PREDATION MEDIATED CARBON TURNOVER IN NUTRIENT-LIMITED CAVE ENVIRONMENTS

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PREDATION MEDIATED CARBON TURNOVER IN NUTRIENT-LIMITED CAVE ENVIRONMENTS

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. REVIEW OF LITERATURE</td>
<td>5</td>
</tr>
<tr>
<td>Cave Microorganisms</td>
<td>5</td>
</tr>
<tr>
<td>Carbon Cycling</td>
<td>7</td>
</tr>
<tr>
<td>Predatory Bacteria</td>
<td>8</td>
</tr>
<tr>
<td><em>Ensifer adhaerens</em>: Discovery and Identification</td>
<td>9</td>
</tr>
<tr>
<td>III. MATERIALS AND METHODS</td>
<td>13</td>
</tr>
<tr>
<td>Sample Site: Lechuguilla Cave</td>
<td>12</td>
</tr>
<tr>
<td>Collection of Bacterial Isolates</td>
<td>14</td>
</tr>
<tr>
<td>Bacterial Isolates</td>
<td>14</td>
</tr>
<tr>
<td>Cross-Streak and Tracking</td>
<td>15</td>
</tr>
<tr>
<td>Testing Optimal Predation Conditions</td>
<td>18</td>
</tr>
<tr>
<td>Single-Species Growth Curves Via Colony Forming Units</td>
<td>20</td>
</tr>
<tr>
<td>Single-Species Growth Curve Via Optical Density</td>
<td>21</td>
</tr>
<tr>
<td>Growth Curve Data Analysis</td>
<td>23</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Final Predation Assay Protocol</td>
<td>23</td>
</tr>
<tr>
<td>Determining Time of <em>Micrococcus luteus</em> Death via <em>Live/Dead</em> Staining</td>
<td>23</td>
</tr>
<tr>
<td>Stable Isotope Probing and Liquid Chromatography – Mass Spectrometry</td>
<td>25</td>
</tr>
<tr>
<td>Re-Testing LC524 Growth in Metal Base Minimal Media</td>
<td>27</td>
</tr>
<tr>
<td>Scanning Electron Microscopy</td>
<td>29</td>
</tr>
<tr>
<td><strong>IV. RESULTS AND DISCUSSION</strong></td>
<td>31</td>
</tr>
<tr>
<td>Bacterial Strains</td>
<td>31</td>
</tr>
<tr>
<td>Cross-Streak and Tracking</td>
<td>32</td>
</tr>
<tr>
<td>Optimal Predation Conditions for <em>Ensifer adhaerens</em> Strain LC11</td>
<td>41</td>
</tr>
<tr>
<td>LC11 Growth</td>
<td>48</td>
</tr>
<tr>
<td>LC524 Growth in Minimal Media and Glucose Utilization</td>
<td>50</td>
</tr>
<tr>
<td>Time of Death for LC524 Via Predation</td>
<td>58</td>
</tr>
<tr>
<td>Stable Isotope Probing, Carbon Turnover, and Data Analysis</td>
<td>63</td>
</tr>
<tr>
<td>Microscopy Observations</td>
<td>68</td>
</tr>
<tr>
<td><strong>V. CONCLUSIONS</strong></td>
<td>70</td>
</tr>
<tr>
<td><strong>LITERATURE CITED</strong></td>
<td>78</td>
</tr>
<tr>
<td><strong>APPENDICES</strong></td>
<td>82</td>
</tr>
<tr>
<td><strong>APPENDIX A: MEDIA AND SOLUTIONS</strong></td>
<td>83</td>
</tr>
<tr>
<td><strong>APPENDIX B: MINIMAL MEDIA</strong></td>
<td>84</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Single-Species Growth Media</td>
<td>22</td>
</tr>
<tr>
<td>3.2</td>
<td>Stable Isotope Probing Cultures</td>
<td>25</td>
</tr>
<tr>
<td>3.3</td>
<td>LC524 Media Test</td>
<td>28</td>
</tr>
<tr>
<td>4.1</td>
<td>Bacterial Strain Characteristics</td>
<td>31</td>
</tr>
<tr>
<td>4.2</td>
<td>Cross-Streak Results of LC11 with Lechuguilla Cave Strains</td>
<td>40</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.1</td>
<td>“Bar-like” structure or cytoplasmic bridge extended between <em>E. adhaerens</em> (ATCC 33213) cell (arrow) and prey cell (L) stained with uranyl acetate. 50,000x (Casida, 1982)</td>
<td>11</td>
</tr>
<tr>
<td>2.2</td>
<td>Attachment of <em>E. adhaerens</em> (ATCC 33212) (arrow) to <em>M. luteus</em> (darkly stained mass in center) on 1:10 BHI. Shows “picket-fence” arrangement of <em>E. adhaerens</em> cells around prey cells. Crystal violet stain. 3123x (Casida, 1982)</td>
<td>12</td>
</tr>
<tr>
<td>3.1</td>
<td>Cross-Streak Diagram</td>
<td>16</td>
</tr>
<tr>
<td>3.2</td>
<td>96-Well Plate Predation Assay</td>
<td>19</td>
</tr>
<tr>
<td>4.1</td>
<td>Cross-streak of LC524 and LC11 on 1:100 strength BHI at pH 6.0. Tracking is observed to the right (blue arrow) of the cross-streak as well as along (red arrows) the LC524 streak.</td>
<td>35</td>
</tr>
<tr>
<td>4.2</td>
<td>Cross-streak of LC524 and LC11 on 1:100 strength BHI at pH 8.0. Tracking is observed to the right (blue arrow) of the cross-streak as well as along (red arrow) the LC524 streak.</td>
<td>36</td>
</tr>
<tr>
<td>4.3</td>
<td>LIVE/DEAD stained images of LC11 and LC524 cross-streak on 1:100 strength BHI at pH 8.0 after one week of growth at 24°C. A) Sample taken from the intersection of the two streaks. B) Sample taken from the right of the cross-streak.</td>
<td>37</td>
</tr>
<tr>
<td>4.4</td>
<td>Parallel streaks (A-C) and an intersection cross-streak (D) of LC11 and LC524 on 1:100 strength BHI after one week of growth at 24°C demonstrating that LC11 must intersect with LC524 for tracking to be observed.</td>
<td>38</td>
</tr>
<tr>
<td>4.5</td>
<td>Cross-streaks of LC11 with LC54 (<em>E. adhaerens</em>) and LC79 (<em>Nocardia asteroids</em>) on 1:100 strength BHI after one week of growth at 24°C</td>
<td></td>
</tr>
</tbody>
</table>
At the intersection of each cross-streak a clearing of LC54 and LC79 can be observed along with tracking to the right of each intersection.

4.6 Gram stain showing the attachment of LC11 (Gram negative bacillus) to LC524 (Gram positive cocci) in a “picket-fence” arrangement (arrow) after 1 week of growth in 1:100 strength BHI at pH 6.0. 1000x.

4.7 For this graph the y-axis compares the change in predator cells to the change in prey cells from 12 to 48 hours while the x-axis shows the total population change. A greater increase in predator to prey cells compared to the total population for each media condition is demonstrated by points that are further up and to the right on the graph.

4.8 Line graphs showing the variation in CFUs/ml of LC11 and LC524 at different pH levels. (A) 15 mg L⁻¹ TOC at pH 6.0 with a ratio of 1:10 predator to prey. (B) 15 mg L⁻¹ TOC at pH 8.0 with a ratio of 1:10 predator to prey.

4.9 Line graphs showing the variation in CFUs/ml of LC11 and LC524 under the same nutrient conditions but different ratios of predator to prey. (A) 15 mg L⁻¹ TOC at pH 6.0 with a ratio of 1:10 predator to prey. (B) 15 mg L⁻¹ TOC at pH 6.0 with a ratio of 1:100 predator to prey.

4.10 Line graph showing the CFUs/ml of LC11 in full strength BHI with corresponding O.D. at 600 nm over 36 hrs at 24°C.

4.11 Graphs showing the CFUs/ml of LC524 in M9MM + A9 media solution with corresponding O.D. at 600 nm over 36 hrs. Top) TSB starting culture + 0.4% glucose; Middle) TSB starting culture and no glucose; Bottom) Washed starting culture and no glucose.

4.12 Line graph comparing the average O.D. at 600 nm of pre-washed LC524 in MBMM with and without 0.4% glucose along with the addition of various amounts (µl) of TSB over 48 hrs at 24°C.

4.13 Line graph comparing the O.D. at 600 nm of pre-washed LC524 in MBMM with and without 0.4% glucose along with the addition of various amounts (µl) of TSB over 48 hrs at 24°C. Each data point represents the average result of three replicates and error bars represent the standard deviation from the mean.

4.14 Graphs showing the O.D. at 600 nm of LC524 in 1:1000 strength BHI at pH 8.0 with a washed starting culture over 48 hrs at 24°C. Each data point represents the average result of three replicates and error bars represent the standard deviation from the mean.
4.15 Graph showing the O.D. at 600 nm of LC524 in 1:1000 strength BHI at pH 8.0 with a washed starter culture over 48 hrs at 24°C.............................57

4.16 LIVE/DEAD stained time-lapse images of the interaction between LC11 and LC524 in 1:1000 strength BHI at pH 8.0 over the course of 96 hours.
1000x......................................................................................................................................................61

4.17 LIVE/DEAD stained image of LC11 in 1:1000 strength BHI at pH 8.0. (A) After 24 hrs of growth in a slide-a-lyzer placed in a culture of LC524. (B) Grown singly in a test tube. 1000x........................................................................................................................................62

4.18 LC/MS excretome spectra for singly cultured LC524 (unlabeled) and LC524* (D-13C glucose isotopically labeled) at the same retention time.
(* indicates that LC524 was isotopically labeled with D-13C glucose)..................65

4.19 LC/MS excretome spectra comparing substrate abundance and mass at the same retention time between LC11, LC524, and LC11/LC524 co-culture. All samples are unlabeled........................................................................................................................................66

4.20 LC/MS excretome spectra comparing substrate abundance and mass at the same retention time between LC11, LC524*, and LC11/LC524* co-culture.
(* indicates that LC524 was isotopically labeled with D-13C glucose)..............67

4.21 SEM image showing biofilm formation surrounding LC11 and LC524 cells...69
CHAPTER I

INTRODUCTION

Predatory organisms are seen in all phyla, each having specialized traits and employing different mechanisms to efficiently make use of their prey (Hillesland et al., 2009; Pasternak et al., 2013). Even on a microscopic level predation is observed; however, our understanding of bacterial predation and its effect on the ecological development of a system is currently limited (Pasternak et al., 2013). Advances in technology have allowed researchers to decipher predatory bacterial genomes to understand predatory phenotypes and identify predatory microorganisms within an ecosystem (Pasternak et al., 2013). While genomic comparisons such as these begin to discern predatory strategies it does not address how predation activity affects the entire microbial community structure.

