# INVESTIGATION OF ANTIBIOTIC RESISTANCE IN ISOLATED LECHUGUILLA CAVE STRAINS

# INVESTIGATION OF ANTIBIOTIC RESISTANCE IN ISOLATED LECHUGUILLA CAVE STRAINS

By

Kirandeep Bhullar, B.Sc.

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AUTHOR: Kirandeep Bhullar, B.Sc. (McMaster University)
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## Abstract

Antibiotic resistance is often linked to human use of antibiotics. However, antibiotics and antibiotic biosynthetic pathways have been evolving for millions of years suggesting that antibiotic resistance is an ancient phenomenon. As of now, there has been no systematic survey of environmental microbes proven to exist in the absence of human influence and Lechuguilla cave offers such environment. Resistance diversity in strains isolated from this cave was analyzed by a phenotypic screen against a panel of 26 different antibiotics. Resistant strains were further investigated through determination of minimal inhibitory concentration (MIC) and inactivation studies. Of particular interest was strain LC044 (*Brachybacterium paraconglomeratum*), observed to inactivate macrolide antibiotics by phosphorylation. Genome sequencing and bioinformatics (BLAST analysis) identified a putative macrolide phosphotransferase (MPH) in strain LC044 and biochemical characterization of the purified recombinant protein confirmed its macrolide inactivating properties. To investigate if characterized MPH was unique to cave isolate, available terrestrial *Brachybacterium faecium* DSM 4810 genome was mined for presence of MPH-like protein. The top hit to the MPH from LC044 (a protein with 282 amino acids and 72% identity) was heterologously expressed and purified. Complete biochemical analysis of this enzyme revealed (i) MPH-activity, despite its annotation as aminoglycoside phosphotransferase (APH), and (ii) no significant differences in substrate specificities or kinetic parameters between these two enzymes suggesting that these two enzymes were equally effective resistance enzymes. This work

highlights the prevalence of antibiotic resistance in a pristine, cave ecosystem and provides further support for the theory that antibiotic resistance is everywhere. Furthermore, the *mph* resistance determinant found in cave isolate and closely related terrestrial isolate show homology to clinical *mph* genes, suggesting that environmental *mph* genes could have served as reservoir of clinical determinants.

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# Table of Contents

Table of Contents

## List of Figures



## List of Tables

## List of abbreviations

| | |
|---|---|
| AAC | Aminoglycoside Acetyltransferase |
| ANT | Aminoglycoside Nucleotidyltransferase |
| APH | Aminoglycoside Phosphotransferase |
| ATCC | American Type Culture Collection |
| ATP | Adenosine Triphosphate |
| BLAST | Basic Local Alignment Research Tool |
| bp | base pair |
| CLSI | Clinical and Laboratory Standards Institute |
| Da | Dalton |
| DHFR | Dihydrofolate Reductase |
| DHPS | Deoxyhypusine Synthase |
| EDTA | ethylenediaminetetraacetic acid |
| *ere* | erythromycin resistance esterase |
| *erm* | erythromycin methyltransferase |
| FDA | Food and Drug Administration |
| GTP | Guanosine Triphosphate |
| LB | Luria Broth |
| LC | Lechuguilla Cave |
| LC-MS | Liquid Chromatography–Mass Spectrometry |
| LDH | Lactate Dehydrogenase |
| Lin | Lincosamide Adenylytransferase |
| *m/z* | mass-to-charge ratio |
| MDR | Multidrug Resistance |
| *mef* | macrolide efflux |
| mgt | macrolide glycosyltransferase |
| MIC | Minimal Inhibitory Concentration |
| MLS | Macrolide Lincosamide Streptogramin B |
| mph | macrolide phosphotransferase |
| MPH | Macrolide Phosphotransferase |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| NADH | Nicotinamide Adenine Dinucleotide Hydride |
| Ni-NTA | Nickel-Nitrilotriacetic acid |
| NMR | Nuclear Magnetic Resonance |
| PBP | Penicillin Binding Protein |
| PEP | Phosphoenol Pyruvate |
| PK | Pyruvate Kinase |
| PMSF | phenylmethanesulfonylfluoride |
| ppm | parts per million |
| SDS-PAGE | Sodium dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| TE | Tris-EDTA |

| | |
|---|---|
| TSB | Tryptic Soy Broth |
| VRE | Vancomycin resistant *Enterococci* |

# CHAPTER 1. INTRODUCTION

## *1.1 Increasing Antibiotic Resistance: A Problem*

Antibiotics are one of the greatest advances in modern medicine. Since their discovery in 20$^{th}$ century, they have been used for treating as well as preventing bacterial infections, and ultimately saving numerous lives. The period between 1945 and 1960 is often referred to as "The Golden Era of Antibiotic Discovery", since most of the currently used antibiotics were discovered during this period. But soon after antibiotics were introduced in clinics, the emergence of antibiotic resistant bacteria was seen, making antibiotic resistance one of the greatest problems in the health care sector (1, 2).

Over the course of the last few decades, there has been a significant increase in the prevalence of antibiotic-resistant bacteria. Resistance is on the rise in pathogenic bacteria and there is an increase in the range and diversity of resistant microorganisms (3, 4). It is challenging our ability to combat bacterial infections that are highly prevalent in clinical settings. Additionally, the emergence of multidrug-resistant (MDR) bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), MDR *Acinetobacter baumanni, Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* poses a serious threat to human health, as these "super-bugs" are inevitably resistant to most of the available therapies (1, 5, 6).

Therefore, it is extremely important to understand the origin, evolution and scope of antibiotic resistance. Understanding these concepts will be crucial for achieving a broader perspective on the problem of antibiotic resistance and help the fight against resistant-microorganisms.

### *1.2 Link between Environmental Resistance and Clinical Resistance*

The big questions facing the scientific community are where does antibiotic resistance come from and what can we do to understand its origin? In recent years, interesting insights into the environmental origin of antibiotic resistance have been provided (7, 8, 9). There is growing evidence to suggest that many resistance genes evolved in non-clinical settings (e.g. in natural ecosystems) and there is an increasing appreciation for the contribution of environmental microorganisms towards the problem of antibiotic resistance (8, 9, 10). The presence of resistance in environments not exposed to human use of antibiotics and the identification of resistant determinants which have not evolved under high antibiotic selection pressure (as experienced by clinical isolates) is of great interest for future prospects of studying antibiotic resistance origin and evolution. Unfortunately, much of the focus has been placed on clinical isolates limiting our knowledge of environmental reservoirs of resistance (9). Currently, there is little unambiguous evidence to suggest direct transmission of antibiotic resistance genes from environmental bacteria to pathogenic bacteria. However, studying the environmental reservoirs of resistance can provide useful insights into resistance mechanisms seen in the clinics (2). Discussed below are the well-studied examples of antibiotic-resistance genes which likely originated in environmental microorganisms and have become important antibiotic resistance mechanisms in clinical isolate.

*Case study 1: Aminoglycoside modifying enzymes*

Aminoglycosides are a class of antibiotics that function by binding to the 30S ribosome of bacteria and interrupting protein synthesis. Aminoglycosides are active

primarily against aerobic Gram-negative and Gram-positive bacteria and are widely used for treating severe abdominal and urinary tract infections and endocarditis (11). Enzymatic inactivation of aminoglycosides by intracellular enzymes is an important resistance mechanism in both environmental bacteria and pathogens (12). One of the first studies to show this relationship identified enzymes in species of environmental *Streptomyces* that catalyzed similar reactions to the aminoglycoside modifying enzymes in Gram-negative pathogens such as *P. aeruginosa* and *Escherichia coli* (13). This finding suggested that environmental bacteria could be a potential origin of this clinical mechanism of aminoglycoside resistance itself as environmental bacteria such as *Streptomyces* spp. are known to produce secondary metabolites such as antibiotics, and thus are capable of evolving resistance mechanisms for self-protection (13, 14, 15). This makes natural habitats interesting for studying antibiotic resistance.

*Case Study 2: Vancomycin resistance mechanism*

Vancomycin is a glycopeptide antibiotic used as last line of defense against various life threatening Gram-positive infections. Vancomycin works by binding to D-alanyl-D- alanine portion of the peptidoglycan precursor, thereby inhibiting the cell wall synthesis (16). Resistance to vancomycin is mediated by changes in peptidoglycan structure which can be attributed to the presence of the *vanH*, *vanA* and *vanX* (*vanHAX*) resistance cassette (16). The striking homology between resistance enzymes VanA found in the producer organism *Streptomyces toyocaensis* NRRL 15009 and enzymes found on a transposable element in vancomycin-resistant *Enterococcus faecium* provides further support for the environmental origin of resistance genes (17, 18). It has been established

that *vanHAX* cluster is conserved among pathogenic, environmental non-glycopeptide producers and glycopeptide producer organisms, suggesting a common ancestral origin (17).

*Case Study 3: β-lactamases of the CTX-M family*

CTX-M β-lactamases ($Bla_{CTX-M}$) are extended spectrum enzymes which confer resistance to oxyimino β -lactam antibiotics such as cefotaxime and are widely found in nosocomial as well as community acquired pathogens (19). These enzymes are chromosomally encoded in the environmental bacteria genus *Kluyvera,* an enterobacterial species, which are thought to be a possible reservoir of the CTX-M β-lactamases in pathogenic isolates (e.g. 99% amino acid identity to plasmid-mediated CTX-M from *E. coli*) (20, 21). Additionally, it has been shown that $bla_{CTX-M}$ genes can be mobilized from *Kluyvera* into *E. coli* (20). This provides further support for the environmental origin of resistance elements and suggests a link between environmental and clinical resistance elements.

### *1.3 Prevalence of Antibiotic Resistance in Nature*

Various natural habitats have been investigated for the prevalence of antibiotic resistance bacteria. A systematic study was conducted by D'Costa *et al* , where a collection of approximately 500 *Streptomyces* from soil samples from diverse locations were screened for antibiotic resistance. On average, soil microorganisms were found to be resistant to 7-8 antibiotics and antibiotic resistance was seen to natural, semi-synthetic as well as synthetic antibiotics. This study demonstrated that the soil environment is a reservoir of antibiotic resistance elements (22).

Recent studies have revealed the presence of antibiotic resistance in bacteria coming from natural habitats such as deep terrestrial subsurfaces, where anthropogenic exposure of antibiotics is unlikely which suggests that antibiotic resistance could be a natural state for microorganisms (23, 24, 25). Although these works do not discount the possibility of contamination from the sampling process or from other sources (e.g. influx from other hydrological sources), it does allude to the possibility that antibiotic resistance could be a natural state for microorganisms.

### *1.4 Evolution of Antibiotic Resistance Genes in Natural Ecosystems*

Resistance genes are thought to have an environmental origin as seen in the case of aminoglycosides, vancomycin and β- lactams. Since the majority of antibiotics are produced by environmental bacteria, it is assumed that these antibiotic resistance genes are present to detoxify the antibiotics in producer organisms (26), but there are some other roles proposed for these genes. It has been discussed that microorganisms could have evolved these genes for other metabolic functions, not necessarily for antibiotic resistance such as resistance to heavy metals and toxic compounds produced by associated microbiota or metabolic intermediates (15).

