Transformed *Legionella* for Application in Engineering Process Validation in the Built Environment

by

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ABSTRACT

Legionella pneumophila is a waterborne pathogen that causes Legionnaires' disease, an infection which can lead to potentially fatal pneumonia. In a culture-based technique, *Legionella* is detected using buffered charcoal-yeast extract (BCYE) agar supplemented with L-cysteine, Iron salt and antibiotics. These supplements provide essential and complex nutrient requirements and help in the suppression of non-target bacteria in *Legionella* analysis. *Legionella* occurs naturally in freshwater environments and for their detection; a sample is plated on solid agar media and then incubated for several days. There are many challenges in the detection of *Legionella* in environmental waters and the built environments. A common challenge is that a variety of environmental bacteria can be presumptively identified as *Legionella* using the culture-based method. In addition, proper identification of *Legionella* requires long incubation period (3-9 days) while antibiotics used in BCYE agar have relatively short half-life time. In order to overcome some of the challenges, *Legionella* has been genetically modified to express reporter genes such Green Fluorescent Protein (GFP) that can facilitate its detection in process validation studies under controlled laboratory conditions. However, such studies had limited success due to the instability of genetically modified *Legionella* strains. The development of a genetically modified *Legionella* with a much rapid growth rate (1-2 days) in simulated environmental systems (tightly-controlled water distribution system) is achieved. The mutant *Legionella* is engineered by transforming with a specific plasmid encoding CymR, LacZ and TetR genes. The newly engineered *Legionella* can grow on conventional BCYE agar media without L-Cysteine, Iron salt and only require one antibiotic (Tetracycline) to suppress the growth of other microorganisms in media. To the best of our knowledge, this

is the first report of *L. pneumophila* strain capable of growing without L-Cysteine. We believe that this discovery would not only facilitate the study of the fate and transport of this pathogen in environmental systems, but also further our understanding of the genetics and metabolic pathways of *Legionella*.

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CHAPTER 1

INTRODUCTION

Legionella pneumophila is a waterborne pathogen that causes Legionnaires' disease, an infection which can lead to potentially fatal pneumonia. The first identified *Legionella* case was in 1976 at the American Legion convention, and it caused 182 illnesses and 29 deaths[1]. The bacterium responsible for the deaths was identified in 1977 and named *Legionella pneumophila*. Upon further investigation, the Centers for Disease Control and Prevention (CDC) was able to identify *Legionella* from fifty-year-old tissue samples that had been stored from pneumonia- associated deaths[2]. From 2000, *Legionella* cases have reached an unprecedented level with annually gradual increase reaching more than 6000 cases in 2015 in the US with about 10 % fatality rate. Because *Legionella* is an opportunistic pathogen and is likely underdiagnosed, the accurate number of incidences is believed to be underestimated[3].

Legionella is a facultative intracellular pathogen that can infect a wide range of hosts, from Free-living amoeba to mammalian cells[4]. *Legionella spp.* has a unique fatty acid composition. More than 80% of the fatty acids contain long branched chains[5]. That kind of fatty acids, which is common in Gram-positive bacteria, results in poor staining. Indeed, *L. pneumophila* can be identified by its unique cellular branched-chain fatty acids as a Gram-negative bacterium[5]. Though, *Legionella* identification from environmental samples seems to be associated with some challenges. *Legionella* can grow on media supplemented with L-cysteine and Iron and requires the presence of strong antioxidants and antibiotics to suppress other microorganisms. Proper identification of *Legionella*

requires long incubation period (3-9 days) while antibiotics used in BCYE agar have relatively short half-life time. We believe these challenges and difficulties in the identification have limited many studies in on the characterization of *Legionella* water systems.

Understanding the factors that affect *Legionella* growth and survival in the natural environment and ultimately in manmade systems is an important step in controlling this pathogen. In this study, we attempted to genetically develop transformed *Legionella* that can overcome some or all of these challenges. The specific objectives of the present study were to construct a genetically modified *Legionella* to be distinguishable easy-detected using a plasmid that carries a resistance gene and a selective marker such as GFP or β-galactosidase. To construct such a *Legionella* cell, pQF-lacZ cumate-inducible plasmid that has tetracycline gene and lacZ gene that codes for β-galactosidase was chosen to be used.