In particular, how bacterial predation influences carbon cycling and the identity of the carbon compounds released to the environment for uptake, has not been empirically described for any ecosystem dominated by bacteria, nor have any studies attempted to determine how such behavior could impact the microbial community. Furthermore, few studies have addressed the movement of carbon through a cave microbial ecosystem. One study did follow the movement of nutrients through microbial mats in cave streams using a nutrient spiraling method.
They observed microorganisms downstream incorporating byproducts produced by the upstream communities (Engel et al., 2010). This study, however, does not address how nutrients are cycled in communities where nutrients cannot be transported via the movement of water. How do microbial communities recycle nutrients that colonize cave walls or sediments deep within the cave system away from allochothonous inputs? In a system where nutrients, especially carbon are limiting identifying additional avenues such as, bacterial predation for the movement and turnover of nutrients will aid in understanding how a diverse group of microorganisms is capable of surviving in oligotrophic environments.

In order to begin addressing how carbon is recycled in nutrient-limited cave environments, we have isolated Ensifer adhaerens, a predatory bacterium from Lechuguilla Cave that obtains energy by attacking other bacteria. *We hypothesize that predation-mediated degradation of bacteria may provide a means of recycling carbon compounds.* This study attempts to describe the effects such predation activity could have on nutrient cycling in geologically isolated nutrient-limited microbial ecosystems.

Historically, cave systems were thought to be devoid of microbial life due to extremely low nutrient availability and isolation from surface-derived energy. In examining these environments through culture-dependent techniques and electron microscopy, microbiologists have however, realized that caves are in fact home to diverse microbial populations (Cunningham et al., 1995; Barton & Northup, 2007). The development of molecular-based techniques has expanded our ability to identify the microorganisms within these environments and determine what
microbial processes support them through functional gene analysis (Lavoie & Northup, 2001; Barton, 2006). By combining our understanding of the geochemical conditions in a cave with microbiological methods, researchers have begun to describe the important roles that microorganisms could play in cave ecosystem dynamics (Cunningham et al., 1995; Barton, 2006; Barton & Jurado, 2007; Barton & Northup, 2007). Nevertheless deciphering the interactions among cave microorganisms is still dependent on culture-based techniques to better understand the physiology of these interactions under the intense nutrient limitation of a cave environment.

Life relies on two strategies for energy acquisition and growth: autotrophy and heterotrophy. Autotrophy is the conversion of inorganic carbon to organic carbon using energy harnessed from inorganic sources, such as sunlight or reduced metals. Heterotrophy releases energy through the catabolic breakdown of complex organic compounds (Konhauser, 2007). In caves, the geologic isolation does not permit microorganisms to use sunlight energy for autotrophy (Barton & Northup, 2007), although alternative sources of energy, such as inorganic iron and sulfur found within cave water, rock, and sediment are available. Allochthonous organic input into the cave is generally limited and cave dwelling heterotrophs rely mainly on chemolithoautotrophic organisms to produce the organic compounds needed for growth (Cunningham et al., 1995; Engle, 2010). It remains unclear how this organic carbon is cycled back into the ecosystem for heterotrophic growth. Understanding the mechanisms of carbon turnover in these cave communities will lead to a better
understanding of how nutrients may flow through this simple, microbially dominated ecosystem.
CHAPTER II

REVIEW OF LITERATURE

Cave Microorganisms

Prior to the 1990s there was little recognition within the scientific community that any significant microbial populations existed in caves primarily due to a limited ability to culture representative species (Barton & Northup, 2007; Barton & Jurado, 2007). The cultured bacterial and fungal isolates that were identified were considered non-native species, transported into the cave via air currents, human activity, insects, or other vectors (Barton & Northup, 2007; Barton & Jurado, 2007). Yet one study (Cunningham et al. 1995) found extensive evidence of resident bacterial and fungal growth in Lechuguilla Cave, including fungi and bacteria associated with iron-, manganese-, and sulfur- secondary deposits. Notably these investigators reported population densities of bacteria to be $5 \times 10^5$ colony forming units per gram within corrosion residues (Cunningham et al., 1995). This study went on to identify over 90 bacterial and fungal species within sediment and water from the cave (Cunningham et al., 1995). These researchers hypothesized that primary producing autotrophic bacteria in ferromanganese deposits were providing organic matter to heterotrophic bacteria, potentially allowing for their continued colonization of subterranean habitats (Cunningham et al., 1995).
By the mid 1990s, advances in molecular biology and geochemical analysis permitted researchers to more thoroughly examine and identify microorganisms in geological environments (Barton & Northup, 2007). Microorganisms were found in great diversity, which was contradictory to the accepted ecological principles for the ecology of bacterial organisms where resources are limited (Barton & Jurado, 2007). Ecological principle dictates that under nutrient deprivation “complete competitors cannot coexist” (Hardin, 1960) and yet, high diversity is found in cave systems. While the exact reason for high diversity is still under debate it is believed that diversity is driven by a preference for mutualistic relationships in subterranean environments (Barton & Jurado, 2007).

A model for the flow of energy in nutrient-limiting microbial systems (Barton & Jurado, 2007) suggests that primary producing bacteria release secondary metabolites that are taken up by other organisms to fix nutrients within the environment. These nutrients are then cycled back to the primary producing bacteria resulting in the cyclic movement of energy through the system verses the traditional bottom-up approach (Barton & Jurado, 2007). Since the nutrients moving into this system are complex, it is speculated that one organism does not contain all the necessary metabolic reactions to sustain life; therefore, mutualistic associations among cave microbial communities provides a mechanism for overcoming nutrient-limitation resulting in high diversity (Barton & Jurado, 2007).
Carbon Cycling

Within soil, bacteria and fungi are responsible for the majority of decomposition of organic matter to CO₂ due to the production of enzymes capable of degrading large organic polymers (Nielson et al., 2011). In soils, bacteria have a constant supply of organic carbon from the movement of materials via rainfall, plant and leaf litter, and input from animal excrement (Hopkins & Dungait, 2010). As such, soil microbial decomposers are responsible for at least 50% of the total output of carbon from soil (Hopkins & Dungait, 2010).

The composition of a soil based microbial community is determined by the physical nature of the environment and different organic matter inputs (Brant et al., 2006). Some of the physical and chemical parameters that control the rate of decomposition include microbial community structure, quality and availability of substrates, temperature, acidity/alkalinity, and water content (Hopkins & Dungait, 2010). The rate of decomposition by these microorganisms will increase with higher temperatures (Hopkins & Dungait, 2010). Decomposers require oxygen and water to effectively break down soil organic matter therefore rates of organic carbon decomposition decrease deeper into soils as oxygen becomes less available (Hopkins & Dungait, 2010). Lastly, decomposition often occurs more readily under neutral conditions (Jenkinson & Rayner, 1977; Hopkins & Dungait, 2010).

To understand how organisms cycle nutrients through an ecosystem one must understand all the processes involved in mobilizing compounds, including fixing inorganic nutrients in a bioavailable form and subsequent mineralization through degradation and decomposition. Understanding the processes involved in
nutrient cycling can also aid in understanding the drive of microbial diversity, as different species can take advantage of different nutrient sources. Following such interaction using stable isotopes has allowed researchers to determine that the breakdown of organic compounds in soil is a complex process (Hopkins et al., 2005) yet, there is virtually no information about how microorganisms cycle nutrients including carbon, nitrogen, sulfur, and phosphorus in cave ecosystems (Engel, 2010).

**Predatory Bacteria**

Predatory bacteria are taxonomically diverse and employ a variety of different strategies to obtain energy from other living bacterial cells (Martin, 2002; Jurkevitch, 2007). They have been observed in a variety of environments, including water, soil, and caves, each employing different predation mechanisms (Menne, 1999; Martin, 2002; Jurkevitch, 2007). Predator-prey interactions play an integral role in the evolutionary and ecological development of biological communities, even among bacteria and archaea (Martin, 2002; Morgan et al., 2010); however the extent to which predatory bacteria influence microbial community structure, their involvement in nutrient cycling, and predation mechanisms are not completely understood (Martin, 2002).

Among predatory bacteria are the *Bdellovibrio* and *Bdellovibrio*-like organisms (BALOs). BALOs are obligate predators found in soil and water that employ a dimorphic life cycle, alternating between a free-living stage and a host dependent growth stage (Martin, 2002; Jurkevitch, 2007). Another well-studied predator is *Myxococcus xanthus*, which uses a “group” or “wolfpack” strategy for
predation. These bacteria use quorum sensing to secrete large quantities of antibiotics that degrade host cells leading to the availability of host derived nutrients (Martin, 2002; Jurkevitch, 2007). *E. adhaerens* is hypothesized to employ a cell-to-cell contact strategy for predation, adhering to the prey cell wall and causing damage to the host cell structure (Casida, 1982; Martin, 2002). *E. adhaerens* and its predation strategy are one of the least studied among predatory and pathogenic bacteria, and the exact mechanism of cell killing has not been determined.

**Ensifer adhaerens: Discover and Identification**

*E. adhaerens* was first isolated from soil in 1982 (Casida, 1982). Strain A, as it was designated, attached to and lysed *Micrococcus luteus*, a Gram positive Actinobacteria. After further review and phylogenetic analysis, Strain A was determined to be a new genus designated *Ensifer* or “sword bearer” because of its characteristic “bar-like” structure observed through electron microscopy when it is in close contact with prey cells (Casida, 1982). The species was termed *adhaerens* due to its “sticky” nature. *E. adhaerens* is not an obligate predator, and predation occurs mainly under low-nutrient conditions when a bar of unknown composition, darkly staining with osmium tetroxide in SEM preparations (Figure 2.1) and referred to as a cytoplasmic bridge, extends from the polar end of the predator to the prey cell (Casida, 1982; Martin, 2002). The purpose of this bar remains unknown, but it is believed that either the prey cell wall is punctured, allowing for the transfer of nutrients, or the bar is used solely for attachment to the prey cell (Martin, 2002; Jurkevitch, 2007). Electron micrograph images have shown *E.
E. adhaerens attaching end-on to the prey cell wall, forming a “picket-fence” arrangement (Figure 2.2) around target cells (Casida, 1982; Martin, 2002).

Since the discovery and identification of E. adhaerens, few studies have examined its predation activity in detail, or the genes responsible for predation. Rather, studies have focused on the classification and phylogeny of the species, demonstrating that E. adhaerens is closely related to members of the genus Sinorhizobium, a nitrogen-fixing and nodule forming bacterium within the Alphaproteobacteria (Family Rhizobiaceae) (Martin, 2002 & Young, 2003). Of the few studies that have been conducted on predation, Germida and Casida (1982) used indirect phage analysis to follow the predatory activity of E. adhaerens against other bacteria in soil samples (Casida, 1982; Martin, 2002).