There is evidence suggesting an evolutionary association between resistance enzymes and metabolic proteins or "housekeeping" genes. One well studied example of this are aminoglycoside modifying enzymes. Resistance to aminoglycosides is primarily caused by drug modification: phosphorylation catalyzed by aminoglycoside phosphotransferases (APHs), acetylation catalyzed by aminoglycoside acetyltransferases (AACs), or adenylylation catalyzed by aminoglycoside nucleotidyltransferases (ANTs).

The genomic enzymology approach has provided useful insights into the evolution of these resistance enzymes (27). APH enzymes share striking structural and functional homology with Serine/Threonine/Tyrosine protein kinases and it has been shown that APHs can phosphorylate some protein and peptides which are natural substrates of kinases (28). Despite the low sequence similarity between these enzymes (> 5%), the catalytic core is conserved suggesting a common ancestor. In particular, while the nucleotide binding site and the overall 3D structure of the active site is conserved, the residues involved in substrate binding evolved to bind specific substrates (for example, peptides or antibiotics) (28, 29).

β- lactam antibiotics are widely used as antimicrobial agents for treating bacterial infections. Enzyme catalyzed hydrolysis by β-lactamases is an important resistance mechanism. Extensive structural and phylogenetic analysis has suggested that β -lactamases evolved from penicillin binding proteins (PBPs) which are involved in peptidoglycan synthesis and cell wall metabolism (30). It has been proposed that the first serine β -lactamases emerged around 2.4 billion years ago from PBPs. Mobilization of β -lactamase genes from chromosomes to plasmids and movement between bacterial lineages has been predicted to date back to millions of years (31, 32).

Clindamycin belongs to the lincosamide class of antibiotics, used for treating infections caused by Gram-positive pathogens, especially MRSA. Therefore, emerging resistance to clindamycin and other lincosamides is a growing concern (33). Adenylylation of clindamycin by lincosamide adenylytransferases (Lin enzymes) is an important resistance strategy (34). LinB shares sequence similarity with bacterial DNA

polymerase β-subunit (Pol β) suggesting that nucleotidyltransferases involved in antibiotic modification have evolved from nucleotide polymerases (27, 34).

The examples discussed above provide strong indirect evidence that the origin of some resistance genes can be traced back to environmental bacteria, and that these resistance determinants most likely evolved from genes involved in other metabolic/physiological functions. Genomic enzymology, biochemical studies and extensive genome sequence analysis has helped in understanding the molecular basis of antibiotic resistance and providing insights into origin and evolution of antibiotic resistance.

### *1.5 Role of Humans in Antibiotic Resistance*

Antibiotic resistance has been linked to the human use of antibiotics. Important questions to ask here are: what role humans play in this problem of antibiotic resistance and how important is "anthropogenic" antibiotic selection pressure? Antibiotics are used widely not only in clinics but also in agriculture and farming practices and thus are constantly being released into water bodies and waste waters. This is believed to result in significant antibiotic selection pressure that can contribute to the selection and dissemination of antibiotic resistance genes (35). For example, avoparcin is a glycopeptide which was used as a growth promoter in poultry farms. It was found that there was a direct correlation between the extensive usage of avoparcin and prevalence of vancomycin-resistant *Enterococci* in intestinal flora of poultry animals (36). Withdrawal of avopracin resulted in decreased glycopeptide resistance (36). Similar trends were observed with antibiotics such as tylosin, virginiamycin and other broad spectrum

antibiotics; this supports the argument that humans play an important role in antibiotic resistance selection (36, 37, 38). In addition to the continuous influx of antibiotics into water/aquatic environments, there is mixing of bacteria from different niches, which include pathogenic bacteria, non-pathogenic bacteria, antibiotic-producing bacteria and antibiotic-resistant bacteria. This phenomenon facilitates the transfer of resistance genes between microbes and increases resistance prevalence (39).

A recent study looked at antibiotic resistance in Galapagos commensals, a remote island with limited human contamination. Acquired antibiotic resistance genes were absent in bacteria studied from this environment further suggesting that the presence of high concentrations of antibiotics is an important selective force in evolution and spreading of antibiotic resistance genes and can contribute significantly in altering the natural microbiota (40).

A survey was conducted recently where the soil samples in Netherlands (from 1940-2008) were assessed for the presence of antibiotic resistance genes. It was found that there was a higher relative abundance of resistance genes for major antibiotic families of drugs in recent soil samples as compared to samples collected in 1940s, and this trend was correlated to increased use of antibiotics in clinics and farming practices (41).

There is also evidence suggesting a correlation between metal resistance and antibiotic resistance in the environment (42). It is not uncommon to find metal resistance genes and specific antibiotic resistance genes on the same plasmid. Therefore, heavy metal contamination and industrial pollutants in environment could also serve as an

important mechanism for selecting antibiotic resistance genes (42, 43).

In summary, past and current usage of antibiotics, release of industrial waste and other contaminants such as heavy metals and biocides could play a critical role in altering resistance levels in nature by providing antibiotic selection pressure.

### *1.6 Lechuguilla Cave: A pristine, isolated ecosystem*

As previously discussed, antibiotics from human-implemented sources are constantly being released into water bodies and waste waters, a release that has been demonstrated to result in a selection pressure sufficient to contribute to the dissemination of antibiotic resistance genes (35-42). While numerous studies provide insights into role of humans towards dissemination of resistance, little is known about its origin. Antibiotics that have found human and agricultural applications are largely derived from environmental bacteria, whose biosynthetic pathways are believed to have evolved over millions of years (1, 2). Several studies have been conducted in the past to look at the widespread nature of environmental antibiotic resistance in the absence of obvious human influence, investigating natural habitats such as soil, deep terrestrial subsurface and aquatic environments (22-25). One of the intrinsic limitations of these studies is the inability to control for bacteria transported from surface environment, or through water systems exposed to the surface that may have been impacted by clinical or industrial antibiotic use. As of now, there has been no systematic survey of a population of microbes not exposed to human-implemented antibiotics. Lechuguilla cave, one of the deepest caves in continental United States, offers such environment.

Lechuguilla cave is classified as an "isolated cave" which means that it is hydrologically isolated from input and output boundaries of aquifers (44, 45). This cave is thought to be isolated from the surface environment for the last 4-6 million years and thus is presumably free of any surface influences such as exposure to antibiotics through water bodies (46). Lechuguilla cave is hypogene in origin, which means that there is no connection to the surface and is not heavily invaded by seepage water further suggesting that the cave has been isolated from surface influences (44). Furthermore, cave geology consists of tight impermeable siltstone which prevents any rapid influx of surface water into the cave (44- 47). Lechuguilla's location within a US National Park also means that human impact is minimized, with designated trails, careful collection of detritus and the removal of solid (and as much liquid) human waste as possible (48). This is a limited access environment where exploration has occurred through a comprehensive permitting system, which allows the generation of an extensive record of human travel, exploration and impact within the cave (49).

All the geological evidence supports the notion that Lechuguilla Cave is a pristine, isolated ecosystem that has not been exposed to anthropogenic antibiotics. Studying bacteria isolated from this cave is of particular interest as it may be useful in understanding trends of antibiotic resistance in environment not exposed to human antibiotic usage. Finding antibiotic resistance determinants in strains coming from an ecosystem, isolated for millions of years will provide further support to the idea that antibiotic resistance genes have been around for millions of years, even in the absence of human influences.

### *1.7 Macrolides as Antimicrobial Agents*

#### *1.7.1 Introduction to Macrolide Antibiotics*

Macrolides are widely used for treating infections caused by Gram-positive bacteria such as *Streptococcus pneumoniae*, group A *Streptococci*, and *Haemophilus influenzaea*. They work by binding to the 50S ribosome and blocking the exit tunnel which cause premature dissociation of peptidyl-tRNA from ribosome, thereby inhibiting protein synthesis (50, 51). Macrolides in clinical use are either natural products, produced by *Streptomyces* bacteria, or semi-synthetic derivatives (e.g. ketolides (telithromycin), roxithromycin, clarithromycin and dirithromycin). Macrolides contain a large lactone ring (12-16-membered) with one or more sugars attached to it. There are four "generations" of macrolide antibiotics with improved pharmacokinetic activity and antibacterial activity (50).

#### *1.7.2 Macrolide Resistance Mechanisms*

Emerging resistance to macrolides is a growing concern as macrolides are clinically important drugs. The most common mechanisms of resistance to macrolides are as follows: (i) target modification (ii) efflux of the drug, and (iii) chemical modification of the antibiotic (52). Target modification occurs by methylation of adenine residue in 23S rRNA catalyzed by the Erm class of methyltransferases. *erm* genes can be found in numerous clinical pathogens, and confer cross-resistance to lincosamides as well as streptogramins (MLS phenotype)(53). Efflux of macrolides is generally mediated by *mef* genes (macrolide efflux) in Gram- positive isolates. Gram-negative isolates are intrinsically resistant to macrolides due to the presence of chromosomally encoded efflux

pumps (54). Macrolides can be chemically modified by phosphotransferases (encoded by *mph* genes) (49), esterases (encoded by *ere* genes) (55, 56), or glycosyltransferases (encoded by *mgt* genes) (57, 58). Esterase-catalyzed drug modification causes hydrolytic cleavage of antibiotic, hence inactivating it. Genes *ereA* and *ereB* encode for erythromycin esterase I and II respectively, found in *E. coli* and are responsible for hydrolyzing 14-membered lactone rings. These two enzymes share poor identity at amino acid sequence level (17 % identical). Based on G+C content and codon usage analysis, *ereA* gene is believed to be indigenous to *E. coli*, where as *ereB* gene is thought to be exogenous (acquired) in origin (55, 56).

Macrolide glycosyltransferases are enzymes encoded by macrolide glycosyltransferase (*mgt*) genes which inactivate macrolides by glycosylation at 2'-OH of desosamine sugar moiety (57). They are predominantly found in macrolide producers (e.g. *Streptomyces ambofaciens* and *Streptomyces antibioticus*) where these enzymes are used for self-resistance (57, 58). These enzymes can also be found in non-producer organisms (e.g. *Streptomyces lividans*) (59).

Macrolide phosphotransferases inactivate macrolides by phosphorylation in nucleotide triphosphate-dependent manner. Enzymatic modification of macrolides is a resistance mechanism rarely seen in clinics, but there have been a growing number of reports of *mph* genes in clinical isolates, suggesting its potential to become an important resistance strategy (53).

*1.7.3 Macrolide Phosphotransferases*

There are two groups of macrolide phosphotransferases (MPHs) with different

substrate specificities and primary amino acid sequence (37% identical): MPH(2')-I and MPH(2')-II. MPH(2')-I preferentially phosphorylates 14-membered macrolides over 16-membered macrolides and is encoded by *mphA* (in *E. coli*) and *mphD* (in *P. aeruginosa*) (60-62). MPH(2')-II shows no preference for 14-membered or 16-membered macrolides and is encoded by *mphB* (*E. coli*) and *mphC* (*S. aureus*) (63-65). As of now, there have been no extensive biochemical or structural characterization of this family of enzymes. It is important to note here that reported *mph* genes are commonly found associated with plasmids and other mobile genetic elements. Therefore, finding chromosomal variants of these genes is intriguing because ancestral genes could be a source of resistance determinants found in clinics. Primary amino acid sequence alignments have shown that MPHs and APHs have conserved C-terminal motifs which are involved in nucleotide binding (66).