In addition, we studied *Legionella* concentrations in central Arizona source water including reservoirs and rivers in a monthly monitoring program starting 2016.
The goal of the program is to better understand the seasonality of *Legionella* in surface waters and the conditions that affect its occurrence and growth.

CHAPTER 2

LITERATURE REVIEW

2.1. Legionella Detection and Identification

Legionella in the environment is a facultative intracellular parasite that can only replicate inside a host such as amoebae, which not only can serve as a host but also protect *Legionella* from environmental stresses such as pH and chlorination[6]. *Legionella* is a Gram-negative non-spore forming parasite which may become filamentous on mature stage when grown *in-vitro*[7]. The bacterium is not encapsulated, and its cell wall consists of an outer cell membrane that has unique high lipopolysaccharide content, which results in a poor staining Gram-negative organism[2]. Iron compounds stimulate the growth of *Legionella*, and the amino acids are believed to be the carbon and nitrogen sources. *Legionella* is also non-fermentative meaning that it does not hydrolyze gelatin nor utilize carbohydrates[2]. *Legionella* can be grown using buffered charcoal-yeast extract (BCYE) agar supplemented with L-cysteine, Iron salt and antibiotics. However, not all *Legionella spp.* can be grown in BCYE, and sometimes can be detected using other molecular techniques or co-culturing with a cell host such as amoeba[6].

2.1.1. Legionella under the microscope

Legionella grows on BCYE or any other *Legionella* selective media with L-cysteine incubated for up to 10 days at 35-37°C with or without 2-5% CO_2. Mature *Legionella* colonies appear as convex, circular white colonies with frosted glass appearance and are 3-4 mm in diameter based on the number of colonies on a plate. The colony iridescence is lost as the colonies get older[8]. Young colonies of *Legionella* show a variety of shades of

pink, purple, green or blue depending on the type and the thickness of the agar plate and the age of the culture as they tend to become gray with age[9]. *Legionella* appears under the microscope as Gram-negative with faintly staining thin rods, which may be filamentous in mature cells.

2.1.2. Identifying *Legionella* colonies

Identifying *Legionella* colonies could be done based on the colonial appearance. Since *Legionella* take at least 36 hours before colonies can be detected, it is a good idea to check the plates that have the potential of having suspect colonies after 24 hours to mark the grown colonies as non-*Legionella* colonies. Another quick way to narrow down the suspected colonies is to add a part of the suspected colony into 3% Hydrogen Peroxide, H_2O_2, solution to check the presence or the absence of Catalase enzymes. All *Legionella spp.* have Catalase Enzyme[10], and oxygen (O_2) bubbles will be formed when a *Legionella* colony subjected to an H_2O_2 solution. Comparative genomics of *L. pneumophila* and a close relative bacterium, *Coxiella burnetii*, which contain two peroxide-scavenging alkyl hydro-peroxide reductase shows that *Legionella* has the same enzymes and they are used as a defense against oxidative stress[11].

2.1.3. Identifying *Legionella* Serotype

Because *L. pneumophila* serogroup 1 causes most cases of Legionnaires' disease, techniques have been developed to specifically detect *L. pneumophila* serogroup 1. Techniques include an enzyme immunoassays (EIA), an indirect fluorescent antibody test (IFA), ELISA, latex agglutination screening test all can be used with high relative sensitivity and specificity[9]. However, there are some cross-reactions with antibodies that have been reported with *Campylobacter* species, *Pseudomonas aeruginosa, Haemophilus*

species and potentially other bacteria[12]. However, since the clinical detection is only for *L. pneumophila* serogroup1, *Legionella longbeachae* and other *Legionella* species may be under-diagnosed[13].

