positive bacterium. Although there has been extensive research on this organism, little is known about its secondary metabolites. We hypothesized that the production of secondary metabolites in this organism could be induced by replacing promoters controlling the expression of genes within the identified clusters. In this study, gene clusters that are predicted to control the production of secondary metabolites were identified using the antiSMASH bioinformatics platform. Phenotypic changes in the secondary metabolite profile of *B. megaterium* were observed when culture conditions were varied indicating that target metabolites are accessible for chemical analysis. To increase production of targeted secondary metabolites, the native promoter of identified secondary metabolite gene clusters will be replaced by an inducible promoter using plasmid mediated chromosomal integration.

Background

Due to the energetic constraints of storing genetic information, bacteria typically maintain only genetic code that is critical for their survival and propagation in a changing environment. There is extensive evidence that many bacteria possess the genetic capability of producing many secondary metabolites that are never observed under laboratory culture conditions.[1-4] Therefore, gaining access to these cryptic secondary metabolites represents a great opportunity for expanding the known chemistry of previously studied bacteria. In this project we have used the widely studied bacterium *Bacillus megaterium* to explore new methods of accessing secondary metabolites that have not been previously identified from this species.

Hypothesis

1) The production of secondary metabolites in *B. megaterium* can be induced by altering the laboratory culture conditions of the bacterium.
2) The production of secondary metabolites in *B. megaterium* can be induced by replacing promoters that control the expression of genes associated with secondary metabolite biosynthesis.

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Results

1) Genomic data mining revealed that *B. megaterium* has the genetic potential to produce previously unreported secondary metabolites.

- Eight gene clusters were identified by the antiSMASH program as having potential to produce secondary metabolites.
- Two gene clusters (designated by *) were identified that are predicted to produce types of secondary metabolites that have not been previously reported from *B. megaterium*.

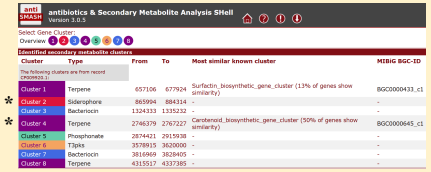

Figure 1. Results of analysis of *B. megaterium* genome using antiSMASH program.

2) Modulation of culture conditions indicates that previously unreported secondary metabolites from *B. megaterium* are accessible.

- Growth of *B. megaterium* on minimal defined media altered its secondary metabolite profile.
- Exposure of *B. megaterium* to light during incubation induced the production of a yellow pigment.

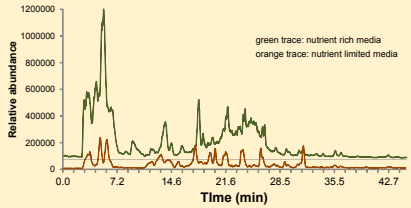

Figure 2. Comparison of *B. megaterium* metabolite profiles from liquid cultures grown in nutrient rich media (green) and nutrient limited media (orange).

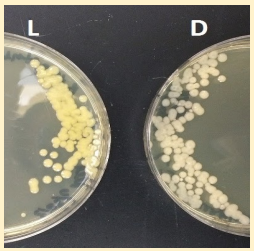

Figure 3. Comparison of *B. megaterium* colonies grown under fluorescent lights (left, L) and in the dark (right, D).

3) Silent gene clusters that are anticipated to control the production of secondary metabolites are accessible for genetic manipulation.

- A promoter replacement protocol has been designed using a Cambell-type integration method.
- Integration sequences from *B. megaterium* genomic DNA have been amplified using PCR.
- Initial attempt to assemble integration plasmid was not successful.

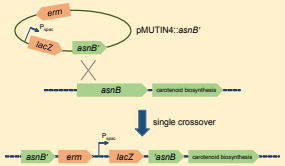

Figure 4. Diagram showing scheme for insertion of controllable promoter (P_{spac}) into *B. megaterium* chromosome.

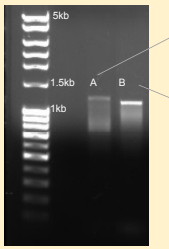

Figure 5. Integration sequences for targeted gene clusters were amplified by PCR from *B. megaterium* genomic DNA. Gel electrophoresis shows that amplification of targeted integration sequences were successful for both the carotenoid gene cluster (A, 1246 bp) and siderophore gene cluster (B, 1204 bp).

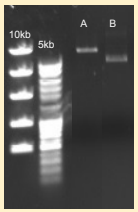

Figure 6. Ligation products of integration sequences into plasmids (pMUTIN4) were amplified in *E. coli* and analysed using restriction mapping. Gel electrophoresis shows ligation reactions were unsuccessful. Restriction fragments of 2466 bp and 7290 bp were expected in lane A, and a fragment of 9756 bp was expected in lane B.

Methods

General methods. LC-MS data were recorded on a Thermo Finnigan MAT LCQ ion-trap ESI mass spectrometer coupled to a Shimadzu LC system with a diode array detector (5 µm C18 column, 100 Å, 250 × 10.0 mm). Polymerase chain reaction (PCR) was performed using a Bio-Rad MODEL thermal cycler.
Genomic data mining. The antiSMASH platform[5] was used to analyze the chromosomal genomic sequence for *Bacillus megaterium* de Bary (ATCC14581). The genomic sequence was accessed from the NCBI GenBank database (accession ID CP009920.1).
Bacterial cultures. *B. megaterium* liquid cultures were inoculated in triplicate in either nutrient-rich tryptic soy broth medium or in a nutrient-poor defined broth medium in Erlenmeyer flasks. Defined media was prepared based on a published recipe.[6] For light/dark cultures, *B. megaterium* on tryptic soy agar petri plates were incubated at room temperature under either white fluorescent light or in the dark, for 48 hours.
Generation of metabolite profiles. Cultures were extracted with 100 mL ethyl acetate. The ethyl acetate was separated from polar contents using a separatory funnel and removed by rotary evaporation to generate a crude extract of each bacterial culture. The extract was re-dissolved in methanol for analysis using liquid chromatography with mass spectrometry (LCMS). A methanol-water gradient mobile phase was used (30-100% methanol over 45 minutes, 100% methanol for 25 minutes) with a 3 µm C18 150×4.6 mm column and a flow rate of 0.5 mL per minute.
PCR conditions and primers. Each reaction was performed using temperatures of 94° C, 64°C, and 72°C for the melt phase, annealing phase and extension phase, respectively. Primers were created using *B. megaterium* genomic sequences at -5 bp and ~1000 bp relative to the first gene within identified clusters, with the addition of restriction enzyme cut sites.

Conclusions and Future Directions

- *B. megaterium* possess genetic potential for making previously unreported secondary metabolites
- Secondary metabolites from *B. megaterium* are accessible for isolation and characterization
- Future work will seek to isolate and characterize secondary metabolites following successful promoter replacement for silent gene clutsters

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