### *1.8 Project Goals*

The aim of this thesis was to investigate antibiotic resistance in strains from an isolated cave ecosystem, Lechuguilla Cave (LC). To explore the resistance diversity, LC strains were screened for antibiotic resistance against 26 antibiotics belonging to major drug families. Resistance catalyzed by enzymes that modify antibiotics is of particular interest as studying the function of these enzymes can provide useful insights into their origin and evolution. Hits from the primary screen were investigated for enzymatic modification through inactivation studies. Macrolide phosphorylation was observed suggesting the presence of an *mph* in cave strain LC044 *Brachybacterium paraconglomeratum* and this mechanism was characterized *in vitro*. Characterization of

the candidate MPH, identified from the draft genome sequence, confirmed its macrolide phosphorylating activity. To understand if the MPH enzyme is unique to *Brachybacterium* spp. found in the cave environment, we characterized a MPH from deep poultry litter isolate *Brachybacterium faecium* DSM 4810 (annnoted as predicted APH based on the available genome sequence). Genome context of each MPH was investigated by comparison of 10 kbp regions upstream and downstream of the *mph* genes using BLAST. The goals of this project were achieved using genome sequencing, bioinformatics and various biochemical and molecular biology approaches.

# CHAPTER 2. MATERIALS AND METHODS

## *2.1 Isolation of Cave Strains (Dr. Hazel Barton)*

This work was done by Dr. Hazel Barton's group (Northern Kentucky University, Kentucky). Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich. Nine different types of media were used for cultivation, based on what we imagined to be potential carbon and energy sources available within the environment. To a subset of these media we added antibiotics (20 µg/ml tetracycline; 10 µg/ml nalidixic acid, or 20 µg/ml chloramphenicol) to limit fast growing species, such as *Pseudomonads,* which can rapidly overwhelm culture plates and make it difficult to isolate unique species, or to inhibit the growth of fast-growing Gram-negative species (40 µg/ml ampicillin). Water was collected from a pool near each sample site and filter-sterilized using a 0.22 mm nitrocellulose filter. A Dremel tool was used to cut ~ 5 g piece of wall rock at each sample site, which was crushed *in situ* using a steel pestle and mortar. Approximately 1 g of this material was then placed in 2 ml of sterile water and mixed by shaking. The course material was then allowed to precipitate out, and the supernatant was used to inoculate each of the prepared culture media. To prevent cross-contamination of carbon sources/nutrients, fresh swabs were used for each plate. The cultures were then carried out of the cave and shipped to the laboratory at 4°C (2 days), before incubating in the dark at room temperature (21°C) for anywhere from 2 – 6 weeks. Unique colonies were then single colony isolated, initially on the same media on which they were isolated, but after several passages on media, these strains were transferred to a 50% concentration of trypticase soy agar (TSA; (Difco™, Becton Dickinson, Franklin Lakes, NJ).

### *2.2 DNA Extraction/16S Analysis of Cave Isolates (Dr. Hazel Barton)*

To identify each isolate, the genomic DNA was extracted using a ZR Fungal/Bacterial DNA Kit (Zymo Research, Orange, CA) and the 16S ribosomal RNA gene sequence identified as previously described (67). Sequences that were difficult to identify in this manner were cloned into a pTOPO-TA Cloning Vector (Invitrogen Corp., Carlsbad, CA) before sequencing. All sequencing was carried out at the University of Kentucky Advanced Genetic Technologies Center (UK-AGTC; http://www.uky.edu/Centers/AGTC). DNA alignments were carried out using the ARB Software Package (http://mpi-bremen.de/molecol/arb). For distance calculations, the evolutionary model used was determined using MODELTEST (68). Distance and parsimonious phylograms were generated in PAUP* using a heuristic search (69). The highest scoring trees were tested by boostrap analysis using 1000 replicates. In all cases, sequences from *Aquifex pyrophilus* and *Thermoplasma acidophilium* were used as outgroups.

### *2.3 Antibiotic Resistance Screen of Cave Strains*

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich. A collection of 93 cave strains (Appendix A), provided by Dr. Hazel Barton (Northern Kentucky University), were grown in 50% Tryptic Soy Broth (TSB) at 30°C for 3-5 days. From these cultures, frozen stocks were prepared in 96 well plate format (1:10 dilution of cultures in 80% glycerol). Master plates containing 150 µl 50% TSB were inoculated from the frozen stock plates using a replica head plater and grown at 30°C for 3-5 days. The inoculum plate was prepared from the master plate as a 1:100 dilution in 50% TSB.

Assay plates containing 50% TSB (140 µl volume) in the presence and absence of 20 µg/ml antibiotic (named resistance plate and growth plate respectively) were inoculated with 10 µl/well of the inoculum plate and incubated at 30°C for 5 days. Note that for all studies containing the antibiotic daptomycin (Cubist Pharmaceuticals), cultures were supplemented with 1.25 mM $CaCl_2$ according to manufacturer's instructions. The screen was performed in duplicates and data was collected as $OD_{600}$ after 5 days. Resistance plate data was normalized to the growth control plates and resistance was defined as more than 50% reproducible growth in the presence of an antibiotic.

***2.4 Antibiotic Inactivation Screen***

Cultures containing 5 ml of 50% TSB cultures in the presence and absence of 20 µg/ml of antibiotic were inoculated with 3-5 single isolated colonies of strain of interest and cultures were grown at 30°C for 5 days. Uninoculated controls were also prepared. After growth at 30°C for 5 days, cultures were centrifuged for 20 minutes at 16,100 ***g*** and conditioned media was collected. One of the following susceptible organisms/test organisms was used for antimicrobial susceptibility testing (antimicrobial disk diffusion assay): *Bacillus subtilis*, *Micrococcus luteus* and *Staphyloccoccus saprophyticus* ATCC 15305. Inocula of test organism were prepared to the 0.5 McFarland standard using the direct colony suspension method according to the Clinical and Laboratory Standard Institute guidelines (CLSI) (70). Susceptible organisms were plated on Luria Broth (LB) Agar, as described and conditioned media (20-30 µl) was spotted on sterile paper disks prior to incubation at 37 °C overnight. Inactivation was defined as complete absence of a zone of inhibition.

### *2.5 Determination of Minimal Inhibitory Concentrations* (MICs)

Cultures (5 ml) containing 50% TSB were inoculated with a single isolated colony of each strain of interest and grown shaking at 250 rpm at 30°C for 5 days. MICs were conducted in 96 well U- bottom MIC plates (VWR) containing 50% TSB media and supplemented with antibiotic, (ten 2-fold serial dilutions of antibiotic, final concentration ranging from 0.5 µg/ml to 128 µg/ml). MIC protocol used was adapted from CLSI (70). Cultures were diluted to $OD_{600}$ of 0.08-0.1 in 0.85% NaCl. A second dilution (1:10) was made in 50% TSB media and 50 µl of this second dilution was used as an inoculum. Plates were incubated at 30°C for 3- 5 days. *E. coli* ATCC 29522, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as MIC control strains. MIC testing was done in duplicates, where MIC was defined as the drug concentration showing no visible growth.

### *2.6 Genome Sequencing and Assembly (Nicholas Waglechner)*

*B. paraconglomeratum* LC044 was grown in 5 ml 50% TSB at 30 °C for 5 days. Genomic DNA was isolated using QIAGEN DNeasy Blood and Tissue Kit 250 (Qiagen, Germany) with one modification of the manufacturer's protocol for cell lysis: 4 µl of RNase (100 mg/ml in Buffer TE) was added to the reaction mixture and incubated at room temperature for 2 minutes before washing the spin column. Genomic DNA was submitted for shotgun sequencing to Roche 454 Life Sciences Genome Sequencer at Farncombe Metagenomics Facility, McMaster University. The 262995 reads were assembled by Nicholas Waglechner (Bioinformatician, IIDR McMaster University) , who subsequently performed BLAST comparison analyses with the *E. coli* MPH(2')-Ia

(accession BAA03776) to identify a candidate macrolide phosphotransferase gene.

### *2.7 Cloning, Expression and Purification of Macrolide Phosphotransferases*

The candidate macrolide phosphotransferase (*mph* gene) from *B. paraconglomeratum* (LC044) was synthesized by GenScript (USA) with codon optimization for *E. coli* expression and cloned into pET28b (Novagen) with NdeI and HindIII restriction sites. The plasmid containing MPH was transformed into *E. coli* BL21 (DE3) (Novagen) and the resulting colonies were grown overnight at 37°C in LB media supplemented with 50 μg/ml kanamycin. For overexpression, 1 L LB cultures supplemented with 50 μg/ml kanamycin were subcultured from the overnight culture at 1% (v/v) and grown at 37°C to an $OD_{600}$ of 0.5-0.7. The cells were chilled on ice for 15 minutes. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Bioshop) at 16°C for 16 hours. The cells were harvested using Avanti J25 centrifuge (Beckman) and stored at -20°C until further use.

For protein purification, the cells were resuspended in lysis buffer (50 mM HEPES, 300 mM NaCl, 10 mM Imidazole, pH 7.5), 1 μg/ml pancreatic-bovine DNase and 1 mM phenylmethanesulfonylfluoride (PMSF) (Sigma- Alrich). The resuspended cells were lysed using T-S series cell disrupter (Constant Systems Inc.) at 35,000 pound-force per square inch (p.s.i.) and cell debris was removed via centrifugation at 48,000 ***g*** for 1 hour. The supernatant was applied to 5 ml Ni-NTA column pre-equilibrated with lysis buffer. Elution was performed with a step gradient method using increasing 10% increments of elution buffer (50 mM HEPES, 500 mM NaCl and 500 mM Imidazole pH 7.5). The purity of protein was determined by SDS-PAGE analysis. A second

chromatography step was conducted with the elution fraction using Hi Load Superdex 200 26/60 Gel Filteration Column (GE Healthcare). The column was pre-equilibrated with 300 ml of 50 mM HEPES, 100 mM NaCl pH 7.5. Isocratic elution over 1 column volume was used for eluting MPH. The protein fractions were pooled and concentrated using Amicon-Ultra centrifugal device from Millipore to 2 mg/ml and stored in 10% glycerol at -20 °C until further use.