2.1.4. Matrix Assisted Laser Desorption/Ionization - Time of Flight Mass Spectrometry (MALDI-TOF MS)

Matrix assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) is an ionization technique that uses a laser energy absorbing matrix to create ions from biomolecules, such as DNA, RNA, proteins and sugar, as well as large organic molecules such as dendrimers and polymers[14]. MALDI-TOF MS can be efficiently and accurately used to analyze the protein composition of a bacterial cell. It is an emerging technology for species identification that compares the protein composition of a bacterial cell into current-database of known bacteria protein composition. The identification using MALDI-TOF MS is time efficient with high analysis sensitivity compared to other identification methods. A full analysis of a single colony can be done within few minutes with a high degree of simplicity and low cost which allows for rapid screening of a large number of colonies in a short time[15]. Currently, more than 38 *Legionella* species are already submitted to Whole Cell Mass Spectrometry (WCMS) with MALDI-TOF, and therefore this technology has been used to successfully identify *Legionella* species[16]. The level of accuracy depends on the similarity between the suspected bacteria and the database data. However, the differentiation between *L. pneumophila* serogroups is still not possible using this technology[17].

2.1.5. Nucleic Acid Amplification Tests

Polymerase Chain reaction (PCR) is widely used for bacterial detection as it is simple, rapid, sensitive and specific by designing primers that target 16S rRNA gene which is highly conserved between different species of bacteria and at the same time contains hypervariable regions that can provide species-unique signature sequences useful for identification of bacteria[18 19]. *L. pneumophila* can be identified and differentiated from other *Legionella* species using PCR technology[20]. Real-Time PCR is also known as quantitative polymerase chain reaction (qPCR) is usually considered to be a good method for bacterial detection and quantitation if the *Legionella* concentration in the sample is needed. However, sometimes it is difficult to design species-specific primers when the sequences of the 16S rRNA genes have high similarity among species. Multiplex real-time PCR, that uses multiple primer pairs in a reaction mixture to amplify multiple targets in a single PCR experiment, can be used simultaneously detect and discriminate *L. pneumophila* serogroup 1 from other *L. pneumophila* and *L. pneumophila* from *Legionella* species[21]. The main advantage of using PCR instead or with other diagnostic methods is that PCR can detect viable, non-viable, culturable and non-culturable microorganisms and viruses.

2.1.6. Rapid Molecular Typing methods

There are a variety of rapid molecular genotyping techniques that have been developed to enable subtyping of unrelated strains with some variation of accuracy, discriminatory power, and reproducibility. These techniques include Pulsed Field Gel Electrophoresis (PFGE), Multi-locus Sequence Typing (MLST), Multiple-Locus Variable-number Tandem Repeat Analysis (MLVA) and Whole Genome Sequencing (WGS). Molecular techniques that assist gene sequence analysis have had an enormous impact on *Legionella* taxonomy

and increased our understanding of the phylogenetic relationships between *Legionella* and other related organisms which have resulted in recognition of numerous new species[22].

2.2. Legionella in the Environment

Understanding the factors that affect *Legionella* growth and survival in the natural environment and ultimately in manmade systems is an important step in controlling the bacterium. It will give us an indication of the areas and systems that have a higher risk of *Legionella*, thereby identifying the points at which control measures will be most effective to be identified. In this section, we will discuss the natural sources of *Legionella*, the effect of temperature and microbial communities on *Legionella* growth and how the biofilm formation enhances *Legionella* survival and growth in water systems.

2.2.1. Legionella Natural Sources

Legionella spp. are ubiquitous in the environment, and survive in a variety environmental conditions[23]. The bacteria can tolerate acid (pH of 2.2) exposure for short periods, about 30 minutes, and they have been isolated from environmental sources ranging from a pH of 2.7 to 8.3[24]. *Legionella* has been found in diverse water bodies such as lakes, groundwater and even sea water[25] [26]. *Legionella* may be present below the detection limit in certain natural aquatic environments that have a low temperature which potentially could introduce *Legionella* into a system within the built environment where *Legionella* proliferate. Soil could be a potential source of *Legionella* which might have been underestimated. There are some reported cases of Legionnaires' diseases that were to be caused *L. pneumophila* found in soil [27]. Also, *Legionella* longbeachae cases increased in some parts of the world especially New Zeeland and Australia. *Legionella* longbeachae is abundant in the soil which is known to be less virulent than *Legionella pneumophila*.