In a similar study, Casida (1982) found that when prey was inoculated perpendicularly with E. adhaerens on most media types, E. adhaerens spread along and through the prey streak. He termed this phenomenon “tracking” and noted that E. adhaerens did not deviate from the prey culture line; however, for tracking to be observed, it was necessary for the cultures to intersect (1982). Tracking was not always associated with lysis except when cultured on low nutrient media (Casida, 1982), which is consistent with E. adhaerens as a non-obligate predator.
Figure 2.1. "Bar-like " structure or cytoplasmic bridge extended between *E. adhaerens* (ATCC 33212) cell (arrow) and prey cell (L) stained with uranyl acetate. 50,000x (Casida, 1982)
Figure 2.2. Attachment of *E. adhaerens* (ATCC 33212) (arrow) to *M. luteus* (darkly stained mass in center) on 1:10 BHI. Shows “picket-fence” arrangement of *E. adhaerens* cells around prey cells. Crystal violet stain. 3123x (Casida, 1982).
Sample Site: Lechuguilla Cave

Lechuguilla Cave, Carlsbad Caverns National Park, NM, formed through hypogenic speleogenesis over the last seven millions years via ascending, sulfuric acid-rich groundwater. The hypogenic nature of this system along with the aggressive nature of the sulfuric acid resulted in the formation of a deep (>500m) maze-like cave system (Cunningham et al., 1995; Polyak et al., 1998; Davis, 2000; Northup et al., 2003). Lechuguilla Cave is currently the fifth longest cave in the world and the deepest in the continental United States with more than 90% of the system located 300m or more below the surface (Cunningham et al., 1995; Northup et al., 2003). The environmental conditions of Lechuguilla Cave remain constant, with no diurnal cycle, no seasonal changes, constant humidity (99.9%) and a temperature of 19-20°C is maintained all year round (Northup, 2003; Bhullar et al., 2012).

Lechuguilla Cave is considered a pristine cave due to the protective nature of an overlying layer of impervious sandstone, the Yates Formation (DuChene, 2000), as well as its isolation from human activity by a gate that has controlled access since its discovery in 1986 (Bhullar et al., 2012). The cave is rich in sulfur, iron, and
manganese deposits, while the walls are composed primarily of carbonate (Cunningham et al., 1995; Lavoie & Northup, 2001; Barton & Northup, 2007). The geologic isolation of the Yates Formation limits the amount of allochthonous materials that can percolate into the system with surface water (< 0.02 mg L⁻¹ total organic carbon has been detected in the cave). Thus Lechuguilla Cave represents an oligotrophic environment (Cunningham et al., 1995; Bhullar et al., 2012) and pristine habitat for the study of microbial interactions under extreme nutrient limitation.

Collection of Bacterial Isolates

Bacterial isolates were collected and isolated from wall scrapings in Lechuguilla Cave (under NPS permit # CAVE-00031) located in Carlsbad Caverns National Park, New Mexico. Sites for collection were chosen based on geology, depth, and areas determined to have minimal human impact (Bhullar et al., 2012). National Park records indicates a maximum of 4-6 people have been within the collection areas. The average temperature at the sample site is 20°C, with 99.9% relative humidity and a measured pH of 8.0. The identity of these isolates was confirmed using PCR amplification of the 16S rRNA gene, DNA sequencing, and comparative phylogenetic analysis (Bhullar et al., 2012).

Bacterial Isolates

The bacterial isolates used in this study were the Gram negative bacillus, *Ensifer adhaerens* (LC11 and ATCC 33212) and the Gram positive cocci, *Micrococcus luteus* (LC524), the characteristics of which are summarized in Table 4.1. All strains were cultivated at 24°C on 50% tryptic soy agar or TSA (5 g L⁻¹ NaCl, 2.5 g L⁻¹,
K$_2$HPO$_4$, 17 g L$^{-1}$ enzymatic digests of casein, 3 g L$^{-1}$ enzymatic digests of soybean, 2.5 g L$^{-1}$ glucose with 1.7% noble agar; Fisher Scientific, Sparks, MD). All stock cultures were stored at -80°C in 50% tryptic soy broth (TSB) containing 30% glycerol.

Cross-Streaking and Tracking

To confirm that our cave strain LC11 demonstrated tracking similar to that observed with the soil isolated strain ATCC 33212 (Casida, 1982) it was cross-streaked against LC524 following the procedure outlined by Casida (1980). Dilutions of 1:10 and 1:100 strength brain heart infusion broth (BHI) [Difco, Detroit, MI] with 1.7% agar (Difco, Detroit, MI) were prepared at soil pH (6.0) and cave pH (8.0) using 0.1 M NaOH and 0.1 M HCl. Following Figure 3.1, LC524 was streaked as a single-line down the center of the agar plate. LC11 was then streaked perpendicular to the LC524 line with the two cultures intersecting in the center of the plate (Casida, 1980). All plates were incubated at 24°C for one to two weeks.
Figure 3.1. Cross-Streak Diagram
After one week of growth, a wet-mount was prepared by emulsifying a sample taken from the center of the cross-streak cultured on 1:100 strength BHI at pH 8.0 (Figure 4.2). The sample was stained using the LIVE/DEAD BacLight Cell Viability Kit (Invitrogen, Carlsbad, CA). This process was repeated on a sample taken from the right of the cross-streak (Figure 4.2; blue arrow). Both samples were visualized using fluorescent microscopy and photographed.

Several plates were streaked with parallel perpendicular streaks of LC11 and LC524 to observe the physiology of LC11 on solid media when in close proximity to prey, but not intersecting a streak of LC524. On 1:100 strength BHI LC11 was streaked perpendicular down the plate followed by a parallel streak of LC524 about 1 inch apart. A second plate was inoculated using the same method but the culture lines were streaked ½ inch apart. For the third plate the cultures were stuck only millimeters apart. A fourth plate was inoculated using the cross-streak method on 1:100 strength BHI as a visual comparison between the plates where cultures were streaked parallel verses cross-streaked. All plates were incubated at 24°C for one week.

In addition to testing LC11 and LC524, we also cross-stuck 42 strains (Table 4.2) isolated from Lechuguilla Cave (LC) with LC11 on 1:100 strength BHI to see if LC11 demonstrated tracking and/or predation of bacteria isolated from the same environment. LC strains were again streaked on the plate first followed by a perpendicular streak of LC11 from left to right moving through the LC strain inoculation line. All plates were cultured at 24°C for one week.
Testing Optimal Predation Conditions

To test optimal pH and nutrient concentration for predation activity all assays were carried out in BHI broth containing 6 g L\(^{-1}\) of brain and heart infusions, 6 g L\(^{-1}\) of peptone, and 3 g L\(^{-1}\) of dextrose for a total of 15 g L\(^{-1}\) of total organic carbon (TOC) at full strength preparation.

Optimal predation conditions including nutrient concentration and pH were determined using a 96-well plate assay (Techno Plastic Products AG, Trasadingen, Switzerland). For the first predation assay, twelve solutions of 1:100 (150 mg L\(^{-1}\) TOC) strength BHI was prepared with a pH range from 1 to 12 using 0.1 M NaOH and 0.1 M HCl. These media were autoclaved and steriley aliquoted across the first row of a 96-well plate, with a total volume of 200 μl, then serially diluted down the plate in a 1:10 ratio from 150 to 0.000015 mg L\(^{-1}\) TOC (Figure 3.2). Overnight cultures of LC11 and LC524 were prepared in 100 ml of 1:100 strength BHI broth with shaking (100 rpm) at 24°C. After overnight cultivation, 3 ml was removed from each culture and the optical density (O.D.) was measured at 600 nm using a HACH DR2800 spectrophotometer. Cultures were then diluted to a 1:1 ratio using 1:100 strength BHI broth. Using inoculating pins, the cultures were added to the 96-well plate in a 1:10 ratio (~ 5μl of starting culture). The plate was covered and incubated at 24°C without shaking for one week after which, 5 μl was removed, Gram stained, and visualized using an Olympus BX53F microscope (Tokyo, Japan).
Figure 3.2. 96-Well Plate Predation Assay

Media aliquoted across the first row (A1-A12)

1:10 dilution of media down plate from row A to H
($10^2 - 10^9$)
The second predation assay was carried out on a larger scale using test tubes. BHI broth at varying concentrations (150 mg L\(^{-1}\) TOC \(\sim 0.150 \mu g\) L\(^{-1}\) TOC) was prepared and adjusted from pH 1 to 12 using 0.1 M NaOH or 0.1 M HCl, aliquoted into individual test tubes (5 ml total volume) and autoclaved. LC11 and LC524 were grown separately with shaking (100 rpm), overnight at 24°C in 100 ml of 1:100 strength BHI broth (150 mg L\(^{-1}\) TOC). After 24 hours of growth, 3 ml was removed from each culture and the O.D. was measured. The cultures were diluted using 1:100 strength BHI to achieve ratios of 1:1, 1:10 and 1:100 predator to prey cells and added to the prepared test tubes. At time point 0 hr, 12 hr, 24 hr, 36 hr, and 48 hr, 200 μl of culture was removed to determine cell number via colony forming units (CFUs) for each condition. These results were plotted over time. This assay was repeated at pH 6.0, 6.5, 7.0, 7.5, and 8.0 with nutrient concentrations of 1.5, 15, and 150 mg L\(^{-1}\) TOC.

**Single-Species Growth Curves Via Colony Forming Units**

Overnight cultures of LC524 and LC11 were grown with shaking (150 rpm) at 24°C in 3 ml of full strength TSB and the O.D. was measured after which, 500 μl of LC524 overnight culture (O.D. \(\sim 1.00\)) was added to 50 ml of M9 minimal media (M9MM) (200 ml of 10X M9 salts, 2 ml 1 M MgSO\(_4\), 100 μl 1 M CaCl\(_2\) L\(^{-1}\); See also Appendix B) in a side-arm flask with 0.4% D-glucose (Difco, Detroit, MI) as the sole carbon source. A9 mineral solution (300 mg H\(_3\)BO\(_3\), 50 mg ZnCl\(_2\), 40 mg MnCl\(_2\) 4H\(_2\)O, 200 mg CoCl\(_2\), 10 mg CuCl\(_2\) 2H\(_2\)O, 20 mg NiCl\(_2\) 6H\(_2\)O, 30 mg NaMO\(_4\) L\(^{-1}\); See also Appendix A) was added with a final concentration of 1X to provide additional minerals for productive growth. Two controls were also run including 1) 500 μl of
LC524 overnight culture and 2) 500 µl of washed LC524 overnight culture. Both controls were added to 50 ml of M9MM plus A9 media solution.