### *2.8 Steady State Kinetic Analysis of MPH (2')*

All small molecules and macrolide antibiotics used in this study were purchased from Sigma-Aldrich, with the exception of telithromycin (Sanofi Aventis). Kinetic parameters of MPHs were determined using Pyruvate Kinase/Lactate Dehydrogenase coupled assay (PK/LDH assay). Phosphorylation of macrolides by MPHs was monitored by monitoring the absorbance of NADH (340 nm) in 96 well format using a SpectraMax reader. The reaction was initiated with macrolide or nucleotide (ATP or GTP). When monitoring macrolide dependence, 200 µM GTP was used and the macrolide concentration ranged from 3.2 µM and 400 µM. For nucleotide dependence, 200 µM macrolide was used and the final concentrations of nucleotide (ATP or GTP) ranged from 7.82 µM to 2000 µM. All reactions were done in triplicate and reaction conditions used were adapted from a previous study (71). The initial rates were determined by using Michaelis-Menten kinetics as shown in Equation below:

$v = V_{max}. [S] / K_m + [S]$ (Equation 1)

The rate for substate-inhibited reactions was calculated using Equation below:

$v = V_{max} * S / (K_m + S*(1 + S/K_i))$ (Equation 2)

Grafit 4 software (Erithacus Software, Staines, UK) was used for determining rates and kinetic parameters.

### *2.9 Phylogenetic Analysis of Macrolide Phosphotransferases (Nicholas Waglechner)*

To further investigate the relationship between known and putative MPH sequences to each other and to APH sequences, all of which fall in the more broadly defined APH family, the putative MPH identified from contig 34 along with the top five hits of the previous blastx search were added to a list of APH and MPH sequences previously analyzed (72). The accessions of the additional sequences used are [MPHx accession], YP_003155631, ZP_05915402, ZP_05749839, NP_737713 and ABF87557 respectively. The sequences were aligned to the hidden Markov model for the APH family from PFAM release 4.1 (accession PF01636) using HMMER3 (73, 74). This alignment was refined using MUSCLE v3.7 (75) followed by manual inspection and small corrections to ensure that known phosphotransferase sequence motifs were successfully aligned. This alignment was used to estimate a phylogeny using Mr. Bayes v3.1.2 (75). Two runs of five million generations of MCMC using 4 chains were run. A fixed WAG model was specified along with gamma distributed rate variation and a proportion of invariant sites. A rooted consensus tree was generated using the 50% majority rule from samples drawn every 100 generations using a burn-in of 1000 and specifying the APH sequences as an outgroup.

### *2.10 Identification of Telithromycin Inactivation Product*

For NMR analysis, phosphorylated telithromycin was purified. The reaction mixture (10 ml total) contained 2.5 ml of buffer (500 mM HEPES, 400 mM KCl, 100

mM $MgCl_2$ ), 200 µM GTP, and 3 ml of 2 mg/ml purified MPH(2')-IIe from LC044. The reaction was initiated using 1 ml of 25 mM telithromycin and incubated at room temperature until completion, determined by LC-MS (QTRAP, Applied Biosystems). To purify the reaction product, anion exchange chromatography was performed using Combiflash System (Teledyne Isco). Phosphorylated telithromycin was eluted from a Redi*Sep*® Rf SAX column (40-63 µm, 60 angstroms using 90% Solvent A (dichloromethane) and 10% Solvent B (methanol). Approximately 10 mg of phosphorylated telithromycin was obtained which was submitted for $^{1}H$ and $^{13}C$ NMR analysis by Dr. Kalinka Koteva.

### *2.11 NMR Analysis of Telithromycin Inactivation Product*

NMR analysis was conducted by Dr. Kalinka Koteva as previously described (22). The compound was dissolved in DMSO-$d_6$ (Cambridge Isotope Laboratories Inc.) to a concentration of approximately 10.0 mg/ml. Chemical shifts are reported in ppm relative to TMS using the residual solvent signals at 2.50 and 39.52 ppm as internal references for the $^{1}H$ and $^{13}C$ spectra, respectively.

# CHAPTER 3. RESULTS

## *3.1 Antibiotic Resistance Screening of Cave Strains*

The cave strain library, constructed by the laboratory of Dr. Hazel Barton (Northern Kentucky University) consisted of 93 strains (36% Gram-positives and 64% Gram-negatives) (Appendix A). These strains were screened against 26 different antimicrobials representing a wide spectrum of natural antibiotics (e.g. tetracycline, vancomycin), synthetic molecules (e.g. linezolid, trimethoprim) and semi-synthetic derivatives (e.g. clindamycin, cephalexin). The frequencies of resistance observed in the primary screen, defined as > 50% reproducible growth in the presence of an antibiotic are shown in Figure 3.1. Diverse resistance profiles were observed in the screen (Figure 3.2). Resistant isolates, both Gram-positive and Gram-negative, were detected for most of the major drug families. In the collection of 33 Gram-positive strains, on average, approximately 45% of the strains were resistant to 7-8 antibiotics. Three strains were resistant to 14 antibiotics and phylogenetic analysis revealed that these strains were *Streptomyces* spp. In our collection, there were only 5 *Streptomyces* spp. and all of them revealed a multi-drug resistant profile, consistent with previous work aimed to characterize the prevalence of antibiotic resistance in *Streptomyces*-related soil bacteria (22) that are known for their ability to produce clinically used drugs and have resistance genes (76, 77).

Gram-negative isolates were also screened for antibiotic resistance. It is important to note here that Gram-negatives are known to have innate resistance to antibiotics due to the presence of the outer membranes with a unique lipopolysaccharide structure and the

presence of highly efficient efflux pumps (78, 79). Therefore, only antibiotics which are known to have some activity against Gram-negatives were included in the data analysis.

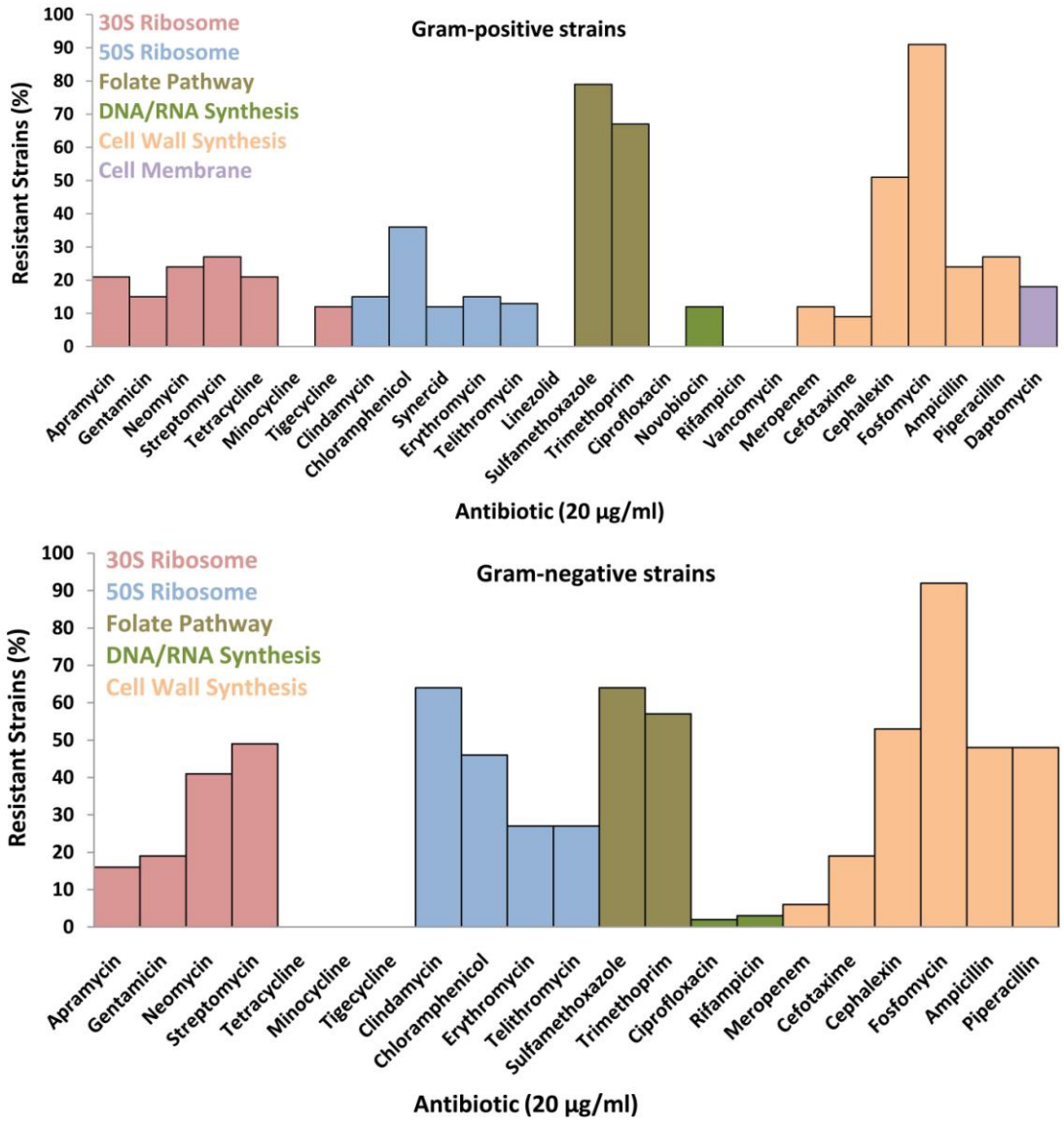


Figure 3.1: Resistance levels of cave strains at 20 µg/ml for (A) Gram-positive (B) Gram-negative strains. Strains were screened in liquid culture against 26 antibiotics and data was collected at $OD_{600}$ on day 5. The screen was performed in duplicates. Antibiotics are grouped according to their mode of action/target, where each color represents a different target.


Figure 3.2: Resistance Profiles for Gram- positive (A) and Gram-negative isolates (B). Red shading represents hits, where-resistant strains were defined as > 50% growth in the presence of antibiotic in comparison to the absence and yellow shading indicates sensitive strains. The X- axis shows antibiotics (grouped according to their mode of action, where each color represents a different target) and the Y- axis lists the strain names. Antibiotics are grouped according to their mode of action/target, where each color represents a different target.

With respect to antibiotics known to exhibit activity against Gram-negative bacteria, most of the Gram- negative strains on average showed resistance to 6 antibiotics. Approximately 27 % of Gram-negative strains were resistant to 10-13 antibiotics. It is of interest to note that no tetracycline resistance was observed in Gram-negative collection, though it is common in surface bacteria (Figure 3.1, Figure 3.2). Antibiotics such as sulfamethoxazole, trimethoprim and fosfomycin were generally not effective against the Gram-positive collection. Bacterial resistance to trimethoprim is most frequently caused by mutational changes in DHFR or overproduction of DHFR resulting in very high level of resistance (80, 81). Sulfamethoxazole resistance is primarily caused by presence of alternative variants of DHPS enzyme which can arise due to chromosomal mutations (82), gene duplication (83) or base pair insertions (84). Sulfamethoxazole and trimethoprim are often used in synergistic combination which might explain the substantial levels of resistance as these drugs were not used in combination.

### *3.2 Quantitative Analysis of Resistant Cave Strains- Determination of MICs*

To gain a quantitative understanding of antibiotic sensitivity levels, resistant and sensitive strains obtained from the primary screen were further analyzed by determination of the minimal inhibitory concentration (MIC). Figure 3.3 summarizes the MIC results determined by a modified broth-dilution method.