However, since the clinical detection is only for *L. pneumophila* serogroup1, *Legionella* longbeachae and other *Legionella* species cases may be under-diagnosed[13].

2.2.2. Effect of temperature and microbial communities on *Legionella* growth

Legionella spp. are known to survive in relative hot-water systems where temperatures are below 66 ^{O}C[28]. However, all *Legionella* species tested were found to decrease at temperatures above 45 °C with a growth limiting about 50 °C[29]. *Legionella* can withstand a temperature of 50 °C for several hours without significant reduction. In the lab, we subjected *Legionella* to elevated shock temperatures up to 62 °C for 1 minute as an attempt to chemically transform a plasmid pQF-LacZ to *L. pneumophila* (see section 3.2.1.), and we found that the difference between *Legionella* concentrations that were heat-shocked with temperatures ranging from to 48 to 62 °C is neglect-able. Complex warm-water systems such plumbing systems, spa/swimming pools and air conditioners encourage *Legionella* growth. Since *Legionella* is L-Cysteine dependent and require a host to grow, warm water may give the bacterium an advantage over the other amoeba-resistant bacteria (ARB) such as *Cryptococcus neoformans, Chlamydophila pneumoniae, Mycobacterium avium, Listeria monocytogenes* and *Pseudomonas aeruginosa*[30]. A study by Yee & Wadowsky (1982) showed that *L. pneumophila* survives and multiply at temperatures range of 25 °C and 45 °C with an optimal temperature between 37-42 °C. At temperatures below 37 °C, the growth rate decreases with almost no growth at temperatures below 20 °C[31]. Warm systems also have higher potential to produce aerosols resulted in increasing of *Legionella* spread. *Legionella* species identification in warm-water tanks or in thermally polluted rivers emphasizes that water temperature is one of the most crucial factors in *Legionella* colonization of water distribution systems[32].

It is believed that *Legionella* cannot grow without a host cell because if their special need to L-cysteine and Iron which present in the environment in very low concentrations. *In-vitro*, *Legionella* are only able to grow if L-cysteine and Iron salt are supplemented. *L. pneumophila* survives for a long time, but do not multiply, in sterile tap water[33], but it is able to survive and multiply in non-sterilized tap water[31]. These results suggest that *L. pneumophila* is completely dependent on other microorganisms grow and to hide from biocides as well. *L. pneumophila* has been associated with many different microorganisms from aquatic sources include protozoa, *Fischerella spp.*, fungus and other bacteria[34 35]. Protozoa are the most likely is the most important factor for the survival and growth of the bacterium within both natural and artificial environments. In the natural environment, *L. pneumophila* proliferates in protozoa within intracellular phagosomes. The pattern of *L. pneumophila* growth in macrophages in human and in some susceptible animals such as pigs, rats and mice is similar to that in protozoa[36].

2.2.3. Biofilm Role in *Legionella* growth in Water systems

Biofilm formation occurs in all natural and artificial environments on different surface types. Microorganisms form biofilm to escape the adverse conditions such as biocides, heat and low nutrients concentrations. Biofilms are complex heterogeneous microbial ecosystems and they usually consist of bacteria, algae and grazing protozoa[37]. *L. pneumophila* is one of these organisms that can form or colonize biofilm[38]. The important role of biofilm in *Legionella* growth and survival can be indicated from swap samples vs. flowing water samples where *Legionella* is relatively easily detected from swab samples than from flowing water [39]. Biofilms that might include *Legionella* and protozoa can form on the surfaces of old or poorly managed buildings and cooling towers. The biofilm

protects microorganisms from biocides and from increases in temperature and pressure. It also facilitates nutrient and gaseous exchange within the microbial communities. Biofilm is most likely to be formed at interface between liquid and solid faces such as pipes internal perimeter where the velocity of water is very slow, and water can stagnate.