In addition to the LC524 conditions an overnight culture of LC11 was grown with shaking (150 rpm) at 24°C in 3 ml of full strength BHI broth. The O.D. was measured and 100 µl was added to 50 ml of full strength BHI broth in a side-arm flask. All cultures were shaken (100 rpm) at 24°C. The O.D. for each LC524 condition and LC11 culture was checked at time zero and every four hours after for a total of 48 hours. For colony counting 100 µl was removed at each time point, diluted from $10^{-9} - 10^{-8}$, and spread onto 50% TSA. Using the recorded CFUs the total number of viable cells in the original culture for each species was calculated and plotted against O.D.

Single-Species Growth Curves Via Optical Density

Using a Versamax Microplate Reader (Molecular Devices, LLC.) single-species growth curves were produced for various conditions of LC524 in metal base minimal media (MBMM) [50 mM Hepes, 25 mM (NH$_4$)$_2$SO$_4$, adjusted to pH 7.0, 1 mM MgSO$_4$, 1 mM K$_2$HPO$_4$, 0.05% NaCl L$^{-1}$; See also Appendix B] (Table 3.1). For each condition 300 µl of test medium was aliquoted into each well and 5 µl of the corresponding starter culture was added. The plate was incubated at 24°C with shaking. The O.D. at 600 nm was measured every 5 minutes for a total of 24 or 48 hours with three replicates for each sample. Readings were stored using Softmax Pro Data Acquisition and Analysis Software (Molecular Devices, LLC) and the O.D. was plotted over time.
An additional growth curve was produced for LC524 singly in 1:1000 strength BHI broth at pH 8.0. This was done to confirm that over the course of the predation assay LC524 was dying from predation and not starvation in the nutrient-limiting media. An overnight culture of LC524 in 5 ml full strength TSB (150 rpm, 24°C) was washed three times with 1:1000 strength BHI broth at pH 8.0 (3500 rpm, 5 min) and re-suspended in 5 ml 1:1000 strength BHI broth at pH 8.0. In a side-arm flask, 25 ml of 1:1000 strength BHI broth at pH 8.0 was inoculated with 300 μl of the washed LC524 culture. All cultures were set-up in triplicate and grown with shaking (125 rpm) at 24°C. The O.D. was measured at 600 nm every 4 hours for 48 hours.

Table 3.1: Single-Species Growth Media

<table>
<thead>
<tr>
<th>Culture</th>
<th>Media</th>
<th>Single Carbon Source</th>
<th>Additional TSB</th>
<th>Total Volume</th>
<th>Starting Culture From</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC11</td>
<td>Full Strength TSB</td>
<td></td>
<td></td>
<td>300μl</td>
<td>TSB</td>
</tr>
<tr>
<td>LC11</td>
<td>1:1000 BHI pH 8.0</td>
<td></td>
<td></td>
<td>300μl</td>
<td>TSB</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>0.4% Glucose</td>
<td></td>
<td>300μl</td>
<td>TSB</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>0.4% Glucose</td>
<td></td>
<td>300μl</td>
<td>Pre-Washed</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td></td>
<td></td>
<td>300μl</td>
<td>TSB</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td></td>
<td>10μl</td>
<td>300μl</td>
<td>Pre-Washed</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>0.4% Glucose</td>
<td>1.0μl</td>
<td>300μl</td>
<td>Pre-Washed</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>0.4% Glucose</td>
<td>0.1μl</td>
<td>300μl</td>
<td>Pre-Washed</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>0.4% Glucose</td>
<td>0.01μl</td>
<td>300μl</td>
<td>Pre-Washed</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>10μl</td>
<td></td>
<td>300μl</td>
<td>Pre-Washed</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>1.0μl</td>
<td></td>
<td>300μl</td>
<td>Pre-Washed</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>0.1μl</td>
<td></td>
<td>300μl</td>
<td>Pre-Washed</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>0.01μl</td>
<td></td>
<td>300μl</td>
<td>Pre-Washed</td>
</tr>
</tbody>
</table>
Growth Curve Data Analysis

All growth curves including O.D. versus time, O.D. versus CFUs/ml, and time versus CFUs/ml were produced using R studio and ggplot2 (R studio, Boston, MA), a free computer program used for statistical computation and graphing.

Final Predation Assay Protocol

The final predation assay conditions required an overnight LC524 culture in full strength TSB washed three times with MBMM, and re-suspended in 5 ml MBMM. A 125 ml flask containing 50 ml MBMM + 0.4% D-glucose + 1.66 ml TSB was inoculated with 300 μl of washed LC524 overnight culture and grown with shaking (150 rpm) at 24°C for 48 hours. On day three 5 ml of full strength TSB was inoculated with LC11 and grown with shaking (150 rpm) overnight at 24°C. Lastly, on day four, the LC524 batch culture was spun down (3500 rpm for 15 min) and washed three times with MBMM to remove all excess carbon sources. It was then re-suspend in 50 ml 1:1000 strength BHI broth at pH 8.0. The O.D. was measured at 600 nm and diluted to 0.079 using 1:1000 strength BHI broth at pH 8.0. In a sterile 125 ml flask 22.5 ml of this culture was combined with 2.5 ml of LC11 overnight culture (O.D. ~0.1). The LC524/LC11 predation assay culture was left undisturbed at 24°C for 24 hours.

Determining Time of Micrococcus luteus Death Via LIVE/DEAD staining

The LIVE/DEAD BacLight Cell Viability Kit was used to microscopically visualize when the LC524 cells began to die via predation. For this method, SYTO 9, a green-fluorescent nucleic acid stain is contrasted with propidium iodide, a red-fluorescent nucleic acid stain that penetrates cells with damaged membranes.
Baclight immersion oil was added to the stains to prevent quenching of both fluorescent stains during visualization and exposure to light.

Stains were first prepared by adding 3 µl of SYTO 9 and 3 µl propidium iodide to 1 ml of baclight immersion oil in a 1.5 ml eppendorf tube. Then 1 ml of the LC524/LC11 predation assay culture was filtered onto a 25 mm anodisc (Whatman International Ltd., Dassel, Germany). The disc was placed onto a clean glass slide and ~10 µl of the SYTO 9/propidium iodide baclight immersion oil mixture was applied to the center of the disc. A cover slip was placed on top of the disc and each end was sealed. Stained cells were immediately visualized using a fluorescent microscope (Zeiss 510 META scanning laser confocal microscope with Argon and HeNe lasers) and photographed. This process was repeated every 24 hours for a total of 96 hours. The number of LC524 and LC11 cells was not counted but death of LC524 was determined by overall color changes from green to yellow, as the cell wall began to degrade and small amounts of propidium iodide moved into the cell, and finally to red with complete cell death.

In order to determine if cell-to-cell contact was causing LC11 to continuously stain red during visualization of the predation assay samples we used a slide-a-lyzer to separate the two cultures during growth. A slide-a-lyzer 10,000 MWCO, 0.5 – 3.0 ml capacity (Thermo Scientific, Rockford, IL) was used to separate LC524 and LC11 cultures during cultivation. The slide-a-lyzer was hydrated in 500 ml of 1:1000 strength BHI broth at pH 8.0. An overnight culture of LC11 in full strength TSB was prepared and 2.5 ml was added to 22.5 ml of 1:1000 strength BHI broth at pH 8.0. Of this mixture 3 ml was injected into the slide-a-lyzer and then placed into 500 ml
of a LC524 culture prepared according to the predation assay protocol. After 24 hours, 1 ml of LC11 was removed from the slide-a-lyzer and stained using the LIVE/DEAD BacLight Cell Viability Kit. The stained LC11 cells were visualized using a fluorescent microscope and photographed.

Stable Isotope Probing and Liquid Chromatography-Mass Spectrometry

Isotopically labeled D-\textsuperscript{13}C glucose (Omicron Biochemicals, Inc., South Bend, IN and Sigma Aldrich, St Louis, MO) was used to label LC524 in MBMM following the final predation assay protocol. The cultures outlined in Table 3.2 were prepared in triplicate according to the final predation assay protocol.

Table 3.2: Stable Isotope Probing Cultures

<table>
<thead>
<tr>
<th>Condition</th>
<th>Prey LC524</th>
<th>Predator LC11</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.5ml LC524 with 2.5ml TSB</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>25ml LC524* with 2.5ml TSB</td>
<td>2.5ml LC11 TSB overnight culture in 22.5ml 1:1000 BHI pH 8.0</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>2.5ml LC11 TSB overnight culture</td>
</tr>
<tr>
<td>4</td>
<td>22.5ml LC524</td>
<td>2.5ml LC11 TSB overnight culture</td>
</tr>
<tr>
<td>5</td>
<td>22.5ml LC524*</td>
<td>2.5ml LC11 TSB overnight culture</td>
</tr>
</tbody>
</table>

After incubation for 24 hours, each sample was transferred into separate 50 ml conical tubes (Corning, Tewksbury, MA) and centrifuged at 3500 rpm for 15 min to separate the media from the bacterial cells. The supernatant was removed from the cell pellet and filtered through a 0.22 μm filter (Millipore, Bedford, MA) to remove remaining cell debris.

The filtered supernatant was then transferred to an acid washed 250 ml round bottom flask, evaporated until about 1 ml of sample remained and transferred to a pre-weighed 19 x 65 mm screw thread sample vial. The remaining
sample was evaporated until all liquid was removed. Vials were weighed again and the total crude cell debris was calculated for each sample and re-suspended to obtain a final concentration of 200 mg/ml in ultra-pure water (Q-Milli Integral Water Purification System, Millipore Corporation, Billerica, MD). From each sample 50 μl was transferred into separate polyspring inserts within a 12 x 32 salanized clear target SC I-D vial (National Scientific, Rockwood, TN). For quality control 50 μl of each sample was combined into a separate eppendorf tube, mixed, and 50 μl was placed into a vial.

To lyse the bacterial cells and extract compounds containing the $^{13}$C isotope the cell pellet was re-suspended in ~ 1 ml methanol optima (Fisher Scientific, Sparks, MD). Tubes were shaken for 15 min then vortexed to ensure the entire pellet was in suspension and a full lysis of the bacterial cells occurred for each sample. The re-suspended pellets were then transferred to an acid-washed 250 ml round bottom flask, one for each sample and evaporated using Büchi rotovap R-200 attached to KNF laboport pump and heating bath B-490 at 35°C until about 1 ml of sample remained. This was transferred to a pre-weighed 19 x 65 mm screw thread sample vial (VWR, Radnor, PA) and evaporated until all liquid was removed. The cell pellet samples were stored at -80°C until liquid chromatography-mass spectrometry (LC/MS) analysis could be run.