### *3.3 Screening Cave Strains for Antibiotic Inactivation*

Enzymes that modify antibiotics are of particular interest as this mode of resistance is a response which most likely evolved to block the activity of specific antibiotics and can provide insights into evolution of resistance (27). Therefore, hits

were investigated for antibiotic alteration as a potential resistance mechanism. Table 3.1 summarizes the results of antibiotic inactivation screen.

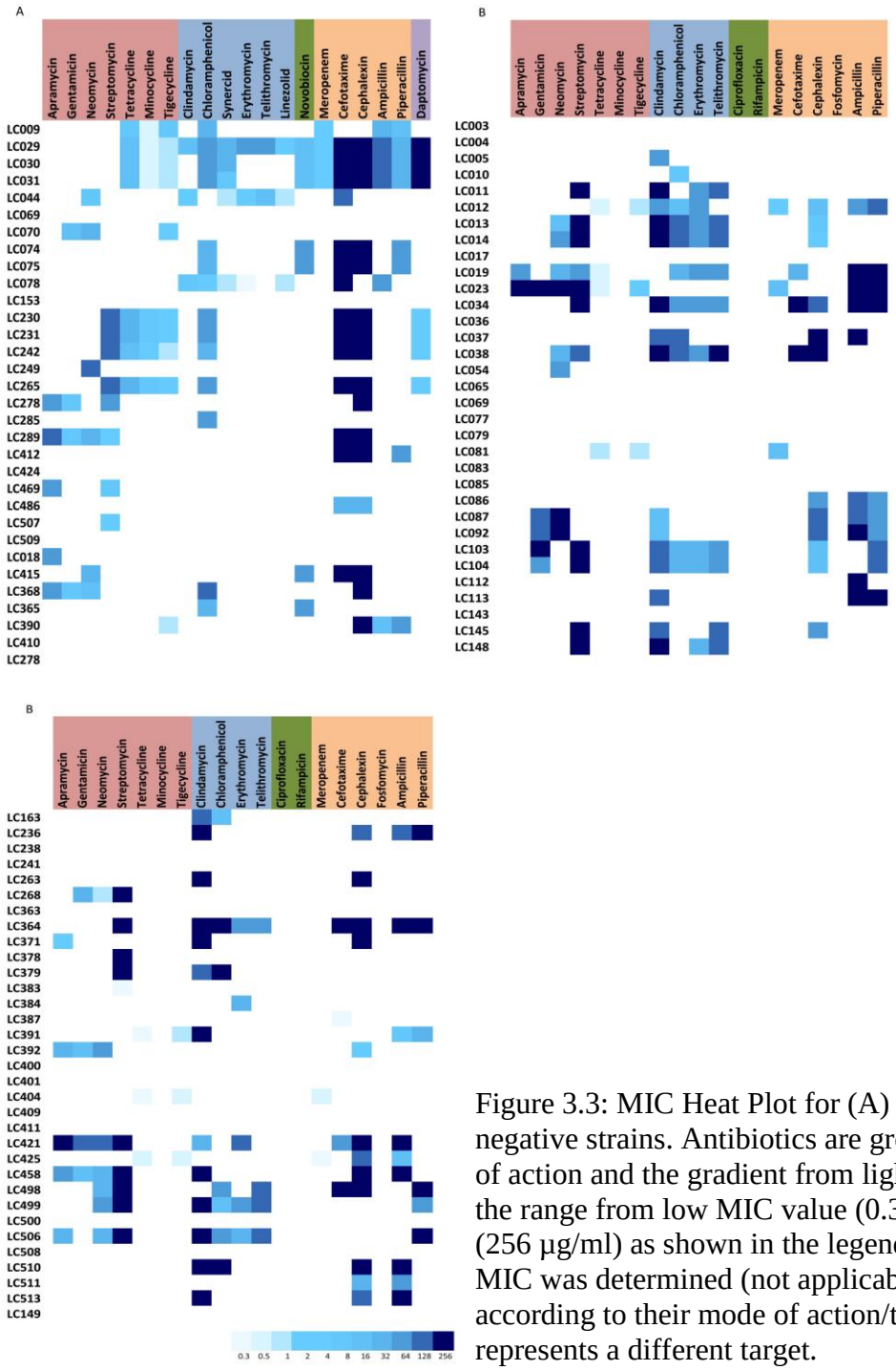


Figure 3.3: MIC Heat Plot for (A) Gram-positive (B) Gram-negative strains. Antibiotics are grouped according to their mode of action and the gradient from light blue to dark blue represents the range from low MIC value (0.3 µg/ml) to high MIC value (256 µg/ml) as shown in the legend (bottom). White means no MIC was determined (not applicable). Antibiotics are grouped according to their mode of action/target, where each color represents a different target.

Table 3.1: Summary of Antibiotic Inactivation Studies for Gram-positive (A) and Gram-negative strains (B). Strains were grown in 50% TSB for 5 days in presence of 20 µg/ml antibiotic. Conditional media was used for setting up disk diffusion assays and LC-MS analyses Inactivation was defined as the absence of a zone of clearance around the disk. Antibiotics of particular interest are highlighted in grey.

A

| Antibiotic | Gram -Positive Strains | | % of Strains Inactivating | |
|---|---|---|---|---|
| | Screened for Inactivation | Completely Inactivating | Of the screened | Mechanism(s) of Inactivation |
| Apramycin | 7 | 0 | 0 | ------- |
| Gentamicin | 5 | 0 | 0 | ------- |
| Neomycin | 8 | 0 | 0 | ------- |
| Streptomycin | 8 | 0 | 0 | ------- |
| Tetracycline | 4 | 0 | 0 | ------- |
| Minocycline | 1 | 0 | 0 | ------- |
| Clindamycin | 6 | 0 | 0 | ------- |
| Chloramphenicol | 12 | 0 | 0 | ------- |
| Synercid | 2 | 0 | 0 | ------- |
| Erythromycin | 7 | 1 | 14 | Phosphorylation |
| Telithromycin | 4 | 4 | 100 | Phosphorylation Glycosylation |
| Linezolid | 2 | 0 | 0 | ------- |
| Novobiocin | 5 | 0 | 0 | ------- |
| Cephalexin | 17 | 5 | 30 | Hydrolysis* |
| Ampicillin | 8 | 5 | 62 | Hydrolysis* |
| Piperacillin | 9 | 2 | 22 | Hydrolysis* |
| Daptomycin | 24 | 7 | 29 | Hydrolysis |

B

| Antibiotic | Gram- Negative Strains | | % of Strains Inactivating | |
|---|---|---|---|---|
| | Screened for Inactivation | Completely Inactivating | Of the screened | Mechanism of Inactivation |
| Apramycin | 10 | 0 | 0 | ------- |
| Gentamicin | 11 | 0 | 0 | ------- |
| Neomycin | 24 | 0 | 0 | ------- |
| Streptomycin | 30 | 0 | 0 | ------- |
| Telithromycin | 17 | 0 | 0 | ------- |
| Clindamycin | 39 | 0 | 0 | ------- |
| Chloramphenicol | 28 | 2 | 7 | Acetylation |
| Trimethoprim | 35 | 0 | 0 | ------- |
| Cephalexin | 36 | 14 | 39 | Hydrolysis* |
| Cefotaxime | 12 | 4 | 33 | Hydrolysis* |
| Ampicillin | 30 | 13 | 43 | Hydrolysis* |
| Piperacillin | 30 | 16 | 53 | Hydrolysis* |

* Not confirmed experimentally

*Drug Inactivation in Gram-positive Isolates:* No inactivation was observed for aminoglycosides, clindamycin and chloramphenicol, where enzymatic inactivation has been reported as an important resistance mechanism (85-89). Notable frequencies of inactivation were seen for β- lactams (ampicillin, piperacillin and cephalexin), which is known to primarily be caused by β -lactamase enzymes that hydrolyze the β -lactam ring (90).

Of particular significance, inactivation was detected for several newly approved clinical therapies. Daptomycin represents the newest class of antimicrobials, the lipopeptides, the only member of its class to be approved for clinical use. Thus far, resistance against this drug is rare and reported cases confer low-levels of daptomycin resistance (91, 92). Among the cave-derived isolates, several *Streptomyces* strains (LC029, LC030 and LC031) were found to be highly resistant to daptomycin (MIC of ≥ 256 µg/ml) and inactivated daptomycin. This result is consistent with a previous study reporting high frequencies of daptomycin inactivation in *Streptomyces*–like bacteria (22). Additionally, several *Paenibacillus lautus* isolates (MICs of 16 µg/ml) completely inactivated daptomycin when grown at sub-MIC concentrations of antibiotic. This finding represents the first reported incidences of daptomycin inactivation within the low G+C content Firmicutes for which daptomycin's use is approved (92).

*Streptomyces* strains LC029, LC030 and LC031, when grown in the presence of the telithromycin (MICs 64 µg/ml), demonstrated complete inactivation of the antibiotic. Analysis of the conditioned media by LC-MS indicated a shift of 162 daltons in comparison to the telithromycin reference (Table 3.2A) suggestive of mono-glycosylation.

This observation is consistent with soil *Streptomyces* isolates, demonstrating the ability to glycosylate telithromycin at the 2'-OH, as well as other macrolides (22, 57-59, 93). Strain LC044, *Brachybacterium paraconglomeratum*, was capable of detoxifying the only ketolide antibiotic to be FDA approved, telithromycin, as well as its parent compound erythromycin (MICs of 4-8 µg/ml for each) (Table 3.2A).

*Drug Inactivation in Gram-negative Isolates:* No significant inactivation was observed in Gram-negative isolates suggesting that there are other molecular mechanisms of resistance in these bacteria such as efflux-based mechanisms, target modification or impermeability barrier (78, 79). Two percent of strains assayed for chloramphenicol inactivation modified the drug through acetylation (Table 3.2B). Chloramphenicol acetylation is a well-studied resistance mechanism both in Gram-positive and Gram-negative bacteria (88, 89).

### *3.4 Characterizing Macrolide Inactivation by Brachybacterium paraconglomeratum (LC044)*

*Brachybacterium paraconglomeratum* isolate (LC044), observed to exhibit a distinct resistance profile, demonstrated macrolide resistance by means of drug inactivation. Of the 21% of isolates screened for erythromycin inactivation and 12% of strains screened for telithromycin inactivation, this was the only isolate which detoxified both macrolides in liquid media. Macrolide inactivation in strain LC044 was analyzed using antimicrobial disk diffusion assay and LC-MS analysis of the clarified culture media. Modification of antibiotic by LC044 was associated with a shift of retention time, an increase in *m/z* ratio and complete loss of antimicrobial activity against indicator

organism, *Bacillus subtilis*. A shift of 80 daltons was suggestive of macrolide phosphorylation as a resistance mechanism (Figure 3.4).

Phosphorylation of macrolides is catalyzed by the macrolide phosphotransferases (MPHs) class of resistance enzymes encoded by *mph* genes (60-65). Bioinformatic analyses performed on a draft genome of LC044 (Nicholas Waglechner) identified a putative gene encoding MPH.