Extracellular matrix that provides structure, nutrients flow, stability and protection from biocides and other toxic substrates is formed by microorganisms embedded within the biofilm. The interactions between *Legionella* and other bacteria are barely understood. There are some microorganisms such as *E. coli, Acinetobacter baumanii,* and *Flavobacterium breve* that are commonly found in the aquatic environments and biofilms that harbor *Legionella*. These microorganisms can be used by *Legionella* to colonize an existing biofilm[40]. Other microorganisms such as *Pseudomonas aeruginosa* and *Klebsiella pneumonia* are believed to compete and inhibit *Legionella* colonization of a biofilm[40]. *Legionella* species may enhance or suppress colonization of other *Legionella* species. Stewart and Burnside (2011) showed that *L. pneumophila* produces a bio-surfactant which has antimicrobial properties, and the one produced by *L. pneumophila* has been shown to inhibit the growth of other *Legionella* species and therefore reduces species competition[41].

Oxygen, pH and nutrients gradient in the biofilm allow the formation of heterogeneous microbial populations within the biofilm matrix[42]. The presence of biofilms in water distribution systems is an important factor in the *Legionella* growth and survival[43]. A single study has shown that *Legionella* can grow in biofilm in the absence of protozoa where a high concentration of Cycloheximide was added to inhibit the growth of eukaryotic cells which increased the heterotrophic and *Legionella* counts[44]. Another study, on the other

hand, using a biofilm model showed that *Legionella* solely multiplies intracellularly in amoeba[45] which in agreement with almost all other studies.

2.3.Legionella Disinfection Methods

Legionella, among others, inhabits the manmade systems such as water distribution systems (WDSs), cooling towers, swimming pools and fountains. In particular, the aerosols of these water systems pose a significant health risk. It is important to prevent the water systems from being contaminated by *Legionella* and to control the risk of exposure[46]. Disinfection and treatment of drinking water from opportunistic pathogens such as *Legionella* are essential, and there are several approaches commonly used such as copper-silver ionization, heat-and-flush, hyper-chlorination, monochloramine, chlorine dioxide point-of-use filtration, ultraviolet (UV) light and Ozone.

2.3.1. Copper-silver ionization

Copper and silver have been used for centuries because of their biocidal mechanisms. Historically, Vikings used copper on their ships to prevent algal growth and shells, and the same mechanism is still applied in the modern ships. Silver was used by some of nomads, Europeans and Russians to improve the water quality for generation[47]. The disinfection principle of copper and silver ionization is to produce positively charged copper and silver ions which will form electrostatic bonds with the negative bacteria cell wall sites. These bonds create stress and ultimately distort cell wall permeability and denature proteins[48]. Copper and silver ions are proven to penetrate biofilms in where protozoans, fungi, *Legionella*, and other bacteria are protected from biocides[49] [50]. The application of this method is by flowing the water into a device that applies low potential electricity to silver and copper electrodes which would result in releasing positively charged copper and silver

ions into the flowing water[51]. The recommended copper and silver concentrations for adequate *Legionella* removal are 0.2-0.4 and 0.02-0.04 mg/L respectively[52]. The efficacy of copper-silver ionization is proven *in-vivo* against *Legionella* and *in-vitro* against a variety of waterborne pathogens such as *Legionella*, *Pseudomonas auruginosa, Acinetobacter baumannii, Stenotrophomonas maltophilia* and *Mycobacterium* species[52]. Another advantage of this method is the long and short-term applicability. However, ionization becomes less efficient when the water pH > 7.6[52]. The accumulation of copper and silver in the sediment of hot water tanks and the development of ions resistant bacteria might become issues in the long-term run[53]. Copper-silver ionization and heat-and-flush are the only methods that can be used to treat the whole system without producing toxic by-product.

2.3.2. Heat-and-flush, Thermal Eradication

The heat and flush method was the first method used to control *Legionella* concentration in hospitals' water distribution systems(WDSs)[54]. It is an easy, fast and cost-effective method to temporarily eradicate pathogenic bacteria from the system. Pathogenic bacteria are killed and biofilm is removed in WDSs when subjected to heat shock greater than 60 °C for 0.5-1 hour. Thermal eradication is carried out by introducing hot tap water, 60-70 °C, into the system and flushing all outlets. The hot water circulates the network and should have at least temperature of 55 °C at the outlets[55]. Long time exposure temperature greater than 60 °C is found to inhibits non-sporulation bacteria including *Legionella*[56]. The efficiency of this method is proven *in-vitro* studies, however, *in-vivo* it is highly dependent on the buildings water networks, and a recent study has shown that heat inactivation was not effective in more than half of the studied buildings (n=718)[57]. Maintaining high water

temperature (60 °C) for a longer period (2 days) can be more effective for controlling *Legionella*[58]. Heat and flush method is effective against wide range of bacteria and protozoa, but it is also temporary disinfection and the system most likely will be recolonized in weeks after thermal disinfection[59 52]. This method does not produce disinfection by-product but can increase the risk of scalding in pipes[59 60].