The LC/MS was performed on a Thermo TSQ Quantum access MAX triple-quad (Thermo Scientific, Waltham, MA) equipped with a Surveyor PC pump and Thermo Pal autosampler. Metabolites were separated on a 250 mm x 4.60 mm Phenomenex C18 (5μm particle size) column and 3 mm I.D. C18 guard column. The
auto-injector sample tray was held at 4°C to minimize sample degradation. A sample size of 20 μl was injected from a sample with a concentration of 200 mg/ml. Chromatographic separation was achieved using a 29 minute gradient at a flow rate of 1 ml/min using a gradient mixer of 10 mM ammonium acetate in 95% water and 5% acetonitrile (mobile phase A) and 10 mM ammonium acetate in 5% water and 95% acetonitrile (mobile phase B). The mobile phase A was held at 100% for 1 minute, followed by a linear ramp to 100% B over a period of 29 minutes and held at 100% B for 15 minutes, followed by a 2 minute gradient to return the system to 100% A and allowed to equilibrate for 3 minutes.

Metabolites were analyzed using positive and negative mode electrospray ionization. Parameters for analysis include capillary voltage of 4.5 kV in positive mode and 3.0 kV in negative mode; tube lens offset was -133 in positive mode and 96 in negative mode. All other parameters were equal for positive and negative mode including source temperature of 275°C, scan time of 1 second, skimmer offset of 5, auxiliary nitrogen gas of 10, sheath nitrogen gas of 54, and spray current of 5 μA. Data was collected over a mass range of 150-2000 Da in centroid mode and evaluated using Thermo Xcalibur 64 bit software (Materials and Methods for LC/MS provided by Cynthia Ruth McNees, Vanderbilt University).

Re-Testing LC524 Growth in Metal Base Minimal Media

Following LC/MS analysis it was determined that LC524 had not incorporated the $^{13}$C-glucose into its biomolecules most likely because it did not switch from metabolizing TSB to the labeled glucose. We therefore needed to establish new single-species growth curves for LC524 in MBMM to determine the
lowest required amount of TSB to provide essential amino acids and encourage initial growth while allowing the cells to switch to metabolizing the labeled glucose. Overnight cultures of LC524 were grown shaking (150 rpm) at 24°C in 5 ml of full strength TSB. The media types outlined in Table 3.3 were set-up in triplicate. The LC524 overnight cultures were washed three times with MBMM and 300 μl was added to 50 ml of each media condition. After inoculation the O.D. was measured and the cultures were grown with shaking (125 rpm) at 24°C, with 3 ml removed every four hours up to 24 hours to measure O.D followed by sampling every 12 hours for a total of 48 hours. The O.D. was then plotted over time for each media condition.

Table 3.3. LC524 Media Test

<table>
<thead>
<tr>
<th>Culture</th>
<th>Media</th>
<th>Single Carbon Source</th>
<th>Additional TSB Added</th>
<th>Total Volume</th>
<th>Starting Culture From</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>0.4% Glucose</td>
<td>160μl</td>
<td>50ml</td>
<td>Pre-Washed</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>0.4% Glucose</td>
<td>16μl</td>
<td>50ml</td>
<td>Pre-Washed</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>0.4% Glucose</td>
<td>1.6μl</td>
<td>50ml</td>
<td>Pre-Washed</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>-</td>
<td>160μl</td>
<td>50ml</td>
<td>Pre-Washed</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>-</td>
<td>16μl</td>
<td>50ml</td>
<td>Pre-Washed</td>
</tr>
<tr>
<td>LC524</td>
<td>MBMM</td>
<td>-</td>
<td>1.6μl</td>
<td>50ml</td>
<td>Pre-Washed</td>
</tr>
</tbody>
</table>

In order to add the prey and predator cells together in the correct ratios we needed to establish the number of viable cells present at a measured O.D. for the new LC524 minimal media conditions. Following analysis of the above growth curves, MBMM + 0.4% D-glucose + 160 μl TSB (Table 3.2) was set-up in triplicate as the amount of TSB was deemed to provide enough nutrients to encourage initial growth but allow turnover to metabolizing 13C-glucose. An overnight culture of LC524 was grown shaking (150 rpm) at 24°C then washed three times with MBMM.
Following the wash step, 300 µl of the washed LC524 overnight culture was added to 50 ml of MBMM + 0.4% D-glucose + 160 µl TSB. The O.D. was measured at time zero and every four hours after for 24 hours followed by a measurement at 36 and 48 hours. For colony counting 100 µl was removed at each time point, diluted and spread onto 50% TSA. The recorded CFUs for the culture was calculated and plotted over the recorded O.D.

**Scanning Electron Microscopy**

We used scanning electron microscopy (SEM) in an attempt to visualize the predation activity of LC11 on LC524 including the “picket-face” arrangement and the “bar-like” structure. A predation assay culture was spun down at 3500 rpm for 15 minutes. The supernatant was removed and the pellet was re-suspended in equal volumes 2% gluteraldehyde (CH₂(CH₂CHO)₂) (Electron Microscopy Sciences, Hatfield, PA) and 0.1 M cacodylic acid sodium salt (C₂H₆AsO₂Na) (Ladd Research Industries, Williston, VT) pH 7.2 followed by fixation for 15 minutes at room temperature (24°C). The sample was again spun down and the supernatant was removed, before washing in 0.1 M C₂H₆AsO₂Na pH 6.8 and re-suspension in equal parts 0.1 M C₂H₆AsO₂Na pH 7.2 and osmium tetroxide (OsO₄) (Electron Microscopy Sciences, Hatfield, PA) followed by fixation for 15 minutes at room temperature (24°C). The samples were then washed three times with distilled water to remove excess OsO₄. A square section of filter paper was folded and saturated with water. The sample pellet was placed in the center of the filter paper, sealed, placed in a glass vial, and covered with water. The vial was then placed in an acetone desiccator and stored at 4°C overnight to allow for the molecular exchange of water.
for acetone. The following day 100% acetone was added drop wise to the vial until all water was exchanged for acetone and the sample was submerged in 100% acetone before critical point drying (Emitech k850, Quorum Technologies Ltd., Kent, United Kingdom). The sample was then sputter coated (Polaron Equipment Ltd. SEM Coating Unit E5000, Watford, England) with gold-palladium (Polaron Equipment Ltd. E5000-314B TS54 Au/Pd Target, Watford, England) and viewed using SEM under high vacuum (FEI Quanta 200 environmental scanning electron microscope).
CHAPTER IV

RESULTS AND DISCUSSION

Bacterial Strains

*E. adhaerens* LC11 grows best on 1:10 and 1:100 BHI at 24°C. When streaked on 1:10 BHI LC11 appears grayish to white, circular, convex, slimy, moist, and opaque to translucent. On 50% TSA LC11 appears extremely mucoid and slimy possibly due to the increase in the salt content of TSA. LC524, our *M. luteus* cave strain, appears small, circular, slightly raised, and yellow in color on 50% TSA.

Table 4.1 Bacterial Strain Characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th><em>Ensifer adhaerens</em></th>
<th><em>Micrococcus luteus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain Designation</td>
<td>LC11 and ATCC 33212</td>
<td>LC524</td>
</tr>
<tr>
<td>Predator/Prey</td>
<td>Predator</td>
<td>Prey</td>
</tr>
<tr>
<td>Gram Reaction</td>
<td>Gram negative</td>
<td>Gram positive</td>
</tr>
<tr>
<td>Morphology</td>
<td>Bacillus (single or in pairs)</td>
<td>Cocci (tetrads)</td>
</tr>
<tr>
<td>Appearance on 50% TSA</td>
<td>Mucoid, white to tan colonies</td>
<td>Round, yellow colonies</td>
</tr>
</tbody>
</table>
Cross-Streak and Tracking

To determine if any physiological adaptations have occurred between our cave strain and the ATCC soil strain we used the techniques described by Casida (1980, 1982) and compared tracking behaviors at different pH values, one to reflect the soil (pH 6.0) and one to reflect the cave (pH 8.0) environment. Tracking was observed within one week of growth at pH 6.0 (Figure 4.1) and pH 8.0 (Figure 4.2) on 1:10 and 1:100 strength BHI. LC11 can be seen growing along the LC524 streak from where the two cultures intersect near the center of the plate. Both cultures can be seen growing to the right of the cross-streak, which may be due to the fact that the LC11 culture line was streaked from left to right through the LC524 culture line. LC11 also appears to be growing along and outward from this line.

A visual inspection of the plate showed little clearing of the prey culture line; therefore we wanted to microscopically observe whether LC11 was killing LC524 via predation activity or if the two were co-existing together. LIVE/DEAD staining analysis of the sample taken from the center of the cross-streak revealed that the majority of the prey cells were alive, fluorescing green (Figure 4.3A) and there was little to no interaction with the predator, LC11. The prey cells from the right of the cross-streak fluoresced yellow and red indicating death for this strain (Figure 4.3B) and there was an increase in the interaction between LC11 and LC524 cells with the majority of the LC11 cells surrounding the prey cells. These results indicated that LC11 was preying upon LC524; however, predation was occurring mainly to the right of the cross-streak intersection for reasons unknown.
In Casida’s original work on *E. adhaerens* ATCC 33212 (1980, 1982) he noted that *E. adhaerens* had to physically intersect with the prey species in order for tracking and predation to be observed. To confirm that our cave strain, LC11, demonstrated the same behavior we inoculated several plates with cultures of LC11 and LC524 but this time the culture lines were parallel to one another and did not touch or intersect at any point along the streak. Figure 4.4 A-C demonstrates that LC11 does not show tracking along the prey streak unless it makes contact or intersects with LC524 (Figure 4.4D).