Table 3.2 (A): Telithromycin Inactivation in Cave Isolates. The strains were grown in presence of 20 µg/ml drug for 5 days at 30 °C. Analysis of the conditioned media by LC-MS indicated a shift of 162 Da (for *Streptomyces* isolates) and 80 Da (for LC044) in comparison to the telithromycin reference, suggestive of mono-glycosylation and phosphorylation respectively.

| Strain | Telithromycin *m/z* (Da) $[M+H]^+$ | Retention Time (min) | Telithromycin Inactivation Product *m/z* (Da) $[M+H]^+$ | Retention Time (min) | Difference *m/z* (Da) |
|---|---|---|---|---|---|
| *Streptomyces flaveus* (LC029) | 813.0 | 13.2 | 974.6 | 12.9 | 161.6 |
| *Streptomyces anulatus* (LC030) | 813.0 | 13.2 | 974.6 | 12.9 | 161.6 |
| *Streptomyces anulatus* (LC031) | 813.0 | 13.2 | 974.6 | 12.9 | 161.6 |
| *Brachybacterium paraconglomeratum* (LC044) | 812.6 | 13.1 | 892.6 | 13.3 | 80.0 |

Table 3.2 (B): Chloramphenicol Inactivation in Cave Strains. The strains were grown in presence of 20 µg/ml drug for 5 days at 30°C. Conditioned media was used for LC-MS analysis. Based on shift in retention time and *m/z* ratio, modification was inferred to be mono-acetylation of chloramphenicol.

| Strain | Chloramphenicol m/z (Da) $[M-H]^-$ | Retention Time (min) | Chloramphenicol Inactivation Product m/z (Da) $[M-H]^-$ | Retention Time (min) | Difference *m/z* (Da) |
|---|---|---|---|---|---|
| *Ochromobactrum intermedium* (LC019) | 321.3 | 5.6 | 363.4 | 6.3 | 42.1 |
| *Agrobacterium tumefaciens* (LC034) | 321.3 | 5.6 | 363.3 | 6.3 | 42.0 |
| *Ochromobactrum intermedium* (LC506) | 321.3 | 5.6 | 363.1 | 6.3 | 41.8 |

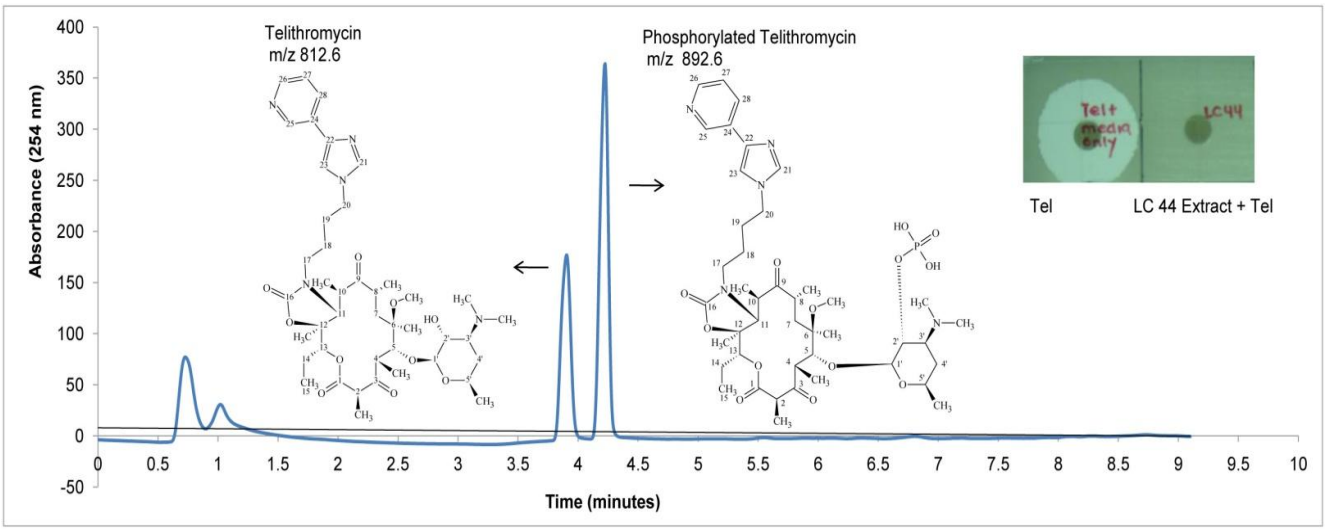


Figure 3.4: Inactivation of Telithromycin by LC044 Strain. Chemical modification was determined by the loss of antimicrobial activity against *Bacillus subtilis* upon incubation of *B. paraconglomeratum* LC044 in the presence of 20 µg/ml telithromycin (Inset). LC-MS analysis of the resulting inactive extract indicated a shift of retention time and an addition of 80 Da. The structures of telithromycin and the inactive product, telithromycin phosphate, as determined by NMR analysis, are shown at each corresponding peak.

To confirm the macrolide modifying activity of this putative enzyme, steady-state kinetic constants were performed on purified recombinant enzyme using a PK/LDH coupled assay (Table 3.3). The reported MPH did not show any selectivity for 14-membered, 15-membered or 16- membered macrolide antibiotics, consistent with previous characterization of MPH(2')-II enzymes from *E.coli* (62, 63). The site of modification for telithromycin was confirmed to be 2'-OH group using multidimensional and multinuclear magnetic resonance analysis (Dr. Kalinka Koteva). Since there have been no previous reports of MPH characterization from *Brachybacterium* spp., we propose to call this enzyme MPH(2')-IIe, according to standard convention.

To determine if functional MPH activity is restricted to cave-associated *Brachybacterium* spp., we characterized a putative MPH ortholog from a deep soil isolate *Brachybacterium faecium* DSM 4810 (94). Despite being annotated as a predicted aminoglycoside phosphotransferase (APH), the translated sequence exhibited 72% identity to MPH from cave isolate LC044. Kinetic constants, particularly $k_{cat}/K_m$ which quantify catalytic efficiency, revealed no significant differences between these enzymes (Table 3.3), suggesting that MPH(2')-IIe activity is not restricted to cave *Brachybacterium* and can also be found in terrestrial isolates. This was further supported by phylogenetic analysis (Nicholas Waglechner) which revealed that MPHs from *Brachybacterium* strains grouped together and showed some relatedness to previously reported MPHs from clinical isolates (Figure 3.5).

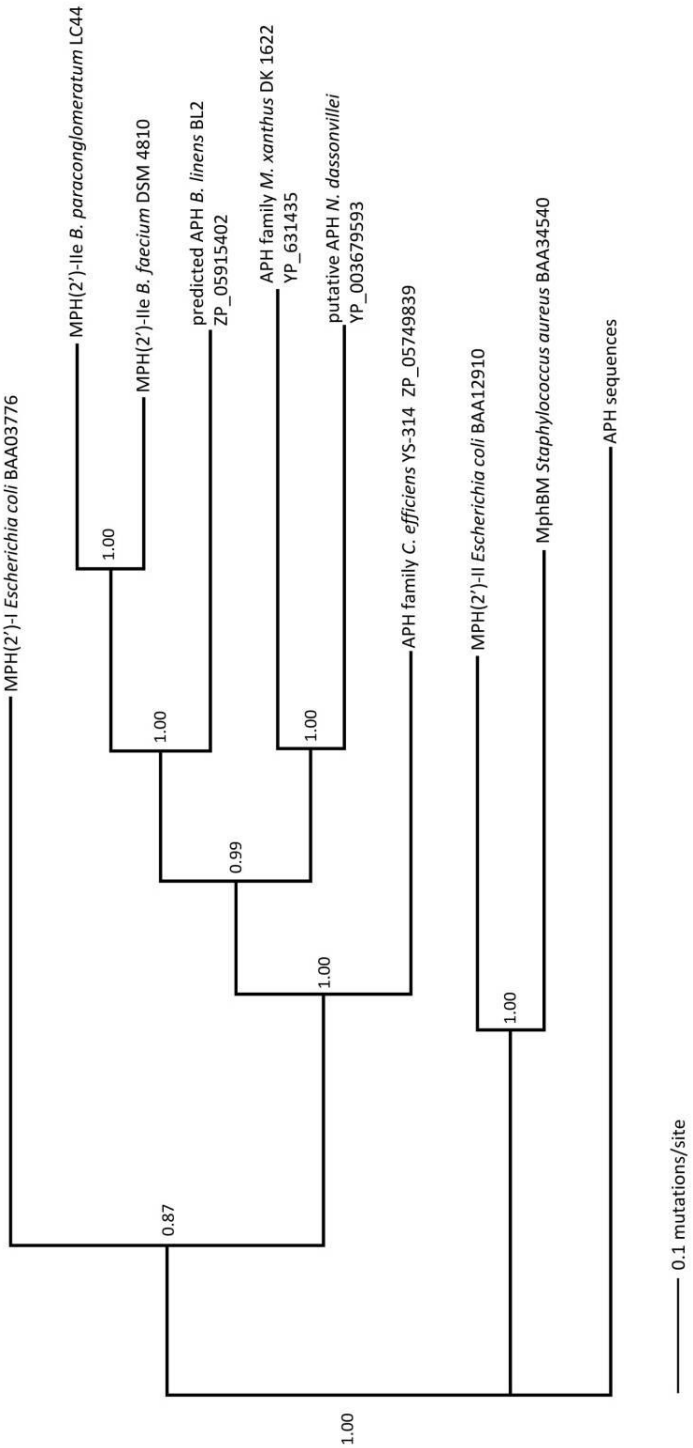


Figure 3.5: Phylogenetic Analysis of MPH/MPH-like proteins. Aminoglycoside Phosphotransferases (APHs) were collapsed into a single branch (APH sequences) which was used as an outgroup. Note that the scale bar represents 0.1 mutations/site. This figure was kindly provided by Nicholas Waglechner.

### *3.5 Understanding Genomic Context of MPHs in Brachybacterium Strains*

We investigated the genomic context of each *Brachybacterium* MPH to (i) gain insights into the physiological/biological function of this gene, and (ii) identify any flanking transposable elements which could facilitate gene transfer to other bacteria. Our analysis revealed considerable conservation of gene organization within 10 kB of the MPHs, with 5 unique regions noted within each isolate. On the amino acid level, conserved genes exhibited on average 70-80% sequence identity (Figure 3.6). It was interesting to find a transposase-like protein flanking MPH in *B. faecium* isolate which may suggest the possibility of mobilization of MPH protein.