2.3.3. Oxidizing Substances

There are several oxidizing agents that are commonly used in disinfection such as Chlorine, Monochloramine, Chlorine dioxide, Ozone and Hydrogen Peroxide. In chemistry, an oxidizing substance is an agent that can oxidize other substances, causes them to lose one or more electrons. Oxidizing substances usually contain oxygen and/or hydroxyl radical group with one or more unpaired electrons. Radicals from these substances are very reactive, and can easily oxidize organic matters such DNA, proteins and lipids which will damage the cell and ultimately kill when there is an adequate concentration of the oxidizing agent.

*2.3.3.1.*Chlorine

Chlorine as a gas or as hypochlorite salts (sodium hypochlorite or calcium hypochlorite) is directly added to water. Alternatively, Free chlorine in the form of hypochlorite can be generated in situ by anodic oxidation device from chloride naturally present in water or from added sodium chloride[61]. Hypochlorous acid (HOCl) has pKa of 7.53, and at pH higher than pKa, the acid appears in its ionic form (H^+ and OCl^-) which was found to be less effective than hypochlorous acid on its natural form (HOCl)[61]. The recommended concentration of free chlorine in the system is 1-2 ppm (particle per million) while for hyper-chlorination, shock treatment, the recommended concentration is10-50

ppm for 12-24 h[60]. For in-situ chlorine generation by anodic oxidation, the concentration of free chlorine should be between 0.3-0.5 ppm[61]. *Legionella* can be readily inactivated using 1.25 mg/L of chlorine for 15 min, and by 5 mg/L for 1 min[62]. In these concentrations, electron transport is impaired and cell lysis is induced. It is however unknown if growing *Legionella in-vitro* where L-cysteine is amply supplied sensitize *Legionella* toward HOCl, H_2O_2 and Ozone. To control *Legionella*, continues hyper-chlorination has been used *in-vivo* and *in-vitro* studies with variable success, and chlorine is found to suppress *Legionella* when associated with protozoans rather than killing it[63]. The limited ability of chlorine to penetrate the biofilm matrix where most of *Legionella* in the system exists is another issue associated with *Legionella* removal by chlorine disinfection[63]. Chlorine is known to be corrosive and compromised by elevated temperatures, and it produces toxic disinfection by-product such as trihalomethanes[63] [59]. However, chlorine is cost-efficient, effective against wide range of pathogens even at low concertation (1 ppm), and a relatively stable oxidizing compound which makes it the most commonly used disinfectant in WDSs.

*2.3.3.2.*Monochloramine

Inorganic chloramine is rapidly generated by mixing hypochlorous acid (HOCL) with ammonia (NH_3). At natural water condition where pH is greater than 7 and no excess hypochlorite, monochloramine which is most effective chloramine compound is the dominant chloramine compound. It is more stable but much less effective disinfectant than HOCl. The recommended concentration is 1-10 ppm[56], and several studies have shown that monochloramine is effective against *Legionella* in simulated plumbing systems[64] [52]. Monochloramine is found to be more effective than chlorine *Legionella* inactivation[65]. Drinking water pH is usually between 7.3 and 8, and monochloramine is can be still

effective compared to copper-silver ionization and chlorination which become less effective with pH more than 7.6[52 59]. monochloramine is not known to have corrosive property compared to other chloride compounds such free chlorine and chlorine dioxide[52]. Chloramine disinfection forms little disinfection byproducts such as trihalomethanes which makes it often used in the US secondary disinfection. However, monochloramine have also some drawbacks such as the complexity associated with on-site generation, increased ammonia and lead concentrations in drinking water, increased the population of other opportunistic pathogens such as *Mycobacterium* species[52]. It is also more difficult to remove from water compared to free chlorine and chlorine dioxide[60].