We were curious to know if LC11 showed predatory activity or tracking behavior with any of the other species isolated from the same cave. In addition to LC11 and LC524, we isolated and identified a number of other species from geologically diverse locations and environments within Lechuguilla Cave, varying mainly in the amount of potential energy. Figure 4.5 shows the cross-streak results of LC54 and LC79, two strains isolated from Lechuguilla Cave, with the cave strain *E. adhaerens* LC11. On these plates there was an observable clearing of the LC54 and LC79 cultures where they intersect with LC11, which showed continuous growth through the cross-streak. These results demonstrate that LC11 is capable of preying upon members outside of the family *Micrococciaceae*, as well as preying upon itself (LC54) in times of great nutrient limitation. Table 4.2 outlines the cultures that LC11 demonstrated tracking along prey when streaked on low nutrient media, of which there were 11 out of a total of 42 cultures tested. In general, the species that LC11 tracked along are classified, either as *Proteobacteria* or *Actinobacteria* and many have been isolated from soil and water. It appears that *E. adhaerens*
demonstrates tracking mainly with secondary metabolite producers or those capable of nitrogen fixation or iron chelation. *E. adhaerens* is a nitrogen fixing bacterium and may target specific bacteria in order to reduce competition for resources or to evade harmful secondary metabolites including degrading antibiotics.
Figure 4.1. Cross-streak of LC524 and LC11 on 1:100 strength BHI at pH 6.0. Tracking is observed to the right (blue arrow) of the cross-streak as well as along (red arrows) the LC524 streak.
Figure 4.2. Cross-streak of LC524 and LC11 on 1:100 strength BHI at pH 8.0. Tracking is observed to the right (blue arrow) of the cross-streak as well as along (red arrow) the LC524 streak.
Figure 4.3. LIVE/DEAD stained images of LC11 and LC524 cross-streak on 1:100 strength BHI at pH 8.0 after one week of growth at 24°C. A) Sample taken from the intersection of the two streaks. B) Sample taken from the right of the cross-streak.
Figure 4.4. Parallel streaks (A-C) and an intersection cross-streak (D) of LC11 and LC524 on 1:100 strength BHI after one week of growth at 24°C demonstrating that LC11 must intersect with LC524 for tracking and predation to be observed.
Figure 4.5. Cross-streaks of LC11 with LC54 (*E. adhaerens*) and LC79 (*Nocardia asteroides*) on 1:100 strength BHI after one week of growth at 24°C. At the intersection of each cross-streak a clearing of LC54 and LC79 growth can be observed along with tracking to the right of each intersection.
<table>
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Optimal Predation Conditions for *Ensifer adhaerens* Strain LC11

Since *E. adhaerens* is not an obligate predator and will only hunt under low nutrient conditions we had to first establish the optimal nutrient concentration and pH level that would promote predation activity. A previous study by Casida (1982) found that *E. adhaerens* ATCC 33212 would predate at pH 6.0-6.5 in 1:10 and 1:100 strength BHI. We used this information as a starting point for testing the optimal nutrient concentration and pH level for our cave strain LC11 while taking into account the fact that caves have a higher pH and less available carbon than soil.

To determine these conditions, we began by culturing the predation assays in 96-well plates, which allowed us to test a large number of conditions at one time. Microscopic analysis revealed that no predation occurred at a pH below 5.0 and in many instances no viable cells could be recovered or visualized using microscopy techniques, most likely due to the increased acidity of the media. At pH 9.0 and above, visible growth (in the form of turbidity) in the media was observed but microscopic analysis revealed no indication of predation. The “picket-fence” arrangement described by Casida was observed in a Gram stain of an LC11 and LC524 culture after one week of growth in 1:100 strength BHI at pH 6.0 (Figure 4.6). The LC11 cells can be seen attaching end-wise to LC524 as well as lining up side-by-side in the “picket-fence” arrangement, which Casida deemed was indicative of predation activity (Figure 4.6). Following this assay we decided to implement a colony counting assay to better determine optimal predation conditions including the change in predator to prey ratios over time.
Figure 4.6. Gram stain showing the attachment of LC11 (Gram negative bacillus) to LC524 (Gram positive cocci) in a “picket-fence” arrangement (arrow) after 1 week of growth in 1:100 strength BHI at pH 6.0. 1000x
After revising our methods to use test tubes instead of 96-well plates for the colony counting assay, we examined the influence of nutrient concentration and pH on predation. We carried out the predation assays under a range of conditions from pH 1 through pH 12. Under these conditions using direct CFU counts we only observed predation under a pH range of 6-8 with a TOC of 0.15-150 mg L⁻¹.

Continuing to use direct CFU counts, we then examined predation at pH 6.0, 6.5, 7.0, 7.5, and 8.0 (Figure 4.7). A comparison of all the results shows that the greatest change between predator and prey cells occurred at pH 8.0 with 15 mg L⁻¹ of TOC; however there were not enough replicates for this data to be statically significant.

We also graphed the results of the direct CFU counts independently for pH 6.0 and pH 8.0 at the same nutrient concentration of 15 mg L⁻¹ of TOC. These graphs demonstrated that at pH 6.0 there was an increase in the number of predator cells over prey by 30 hours (Figure 4.8A) as seen by the increase of predator cells with a correlated decreased in prey cells over time. The predator population exceeded the prey population much earlier under pH 8.0 conditions and there was also a more dramatic increase in population size (Figure 4.8B).

Along with determining the optimal nutrient concentrations and pH levels for predation we also wanted to examine the ratios of predator to prey that allowed for observable predation activity. Predator to prey ratios of 1:1, 1:10, and 1:100 were examined under various concentrations of carbon and pH levels. When grown at 15 mg TOC L⁻¹ at pH 6.0, with a ratio of 1:10 predator to prey CFUs, there was an increase in the number of predator CFUs and a decrease in the number of prey following inoculation (Figure 4.9A). A similar trend was observed at pH 6.0 with a
ratio of 1:100 predator to prey CFUs; however, the shift in population size of the predator to prey took much longer, exceeding 48 hours (Figure 4.9B) most likely due to the increased difference in starting culture cell number between the predator and prey.
Figure 4.7. For this graph the y-axis compares the change in predator cells to the change in prey cells from 12 to 48 hours while the x-axis shows the total population change. A greater increase in predator to prey cells compared to the total population for each media condition is demonstrated by points that are further up and to the right on the graph.
Figure 4.8. Line graphs showing the variation in CFUs/ml of LC11 and LC524 at different pH levels. (A) 15 mg L⁻¹ TOC at pH 6.0 with a ratio of 1:10 predator to prey. (B) 15 mg L⁻¹ TOC at pH 8.0 with a ratio of 1:10 predator to prey.
Figure 4.9. Line graphs showing the variation in CFUs/ml of LC11 and LC524 under the same nutrient conditions but different ratios of predator to prey. (A) 15 mg L\(^{-1}\) TOC at pH 6.0 with a ratio of 1:10 predator to prey. (B) 15 mg L\(^{-1}\) TOC at pH 6.0 with a ratio of 1:100 predator to prey.
LC11 GROWTH

A close analysis of the above predator to prey graphs (Figure 4.7, 4.8, and 4.9) revealed that adding the predator and prey cultures in a 1:1, 1:10, and 1:100 ratio using diluted starting cultures was not in fact a reliable method for observing predation via colony counting and we were not able to obtain consistent results. Realizing that the O.D. and correlating cells numbers were different for LC11 and LC524 in the starting medium we developed single-species growth curves using direct colony counts compared with O.D. measurements. By measuring the O.D. at regular time intervals along with colony counting we could calculate how many cells were present at a particular O.D.

After 24 hours a culture of LC11 in full strength TSB with an O.D. (600 nm) of 0.1 contained ~ 1 x 10^8 CFUs/ml (Figure 4.10), although this value could be low as LC11 tends to clump in broth media. Using the growth curve generated from this experiment we could use the O.D. of a culture of LC11 grown in full strength TSB and determine how many viable cells are present.
Figure 4.10. Line graph showing the CFUs/ml of LC11 in full strength BHI with corresponding O.D. at 600 nm over 36 hrs at 24°C.
LC524 Growth in Minimal Media and Glucose Utilization

We needed to ensure that LC524 could metabolize glucose in order to use D-\(^{13}\)C glucose to isotopically label biomolecules during stable isotope probing, which will aid us in following the movement of carbon through the system. To test this we inoculated 5 ml of M9MM with 0.4% glucose and grew it at 24°C with shaking. After 16 hours the culture appeared turbid and slight flocculation was observed.

In order to establish correct predator to prey ratios we needed to determine how many LC524 cells were present per ml during growth. We accomplished this through colony counting. After 24 hours of LC524 growth in M9MM + 0.4% glucose, the culture had an O.D. of 0.09 which correlated to \(~ 1 \times 10^6\) CFUs/ml (Figure 4.11); however a precipitate was observed that could have affected the O.D. measurements. The precipitation appeared pink in color and was attributed to the A9 mineral solution. In addition to this, the colonies that grew on 50% TSA became smaller in size indicating that LC524 was generating smaller cells in order to grow in this media. As we could not use the information from this data to determine growth we began cultivating LC524 in MBMM.

While no mineral precipitation was observed when LC524 was grown in MBMM, it was noted that without the addition of TSB LC524 did not reach exponential growth even after 24 hours of cultivation. To determine how much TSB was needed to give LC524 the required amino acids for growth, but to also ensure that cells switched over to metabolizing glucose, varied amounts of TSB were added. It was essential not to add too much TSB, as the LC524 cells would metabolize the
TSB rather than glucose and it was critical that LC524 incorporate D-\textsuperscript{13}C glucose in order to track the movement of carbon using stable isotope probing.

We initially believed that the addition of 10μl of TSB to 300μl of total MBMM was sufficient to encourage growth, but still allow for the change to metabolizing glucose (Figure 4.12); however after analysis of our LC/MS data we found that the cultured LC524 had not metabolically incorporated D-\textsuperscript{13}C glucose into biomolecules (discussed in more detail later), indicating that LC524 growth was dependent on TSB. The initial test of determining how much TSB was required to encourage growth was done in a 300 μl tube, while the LC524 was grown in 50 ml for the predation assay; therefore a scaling error may be the reason why LC524 did not switch to metabolizing glucose.

The second trial of LC524 grown in MBMM was performed in 50 ml MBMM over 48 hours. This demonstrated that 160 μl of TSB added to 50 ml of MBMM was sufficient to encourage initial growth of LC524 but still allow it to metabolize D-\textsuperscript{13}C glucose (Figure 4.13). After 48 hours the culture containing 160 μl TSB in addition to the 0.4% glucose had continued to grow while the culture containing only TSB had not. The conditions containing TSB but no glucose demonstrated little to no growth as there was no carbon source once the TSB was consumed. The conditions containing 1.6 μl TSB along with glucose showed minimal growth as well as 16 μl of TSB with glucose.

Since we changed the culture medium for LC524 we had to determine how many LC524 cells would be present per ml under the new conditions in order to add the predator and prey cells in the correct ratio. A colony counting assay was
performed over 48 hours under the above conditions. At an O.D. of 0.1 there was between 1 x 10^7 and 1 x 10^8 cells/ml (Figure 4.14). Throughout the course of the experiment, in MBMM the colonies maintained a uniform size indicating that they were not starving, but growing successfully in the media and utilizing glucose as a carbon source; however we do not have LC/MS data to confirm this.

*Micrococcus luteus* is known to grow under nutrient-limiting conditions, entering into a dormant state without the production of endospores when nutrients become scarce (Young, 2010). A cave strain should also be adapted to nutrient-limiting conditions and be capable of surviving on low amounts of available carbon.