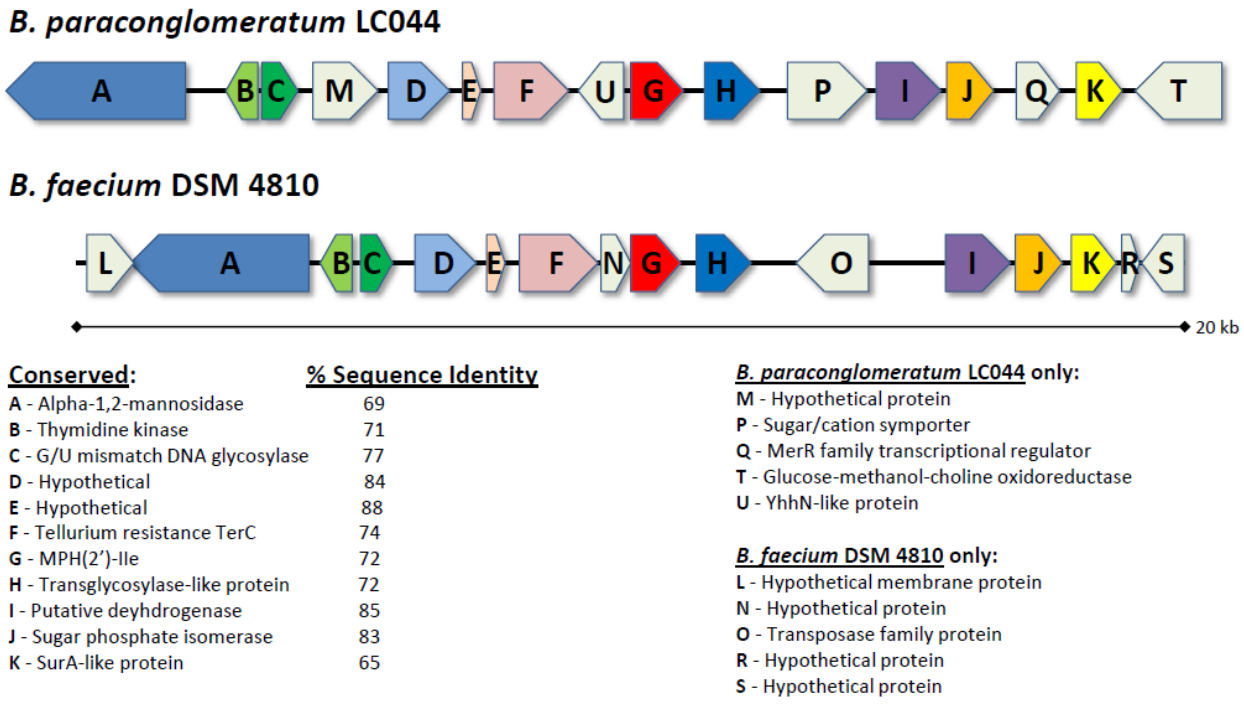


Figure 3.6: Genetic context of *mph* genes in *Brachybacteria*. Genetic map was constructed using available genome sequences of *Brachybacterium* strains and percentage statistics (% identity) shown above are translated protein queries based on BLAST analysis. MPH sequence is shown in red and homologous sequences are marked with identical colours. The length of the genes is proportional to their sizes. Note that this figure was kindly provided by Nicholas Waglechner.

Table 3.3: Kinetic parameters for MPH(2')-II characterized from cave strain LC044 *B. paraconglomeratum* (A) and poultry deep litter isolate *B. faecium* DSM 4810 (B)

A

| Macrolide* | Lactone Ring | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $K_i$ (μM) | $k_{cat}/K_m$ ($s^{-1}$ $M^{-1}$) |
|---|---|---|---|---|---|
| Erythromycin | 14 | 43.8 ± 11.3 | 0.101 | - | $2.30 \times 10^3$ |
| Clarithromycin | 14 | 21.9 ± 5.5 | 0.106 | - | $4.82 \times 10^3$ |
| Telithromycin | 14 | 12.2 ± 5.0 | 0.04 | 600 ± 285 | $3.52 \times 10^3$ |
| Roxithromycin | 14 | 58.98 ± 10.9 | 0.202 | - | $3.42 \times 10^3$ |
| Azithromycin | 15 | 53.97 ± 7.6 | 0.168 | - | $3.14 \times 10^3$ |
| Spiramycin | 16 | 45.6 ± 15.0 | 0.173 | 292 ± 108 | $3.79 \times 10^3$ |
| Tylosin | 16 | 222 ± 29.2 | 0.197 | 66.4 ± 8.9 | $8.87 \times 10^2$ |

* [GTP]= 200 μM

B

| Macrolide* | Lactone Ring | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $K_i$ (μM) | $k_{cat}/K_m$ ($s^{-1}$ $M^{-1}$) |
|---|---|---|---|---|---|
| Erythromycin | 14 | 38.2 ± 6.2 | 0.192 | - | $5.03 \times 10^3$ |
| Clarithromycin | 14 | 42.0 ± 6.3 | 0.286 | - | $6.81 \times 10^3$ |
| Telithromycin | 14 | 23.9 ± 4.2 | 0.168 | - | $7.05 \times 10^3$ |
| Roxithromycin | 14 | 19.3 ± 2.6 | 0.262 | - | $1.36 \times 10^4$ |
| Azithromycin | 15 | 60.6 ± 8.9 | 0.312 | - | $5.18 \times 10^3$ |
| Spiramycin | 16 | 17.3 ± 3.2 | 0.157 | - | $9.10 \times 10^3$ |
| Tylosin | 16 | 14.5 ± 4.0 | 0.088 | - | $6.07 \times 10^2$ |

* [GTP]= 200 μM

C

| Nucleotide | Saturating Antibiotic[C] | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $M^{-1}$) |
|---|---|---|---|---|
| GTP [A] | Erythromycin | 10.2 ± 0.5 | 0.093 | $9.30 \times 10^4$ |
| ATP [A] | Erythromycin | N.C. | - | - |
| GTP [B] | Erythromycin | 16.0 ± 1.3 | 0.203 | $1.27 \times 10^4$ |
| ATP [B] | Erythromycin | N.C. | - | - |

[A] *B. paraconglomeratum* LC044
[B] *B. faecium* DSM 4810
[C] Concentration of antibiotic was 400 μM
N.C. No curve was obtained

## CHAPTER 4. DISCUSSION

This study examined antibiotic resistance in a diverse strain collection derived from the isolated, preserved Lechuguilla cave ecosystem. Cave geology features, such as the impermeable siltstone caprock which prevents rapid influx of surface water, and long surface isolation rules out the possibility of exposure to anthropogenic use of antibiotics as well as antibiotic contamination through water bodies (44-47). Fortunately, Lechuguilla's location within a US National Park also means that human impact is minimized and human exploration of Lechuguilla through a permitting system minimizes human traffic into the cave (49). As a result Lechuguilla cave is an ideal ecosystem for investigating microbes that have not been exposed to humans (i.e anthropogenic use of antibiotics). This is the first systematic study of antibiotic resistance in such ecosystem. Thus the results from this work illustrate that antibiotic resistance is widespread even in ecosystems untouched by human influence.

Macrolides remain an important class of clinically relevant antibiotics and emerging resistance to macrolides is a concern (53). This includes resistance by antibiotic phosphorylation, encoded on plasmids found in clinical isolates of *E. coli* (60, 61, 63, 64), *S. aureus* (65) and more recently in other pathogens such as *P. aeruginosa* (62) and *Pasteurella multocida* (95). Our characterizations of MPH determinants encoded within the chromosomes of cave isolate *B. paraconglomeratum* as well as closely related environmental isolate *B. faecium* provides the evidence to suggest an environmental reservoir of the *mph* genes**.**

Mobile genetic elements play an important role in the dissemination of antibiotic

resistance genes between bacterial strains (1). The presence of a transposase-like protein upstream of *B. faecium mph* but not the cave-derived *B. paraconglomeratum mph* is particularly alarming, as it could suggest the potential for mobilization of this gene. This study provides an early warning that enzymatic inactivation of macrolides through MPHs could be more prevalent in environmental bacterial communities than previously anticipated. Furthermore, characterized MPHs were capable of inactivating three generations of macrolides, which implies that most of the clinically used macrolides are susceptible to MPH-catalyzed inactivation.

It was interesting to find *mph* resistance genes with same biochemical properties despite the long isolation between *Brachybacterium* strains. It is possible that before the cave was sealed off millions of year ago, *mph* gene was a shared trait between these bacteria. Based on the genetic context map of *mph* genes, it is hard to say if these genes are a part of some metabolic pathway, thereby serving some other function, unrelated to antibiotic resistance. Investigating the cave ecosystem for potential macrolide producers will help in understanding the role of microbial ecology (i.e. presence of antibiotic producers) in antibiotic resistance and if these genes are resistance elements meant for conferring antibiotic resistance.

Antibiotics that have found human and agricultural applications are largely derived from environmental bacteria, whose biosynthetic pathways are believed to have evolved over millions of years (1, 30, 97). As such, it has been suggested that resistance is the result of dynamic microbe-chemical interactions over an equally long period of time which predate the human use of antibiotics. The finding of an *mph* gene encoded in

the genome of a cave strain isolated for more than 3 million years provides further support to the growing view in the field that antibiotic resistance elements are ancient and have probably been circulating among bacterial populations for millions of years (31, 96, 97).

It was interesting to find no tetracycline resistance in Gram-negative isolates. Tetracyclines are widely used in clinics and farming practices, which is believed to provide a driving force for the dissemination of resistance (98). Since this ecosystem has not been exposed to tetracyclines through human or agricultural use, this finding provides evidence to support the correlation between antibiotic usage and emergence of resistant bacteria (99). It was equally interesting to find substantial aminoglycoside resistance compared to surface bacteria (22) which may indicate the presence of antibiotic producers in Lechuguilla cave ecosystem. Unlike surface bacteria, no resistance was observed for the synthetic drugs ciprofloxacin and linezolid, semi-synthetic derivatives of natural products rifampicin and minocycline and naturally produced antibiotic vancomycin (22). It is important to note here that lack of resistance against some of these antibiotics may be a result of small sample size and not enough biological diversity within the collection; perhaps looking at strains from more diverse sample sites within the cave will be important for further understanding of these trends.

We showed in this study that despite being annotated as predicted APH in the genome of the environmental *B. faecium* strain, the protein had MPH-like activity, suggesting apparent flaws in the genome annotation process. Complete phylogenetic analysis of MPH/MPH-like proteins suggests that there are other putative sequences that

fall on the MPH-Ia side of the tree possessing MPH activity rather than APH activity. Verification of this would serve to highlight the deficiencies of automatic annotation, or annotation by transfer of incomplete knowledge. The distinct grouping of MPH sequences within the APH tree supports the construction of an MPH-specific profile model which could be used to annotate MPH sequences to assist in the exclusion of the APH sequences in future applications. Functional characterization and experimental validation of proteins annotated as putative resistance determinants, therefore, becomes important to truly understand the nature and prevalence of these resistance proteins in diverse bacteria.

It has been suggested that antibiotic resistance elements evolved from proteins involved in other metabolic functions. For example, 3D structural and biochemical characterization of APHs has revealed links with eukaryotic protein kinases. Previous studies have shown conservation of C-terminal regions between APHs and MPHs and relatedness in their mechanism of action (28, 29). Computational analysis using Pfam protein database (this study) revealed that MPH from cave isolate have protein-kinase like fold and could belong to the same family as Aminoglycoside Phosphotransferase family (29). Comprehensive structure and functional characterization will provide further insights into origin and evolution of MPHs. 3D structural studies will be important for development of MPH inhibitors to overcome this resistance.