While we were confident that LC524 was dying due to predation activity we wanted to confirm that LC524 was capable of growing singly in the nutrient-limiting predation media, 1:1000 strength BHI broth at pH 8.0, since it contained only 15 mg L\(^{-1}\) of TOC. If the prey was not able to survive long enough in the media for predation to occur we would not be able to establish predation activity or determine if carbon turnover was occurring due to predation by LC11. By tracking the O.D. of LC524 in the predation media over time we found that a washed starting culture had an average O.D. of 0.031 (Figure 4.15). This data indicates that LC524 is capable of slowly growing in the nutrient-limited predation media for at least 48 hours and major death of the cells during the predation assay is most likely due to predation by LC11 then starvation.
Figure 4.11. Graphs showing the CFUs/ml of LC524 in M9MM + A9 media solution with corresponding O.D. at 600 nm over 36 hrs. Top) TSB starting culture + 0.4% glucose; Middle) TSB starting culture and no glucose; Bottom) Washed starting culture and no glucose.
Figure 4.12. Line graph comparing the average O.D. at 600 nm of pre-washed LC524 in MBMM with (solid line) and without (dashed line) 0.4% glucose along with the addition of various amounts (μl) of TSB over 48 hrs at 24°C.
Figure 4.13. Line graph comparing the O.D. at 600 nm of pre-washed LC524 in MBMM with and without 0.4% glucose along with the addition of various amounts (μl) of TSB over 48 hrs at 24°C. Each data point represents the average result of three replicates and error bars represent the standard deviation from the mean.
Figure 4.14. Line graph showing the CFUs/ml of LC524 in 50 ml MBMM with 0.4% glucose and 160 μl TSB with corresponding O.D. at 600 nm over 48 hrs at 24°C.
Figure 4.15. Graphs showing the O.D. at 600 nm of LC524 in 1:1000 strength BHI at pH 8.0 with a washed starting culture over 48 hrs at 24°C. Each data point represents the average result of three replicates and error bars represent the standard deviation from the mean.
Time of Death for LC524 Via Predation

While we were originally using cell counts to determine how the LC524 cell population declined during predation, we were unable to get accurate counts because LC11 appeared to form a biofilm around the prey cell, making quantitative analysis difficult. Thus if the cells are stuck together they would not be counted as individual cells in a CFU assay and the number obtained would not be representative of the entire culture. We therefore did not feel confident that our colony counting assay was an accurate way to determine time of death for LC524. Nonetheless, since LC11 and LC524 have different cellular morphologies we could use a staining technique that would allow us to microscopically confirm predation activity and time of death for LC524.

While most staining methods cannot distinguish between a live cell and a dead cell, the LIVE/DEAD staining technique can through the use of specific stains where live cells will stain green and dead or damaged cells will stain red. Figure 4.16 shows time-lapse images of the LIVE/DEAD stained co-culture of LC11 and LC524 in the predation media, 1:1000 strength BHI broth at pH 8.0. At time 0 hr both LC11 and LC524 stained green, with the total population of LC524 cells being higher than that of LC11. After 24 hours, the LC11 cells were observed attaching to and associating with LC524 cells. At this time point LC524 cells began to turn yellow indicating that their cell wall was damaged and small amounts of propidium iodide were penetrating into the cell. Between 48 and 96 hours the red fluorescence of LC524 cells began to increase as cell wall damage continued most likely due to predation activity. With an increase in the number of red-staining LC524 cells, we
also saw an overall increase in the number of LC11 cells, indicating that they were surviving on carbon and energy sources released from the dead LC524 cells.

While propidium iodide is made to stain cells with damaged cell walls, we noticed that when LC11 cells were associated with or attached to LC524 cells they also stained red (Figure 4.16, time point 24 hours) while generally staining green when not associated (Figure 4.16, time point 0 hr & Figure 4.17B). The red staining was not due to death because the CFU counts did not drop through the course of the experiment. To determine if this phenomenon was due to direct cell contact with LC524 we separated the two cultures using a dialysis membrane, which allowed the movement of small molecules between cultures but did not allow direct cell-to-cell contact. This assay demonstrated that LC11 was not turning red during the LIVE/DEAD staining assay when they were unable to come into contact with prey (Figure 4.17A). In addition to this, microscopic analysis of LIVE/DEAD stained LC11 grown singly in the predation media as well as plate counts indicated LC11 cells were viable and cell numbers were increasing.

These findings suggest that the red staining of LC11 occurs as a result of direct cell-to-cell contact with LC524. It is believed that LC11 produces the “bar-like” structure only when in close proximity to prey cells most likely in an effort to attach to the prey organism in preparation for predation and consumption of host derived nutrients. When this structure is produced we believe it may result in small pores or openings within the cell membrane as the “bar-like” structure extends outward from the polar ends toward the prey cell. These openings may provide an avenue where propidium iodide could enter the predator cell. If LC11 produces this
structure during predation in order to attach to prey cells it would explain why we saw the majority of LC11 cells staining red when in direct cell contact with LC524.
Figure 4.16. LIVE/DEAD stained time-lapse images of the interaction between LC11 (Bacillus) and LC524 (Cocci) in 1:1000 strength BHI at pH 8.0 over the course of 96 hours. 1000x
Figure 4.17. LIVE/DEAD stained image of LC11 in 1:1000 strength BHI at pH 8.0. (A) After 24 hrs in a slide-a-lyzer placed in a culture of LC524. (B) Grown singly in a test tube. 1000x
Stable Isotope Probing, Carbon Turnover, and Data Analysis

Once optimal predation conditions were established we employed stable isotope probing (SIP) to track the movement of carbon throughout the predation process. To identify compounds containing the carbon isotope we used LC/MS, a technique that separates high molecular weight compounds based on mass and charge. Figures 4.18-4.20 illustrate the results of the LC/MS analysis of the predation assay SIP samples. The top spectra (shown in black) are the result of PDA analysis, the middle or red spectra are the result of the positive mode (positive ions) and the green spectra are the result of the negative mode (negative ions). All spectra are from the same retention time of 0 to 30 min and the height of the peak correlates to relative abundance.

Unfortunately, analysis of the LC/MS data revealed that LC524 did not incorporate the D-13C glucose, as there was not a quantitatively relevant shift in the mass of the peaks between the labeled and unlabeled prey excretome (Figure 4.18). As a result we could not use the C-13 isotopes as an indicator of carbon turnover. Instead we looked for peak differences between the predator excretome, the prey excretome, and the predator/prey co-culture excretome. This was done for the unlabeled (Figure 4.19) and the labeled samples (Figure 4.20).

Even though there was no significant incorporation of the glucose isotope there were slight differences in the peaks for the predator, the prey, and the co-culture excretome. We cannot definitively conclude that these differences are carbon-containing compounds being recycled through predation, but we can conclude that there are different compounds being produced when the bacteria are
being cultured singly versus together. This can be seen along the spectra for Figure 4.19, especially for the peaks between two and ten minutes. Within this time frame the relative abundance of each compound differs between all three conditions and in general compounds in the co-culture are slightly lighter than compounds from the single species excretome. This trend was consistent for the samples outlined in Figure 4.20 including the predator, the labeled prey, and the predator/labeled prey co-culture. While this assay will need to be repeated to confirm these results and identify potentially released carbon, this experiment did show that co-culturing a predator and prey species does lead to the production of different compounds.
Figure 4.18. LC/MS excretome spectra for singly cultured LC524 (unlabeled) and LC524* (D^{-13}C glucose isotopically labeled) at the same retention time.

(* indicates that LC524 was isotopically labeled with D^{-13}C glucose)
Figure 4.19. LC/MS excretome spectra comparing substrate abundance and mass at the same retention time between LC11, LC524, and LC11/LC524 co-culture. All samples are unlabeled.
Figure 4.20. LC/MS excretome spectra comparing substrate abundance and mass at the same retention time between LC11, LC524*, and LC11/LC524* co-culture. (* indicates that LC524 was isotopically labeled with D-13C glucose)
Microscopic Observations

Light microscopy, LIVE/DEAD staining, and SEM images demonstrated extensive biofilm production surrounding LC11 and LC524 cells, with LC11 most likely responsible for the biofilm production (Figure 4.21). While further research is required to understand the exact mechanism of *E. adhaerens* predation activity, biofilm production could be a result of predation activity or production of secondary metabolites useful during predation and the killing of host cells.
Figure 4.21. SEM image showing biofilm formation surrounding LC11 and LC524 cells.
CHAPTER V

CONCLUSIONS

During our cultivation studies we isolated *E. adhaerens* from numerous oligotrophic cave systems across the United States, including Lechuguilla Cave in New Mexico (Bhullar *et al*., 2012), Wind Cave in South Dakota, and Jack Bradley Cave in Tennessee (Pemberton *et al*., 2005). The presence of *E. adhaerens* in these geologically diverse caves amongst others suggests that cave-isolated *Ensifers* may play an important role in cave ecosystem dynamics. To date, this is the first study of a cave isolated strain of *E. adhaerens* and the role this species may have in carbon turnover. Although we were not able to definitively demonstrate carbon turnover by *Ensifer*, we were able to provide four significant physiological findings about a cave strain of *E. adhaerens*.

First, we were able to demonstrate the similarities and differences between the cave isolated strain *E. adhaerens* LC11 and the soil isolated strain *E. adhaerens* ATCC 33212. Casida’s initial identification of *E. adhaerens* laid much of the foundation for understanding the physiology of *E. adhaerens* and provided detailed information about predation requirements. Comparing our findings with Casida’s (1982) we demonstrated that LC11 and *E. adhaerens* ATCC 33212 are similar in their ability to track along a prey culture on a solid medium with tracking and
predation observed only when the predator and prey cultures intersect (Figure 4.4). This was important considering the differences we found in the nutrient requirements and pH levels suitable to predation activity between soil and cave strains. It also points to the idea that *E. adhaerens* requires direct cell-to-cell contact with the prey species in order for its specific predation mechanism to be effective.

These findings suggest that LC11 has adapted to the cave environment, which is supported by *E. adhaerens* cave isolates prey more readily under cave conditions (pH 8.0, Figure 4.7 and 4.8), while the soil-isolated strain, *E. adhaerens* ATCC 33212 preys at a pH of 6.0-6.5 (Casida, 1982). It is possible that *E. adhaerens* requires a specific pH in order to maintain an interaction with its host or to stabilize protein structures. Adaptation to the cave environment is further supported by predation occurring under lower nutrient conditions (Figure 4.7 and 4.8) of 15 mg L\(^{-1}\) TOC. Casida observed a similar trend where *E. adhaerens* ATCC 33212 would predate only under starved conditions including 1:10 and 1:100 strength BHI (1982). These observations confirm Casida's original finding that *E. adhaerens* is a non-obligate predator, searching for prey only when nutrients become scarce (1982; 1988). These observations also provide useful information for cultivating *E. adhaerens* under conditions favorable to predatory activity in order to study various physiological interactions.

Under the conditions tested we also observed LC11 cells organizing into the “picket-fence” arrangement, which Casida deemed indicative of predation (Casida, 1982). This demonstrated that LC11 and *E. adhaerens* ATCC 33212 most likely utilize the same predation strategy of direct cell-to-cell contact; however the exact
predation mechanism and purpose of the cytoplasmic bridge remains unclear. While we observed the “picket-fence” arrangement we did not observe the “bar-like” structure or cytoplasmic bridge using microscopic techniques. Several reviewers have made reference to the “bar-like” structure Casida observed with electron microscopy, but no other researchers including us have observed this structure during microscopic analysis of *E. adhaerens* (Casida, 1982; Martin, 2002; Jurkevitch, 2007). The lack of an observable bar-like structure and the similarity in structure to the “bar” observed by Casida (1982) may suggest that this structure is in fact an artifact of the osmium tetroxide staining technique used for electron microscopy.

The second significant finding of this study was that LC11 was found to demonstrate tracking against other species isolated from Lechuguilla Cave. In general the majority of the microorganisms LC11 tracked along were classified as *Actinobacteria* including *Streptomyces anulatus* and *Dietzia maris*, *Alphaproteobacteria* including *Sphingobium yanoikuyae*, *Sphingomonas pseudosanguinis*, *Tetrathiobacter kashmirensis*, *Brevundimonas aurantiaca*, or *Gammaproteobacteria* including *Pseudoxanthomonas mexicanas* (Table 4.2). Many of these species are capable of nitrogen-fixation (Al-Mailem *et al.*, 2010; Videira *et al.*, 2009; Rasolomampianina *et al.*, 2005), an energetically expensive mechanism of acquiring nitrogen, although *E. adhaerens* is also a nitrogen-fixing bacterium common in the root nodules of plants (Balkwell, 2005). One explanation for why LC11 demonstrated tracking with nitrogen-fixing organisms may be because
E. adhaerens prefers to obtain nitrogen from other organisms and fix nitrogen themselves as a last resort.

The majority of the tested Lechuguilla Cave strains, including the Actinobacteria have been studied for their ability to produce secondary metabolites (Holmalahti et al., 1998; Krastel et al., 2002). Why E. adhaerens targets organisms that can produce secondary metabolites may be to decrease competition for host derived nutrients in the area as well as potential prey targets. Casida even claimed that some bacteria control the types of bacteria present within a population, which could be the case here with a predatory bacterium (1988). While LC11 merely tracked along the majority of the cultures tested it was found to clear the Actinobacteria, Nocardia asteroides (LC79), a saprophytic microorganisms found to colonize soil and water habitats throughout the world (Saubolle & Sussland, 2003). In addition to their ability to biosynthetically produce secondary metabolites, Nocardia sp. are also iron-chelating bacteria Feistner & Beaman, 1987). Similar to LC11 tracking along nitrogen-fixing bacteria, it may have targeted N. asteroides as a potential source of iron. From this research it is clear that LC11 tends to predate upon Actinobacteria and those capable of nitrogen-fixation or iron chelating; however, this should be verified with additional organisms

A study by Zeph and Casida (1986) on predatory bacteria in soil found that in general Gram-negative predatory bacteria were at the top of the predatory bacterial hierarchy. They also observed the trend of Gram negative predators attacking both Gram positive and Gram negative species including the Actinomycetes; therefore, they concluded that Gram negative predator species could play a role in determining
the species type and population within the community (Zeph & Casida, 1986). This could begin to explain why we observed LC11 tracking along some of the organisms that were Gram positive. Further research needs to be carried out to fully understand the scope of desired targets and the purpose behind tracking behaviors.

Thirdly, along with N. asteroides, LC11 was found to predate upon another isolate of E. adhaerens, LC54, which was a surprising result. We believe that LC11 and LC54 are in fact two different species of Ensifer. If this is the case we postulate that LC11 and LC54 have specific territories where they “hunt” for prey. When one Ensifer species crosses into the territory of another, it is capable of predating upon the invading Ensifer by competitive exclusion (Hardin, 1960). Predator-on-predator predation has been found among soil bacteria but only when the predators occupy different genera, thereby creating a bacterial hierarchy within the present bacterial community (Zeph & Casida, 1986; Casida, 1988). For example, Agromyces ramosus, a non-obligate bacterial predator found in high numbers among soil samples has been shown to be susceptible to predation by other predatory bacteria (Zeph & Casida, 1986; Casida, 1988). This same study found E. adhaerens ATCC 33212 susceptible to predation by other predatory bacteria as well (Zeph & Casida, 1986); however, the results we observed where an Ensifer isolate attacked another Ensifer has not been reported. This demonstrates an interesting ecological finding for predatory bacteria and warrants further study to understand how this type of predation could affect predatory bacteria as well as the resulting prey community.

Finally, the main objective of this study was to determine if predation by E. adhaerens LC11 on a prey species would result in a pathway for carbon turnover.
Our preliminary results suggested that under the co-cultured conditions, different metabolites are produced by *E. adhaerens*, which could be due to predation activity (Figure 4.19 and 4.20) (Pasternak *et al.*, 2013). Continued work using the revised LC524 cultivation conditions and predation assay along with SIP will hopefully reveal how carbon is cycled through this system and help explain how cave microorganisms survive under nutrient-limiting conditions.

The research presented on the interactions between *E. adhaerens* LC11 and its targeted prey species has opened a number of avenues for future research and discussion. One such avenue would be continuing to examine predation as a mechanism for carbon turnover in cave environments by utilizing SIP and mass spectrometry. Demonstrating carbon turnover via predation activity would illustrate a niche that could be exploited by other bacteria within the community for the uptake of limiting nutrients. Analysis of the excretome produced after predation activity may also reveal the production of secondary metabolites. These metabolites could be extracted and identified to help us understand the compounds *Ensifer* employs to degrade their target and obtain desired nutrients.

Additional studies could be conducted on the putative “bar-like” structure including determining the mechanism of production, composition, and purpose along with its potential involvement in LC11 cells staining red when in direct cell-to-cell contact with LC524. Improved analysis of electron micrograph images, perhaps using Field-Emission microscopy would not only aid us in developing a better understanding of the ability of *Ensifer* to kill prey, but also provide information
about a mechanism that has only been described in a single publication (Casida, 1982).

Additional experiments could continue to cross-streak *E. adhaerens* isolates with other isolates collected from Lechuguilla Cave. In a future study, cross-streaking could be reversed to see if tracking behaviors differ depending on which organism is applied to the plate first, or streaking two host species on a plate along with LC11 to see if *E. adhaerens* demonstrates prey selection, preferring to predate or track along one species before another. Such information could help us understand which nutrients *E. adhaerens* prefers to obtain from a host, what species it targets, and how this behavior could affect the surrounding community. It may also lead to experiment that deciphers how *E. adhaerens* identifies its targets.

One of the most interesting findings of this study was that LC11 was shown to predate on other isolates of *E. adhaerens* from the same cave. A number of studies could be generated from this result. First, different isolates of *E. adhaerens* could be cross-streaked under various nutrient-limiting conditions to see which isolate predates on the other if at all. This information could be combined with collection location to determine if there is a correlation between which organism predates on the other, location within the cave, and level of available nutrients within that area. Second, genomic analysis could be conducted to determine percent similarity between the different isolates to determine if they are in fact different species. Third, experiments could be generated to determine if *E. adhaerens* is capable of self-recognition and if so why does it predate upon other *E. adhaerens* isolates (could it be a result of competitive exclusion?).
With this study we were able to learn a great deal about a cave isolated strain of *E. adhaerens*, including several surprising results that have generated additional questions about this organism’s physiology. We were able to further our knowledge about a cave isolated non-obligate bacterial predator and understand its requirements for predation. By understanding the physiology of *Ensifer* we can better understand how they obtain nutrients from the environment and other species, and how the carbon cycle turns under near starving conditions. Results such as these could demonstrate an ecological need to maintain the presence of predatory bacteria in caves.
LITERATURE CITED


APPENDICES
APPENDIX A

MEDIA AND SOLUTIONS

Tryptic Soy Broth (TSB)

Contents: 16.6% sodium chloride, 8.3% potassium phosphate, enzymatic digests of casein, enzymatic digests of soybean, 8.3% dextrose (for Tryptic Soy Agar add 1.7% agar)

Company: Fisher Scientific, Sparks, MD

Brain Heart Infusion (BHI)

Contents: 6.0g L⁻¹ brain heart, infusion from (solids), 6.0g L⁻¹ peptic digest of animal tissue, 5.0g L⁻¹ sodium chloride, 3.0g L⁻¹ dextrose, 14.5g L⁻¹ peptic digest of gelatin, 2.5g L⁻¹ disodium phosphate

Total Organic Carbon: 6g L⁻¹ of brain and heart infusions from solid, 6g L⁻¹ of peptic digests of animal tissue, and 3g L⁻¹ of dextrose for a total of 15g L⁻¹ of total organic carbon (TOC) at full strength preparation

Company: Difco, Detroit, MI

A9 media solution

Contents (1X): 300mg H₃BO₃, 50mg ZnCl₂, 40mg MnCl₂ 4H₂O, 200mg CoCl₂, 10mg CuCl₂ 2H₂O, 20mg NiCl₂ 6H₂O, 30mg NaMO₄ L⁻¹
APPENDIX B

MINIMAL MEDIA

M9 Minimal Media (M9MM) Recipe

- Aliquot 800ml H₂O and add
  - 64g Na₂HPO₄·7H₂O
  - 15g KH₂PO₄
  - 2.5g NaCl
  - 5.0g NH₄Cl
  - Stir until dissolved
  - Adjust to 1000ml with distilled H₂O
  - Sterilize by autoclaving

- Measure ~700ml of distilled H₂O (sterile)
- Add 200ml of M9 salts
- Add 2ml of 1M MgSO₄ (filter sterilize)
- Add 20 ml of 20% glucose (filter sterilize)
- Add 100μl of 1M CaCl₂ (filter sterilize)

- Adjust to 1000ml with sterile distilled H₂O

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M9 Salts

Carbon Source

Final M9 Minimal Media
Metal Base Minimal Media (MBMM) Recipe

**Base Media:**
- 50mM Hapes
- 25mM (NH₄)₂SO₄
- Adjust to pH 7.0

Add carbon source before inoculation:
- 1mM MgSO₄
- 1mM K₂HPO₄
- 0.05% NaCl
- Filter sterilize

**Final Metal Base Minimal Media**
- Add glucose to 0.4% final concentration