One of the intrinsic limitations of this study was that antibiotic resistance was investigated only in culturable bacteria and since cave microbes are adapted to survive in oligotropic (near starvation) conditions, cultivating them in nutrient conditions is

extremely challenging (100). The majority of strains from these ecosystems are not amenable to culturing, therefore our sample size represents only a fraction of microbial diversity and resistance diversity which can be found in the cave ecosystem. Metagenomic analysis will be a useful approach to further explore the depth and extent of cave resistome (8). Although these isolates have never been exposed to clinical antibiotics, natural exposure is likely as there may be antibiotic producing species in the cave environment. Therefore, understanding the role of microbial ecology will be crucial for getting a complete picture of antibiotic resistance in cave ecosystem. Furthermore, the criteria used for defining hits/resistant strains excluded phenotypes which exhibited low levels of resistance (i.e. < 50% growth with an antibiotic) which likely resulted in an underestimation of level of resistance in the cave ecosystem.

## SIGNIFICANCE

Numerous studies have shown that environmental bacteria are a reservoir of previously unanticipated antibiotic resistance determinants and this study lends further support to the idea that even in absence of obvious human sources of contamination, antibiotic resistance is prevalent. Although resistance catatylzed by MPHs is not a prevalent mode of resistance utilized by pathogenic bacteria, increased usage of macrolides and introduction of new derivatives could eventually serve to provide resistance pressure for MPH-mediated resistance. Furthermore, finding *mph* genes in *Brachybacterium* strains and *mph*-like genes in diverse set of bacteria (based on phylogenetic analysis) highlights the extent and depth of antibiotic resistome and understanding the scope of this resistance mechanism can assist in developing strategies for overcoming this resistance mechanism. Finally, the information from this study sheds some light on the origin and dissemination of *mph* resistance genes.

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## APPENDIX A. List of Strains

| Isolate | Sample site | Isolation media | Antibiotics * | 16S ID | Gram |
|---|---|---|---|---|---|
| LC 3 | LCECE | FC | | | |
| LC 4 | LCECE | FC | | Bosea sp. PD 23 | - |
| LC 5 | LCECE | FC | | Devosia neptuniae | - |
| LC 9 | LCECE | VM/C/NPS/P/CAS | | Streptomyces mauvecolor | + |
| LC 10 | LCECE | VM/C/NPS/P/CAS | | Devosia neptuniae | - |
| LC 11 | LCECE | VM/C/NPS/P/CAS | | Ensifer adhaerens | - |
| LC 12 | LCECE | VM/C/NPS/P/CAS | | Sphingobium yanoikuyae | - |
| LC 13 | LCECE | VM/C/NPS/P/CAS | | Same as LC11 | - |
| LC 14 | LCECE | VM/C/NPS/P/CAS | | Same as LC11 | - |
| LC 17 | LCDS1B | VM/C/NPS/P/CAS | | Massilia timonae | - |
| LC 18 | LCDS1B | VM/C/NPS/P/CAS | | Agrococcus jenensis | + |
| LC 19 | LCDS1B | VM/C/NPS/P/CAS | | Ochrobactrum intermedium | - |
| LC 23 | LCDS1B | C/P/CAS | | Beta proteobacterium HI-B12 | - |
| LC 29 | LCECE | VM/NPS/P/CAS | | Streptomyces flaveus | + |
| LC 30 | LCECE | HC | | Streptomyces anulatus | + |
| LC 31 | LCECE | H | | Streptomyces anulatus | + |
| LC 34 | LCECE | VM/NPS/P/CAS | | Agrobacterium tumefaciens | - |
| LC 36 | LCECE | VM/NPS/P/CAS | | Brevundimonas vesicularis | - |
| LC 37 | LCECE | VM/NPS/P/CAS | | Acinetobacter calcoaceticus | - |
| LC 38 | LCECE | VM/NPS/P/CAS | | Ochrobactrum anthropi | - |
| LC 44 | LCDS1B | VM/NPS/P/CAS | | Brachybacterium paraconglomeratum | + |
| LC 54 | LCECE | HC | | Ensifer adhaerens | - |
| LC 65 | LCDS1B | NPS | | Ochrobactrum anthropi | - |
| LC 69 | LCECE | NPS | | Arthrobacter sp. M4 | + |
| LC 70 | LCDS1B | H | | Microbacterium sp. AC35 | + |
| LC 74 | LCDS1B | H | | Rhodococcus erythropolis | + |
| LC 75 | LCECE | H | | Rhodococcus erythropolis | + |
| LC 77 | LCECE | H | | Sphingomonas sp. HI-K4 | - |
| LC 78 | LCECE | H | | Streptomyces mauvecolor | + |
| LC 79 | LCECE | H | | Nocardia asteroides | + |
| LC 81 | LCECE | H | | | |
| LC 83 | LCECE | H | | Same as LC77 | - |
| LC 85 | LCECE | F | | Bosea thiooxidans | - |
| LC 86 | LCECE | F | | Same as LC85 | - |
| LC 87 | LCECE | F | | Same as LC85 | - |
| LC 92 | LCECE | F | | Same as LC85 | - |
| LC 103 | LCECE | VM/C/C1 | | Mesorhizobium plurifarium | - |
| LC 104 | LCECE | VM/C/C1 | | Alpha proteobacterium clone RR4A6 | - |
| LC 112 | LCDS1B | F | | Uncultured Roseomonas sp | - |
| LC 113 | LCDS1B | F | | Same as LC112 | - |
| LC 143 | LCEA1 | FC | | | |
| LC 145 | LCEA1 | FC | | Rhizobium galegae | - |
| LC 148 | LCEA1 | FC | | Rhizobium sp. ORS 1465 | - |
| LC 149 | LCEA1 | FC | | | |
| LC 153 | LCEA1 | FC | | Kocuria rosea | + |
| LC 163 | LCEA1 | DW | | Ensifer adhaerens | - |
| LC 230 | LCEA1 | VM/C/NPS/P/CAS | | Paenibacillus lautus | + |
| LC 231 | LCEA1 | VM/C/NPS/P/CAS | | Same as LC230 | + |
| LC 236 | LCEA1 | VM/C/NPS/P/CAS | | Pseudoxanthomonas mexicana | - |
| LC 238 | LCEA1 | VM/C/NPS/P/CAS | | Naxibacter varians | - |

| Isolate | Sample site | Isolation media | Antibiotics* | 16S ID | |
|---|---|---|---|---|---|
| LC 241 | LCEA1 | PCAS | | Sphingomonas pseudosanguinis | - |
| LC 242 | LCEA1 | PCAS | | Paenibacillus lautus | + |
| LC 249 | LCECE | VM/C/NPS/P/CAS | | Microbacterium esteraromaticum | + |
| LC 263 | LCEA1 | PCAS | | Pseudomonas stutzeri | - |
| LC 265 | LCEA1 | PCAS | | Same as LC242 | + |
| LC 268 | LCDS1B | P/C/CAS | | Massilia timonae | - |
| LC 278 | LCEA1 | VM/C/C1 | AMP | Microbacterium phyllosphaerae | + |
| LC 285 | LCEA1 | FC | TN | Brevibacterium casei | + |
| LC 289 | LCEA1 | FC | AMP | Arthrobacter sp. Ellin159 | + |
| LC 363 | LCECE | DW | | Sphingopyxis alaskensis | - |
| LC 364 | LCEA1 | DW | | Ochrobactrum anthropi | - |
| LC 365 | LCEA1 | VM/NPS/P/CAS | AMP | Actinobacterium CH21i | + |
| LC 368 | LCEA1 | VM/NPS/P/CAS | AMP | Microbacterium hominis | + |
| LC 371 | LCEA1 | VM/NPS/P/CAS | | Pseudomonas stutzeri | - |
| LC 378 | LCEA1 | VM/NPS/P/CAS | | Mesorhizobium mediterraneum | - |
| LC 379 | LCEA1 | DW | | Sphingomonas sp. HI-K4 | - |
| LC 383 | LCDS1B | HC | | Sphingomonas dokdonensis | - |
| LC 384 | LCDS1B | HC | | Ensifer adhaerens | - |
| LC 387 | LCEA1 | VM/NPS/P/CAS | AMP | Tetrathiobacter kashmirensis | - |
| LC 390 | LCEA1 | VM/NPS/P/CAS | AMP | Leucobacter alluvii | + |
| LC 391 | LCEA1 | DW | | Variovorax sp. HI-I4 | - |
| LC 392 | LCEA1 | VM/C/NPS/P/CAS | | Bosea sp. CRIB-12 | - |
| LC 400 | LCEA1 | VM/C/NPS/P/CAS | AMP | Pseudomonas stutzeri | - |
| LC 401 | LCEA1 | VM/C/NPS/P/CAS | AMP | Dietzia maris | + |
| LC 404 | LCEA1 | VM/C/NPS/P/CAS | AMP | Brevundimonas aurantiaca | - |
| LC 409 | LCEA1 | VM/C/NPS/P/CAS | AMP | Microbacterium esteraromaticum | + |
| LC 411 | LCDS1B | HC | | Sphingomonas sp. HI-K4 | - |
| LC 412 | LCECE | VM/C/NPS/P/CAS | | Rhodococcus erythropolis | + |
| LC 415 | LCEA1 | VM/NPS/P/CAS | AMP | Rhodococcus erythropolis | + |
| LC 421 | LCEA1 | FC | | Achromobacter sp. LMG 5911 | - |
| LC 424 | LCEA1 | VM/NPS/P/CAS | AMP | Microbacterium sp. AC35 | + |
| LC 425 | LCEA1 | VM/NPS/P/CAS | | Pseudoxanthomonas mexicana | - |
| LC 458 | LCEA1 | H | | Bacterium RRP-E5 | - |
| LC 469 | LCEA1 | F | | Microbacterium esteraromaticum | + |
| LC 485 | LCEA1 | PCAS | | Brevibacterium casei | + |
| LC 486 | LCEA1 | PCAS | | Brevibacterium casei | + |
| LC 498 | LCECE | VM/NPS/P/CAS | | Ochrobactrum anthropi | - |
| LC 499 | LCECE | VM/NPS/P/CAS | | Ensifer adhaerens | - |
| LC 500 | LCECE | VM/NPS/P/CAS | | Sphingomonas yanoikuyae | - |
| LC 506 | LCDS1B | P/C/CAS | | Ochrobactrum intermedium | - |
| LC 507 | LCDS1B | P/C/CAS | | Microbacterium esteraromaticum | + |
| LC 508 | LCECE | VM/C/NPS/P/CAS | | Devosia neptuniae | - |
| LC 509 | LCEA1 | FC | | Micrococcus luteus CV39 | + |
| LC 510 | LCECE | VM/NPS/P/CAS | | Acinetobacter calcoaceticus | - |
| LC 511 | LCDS1B | VM/C/NPS/P/CAS | | Brevundimonas vesicularis | - |
| LC 513 | LCDS1B | VM/C/NPS/P/CAS | | Methylobacterium lusitanum | - |

VM = Vitamins Minerals
P = Pyruvate
C = CaCO3
C1 = Methanol/formate
F = Fulvic acid
H = Humic acid
DW = Distilled water
NPS = Nitrogen/Phosphate/Sulfur
CAS= CAS Amino Acids
AMP= Ampicillin
TN= Trimethoprim
* Antibiotics added to the media