*2.3.3.3.*Chlorine Dioxide (ClO_2)

Chlorine dioxide is a chlorine gas that is generally produced in the site since it is dangerous and difficult to transport. Chlorine dioxide is usually by produced mixing chemical precursors such as sodium chlorite ($NaClO_2$) and a strong acid (HCl)[52]. Chlorine Dioxide is usually used as a primary disinfectant with wider working pH than free chlorine, and it is frequently used to remove color, odor, taste, iron and manganese compounds. It is also effective against chlorine-resistance parasites such as *Cryptosporidium*[66]. The targeted concentration to maintain efficacy is 0.3-0.5 ppm at the point of use. Most chlorine dioxide, about 70%, in treated water is converted to chlorite ions (regulated inorganic DBP with MCL of 1 mg/L), therefore, the maximum chlorine dioxide concentration in water is set to be about 1.4 mg/L[67]. Compared to chlorine, chlorine dioxide penetrates biofilm more effectively, less corrosive and less pH dependent[55]. However, total eradication of *Legionella* based on various field studies was not achieved in some of these studies[52]. Planktonic *Legionella* is easily removed using this method, but when associated with

protozoa, *Legionella* can survive the regular dose concentration[68]. Chlorine dioxide is easily removed from water by aeration or carbon dioxide (CO_2). The main two disadvantages of Chlorine dioxide are that it produces a relatively high number of disinfectants by-products (DBPs), especially chlorite and chlorate, and it is difficult to maintain the desired concentration at distal points.

*2.3.3.4.*Ozone (O_3)

Ozone is the most powerful known oxidizing substance used in water disinfection. It is effective against a wide range of bacteria, viruses and parasitic protozoa such as *giardia* and *cryptosporidium* which are hardly removed from water by chlorination. Ozone is generated by applying an extremely high voltage or UV-light that decompose molecular oxygen (O_2) into two oxygen radicals ($^{\bullet}O$) which reacts again with O_2 to form O_3. Organic and inorganic compounds can be oxidized by ozone; therefore, it is widely used to remove odor, color, taste, and pesticides. It also has been found to sufficiently reduce DBPs if followed by Granular Activated Carbon(GAC) treatment. Ozone, and chlorine dioxide to some extent, poses high disinfecting properties against wide range of free-living amoeba (FLA)[69]. Coexistence of *Legionella* and FLA in natural environments has been shown in different field studies[70], and it is believed that reducing the number of FLA would result in a reduction of *Legionella* concentration because of its strictly intercellular growth. Ozone with as little as 0.1 mg/L can inactivate 2-log of *Legionella* in 5 min[71]. However, the main disadvantage of ozone as a disinfectant is that decomposes quickly and it is hard to maintain effective concentrations in the system, and therefore, it can only disinfect at the point of injection.

2.3.4. Ultraviolet light (UV)

The first use of UV to disinfect drinking water was in 1910 in France in a phenotype plant before it was shut down due to poor reliability. UV light is a light that has a shorter wavelength, higher in frequency, than visible light and therefore more energy. UV light in a range between 200 and 300 nm is considered germicidal where the optimum range wavelength for UV energy absorption by DNA is between 240-280 nm. Energy from UV is absorbed by DNA which leads to changes in the base pairing and creates thymine dimers (T=T) and DNA gaps in microorganisms' genes. A low-pressure mercury lamb that produces UV light with a wavelength of 254 nm is commonly used because of its high efficiency and durability. For controlling *Legionella*, UV device is placed at the entry of the system such as building water inlet or can also be placed on the tap-points[72]. Efficacy of UV light to disinfect the system from *Legionella* has been proven *in-vitro* and *in-vivo* studies as well[73] [63]. UV disinfection, similar to ozone, is only effective at the point of use and it does not provide residual protection. Using UV alone is insufficient to control *Legionella* and should be combined with other methods such as hyper-chlorination or heat pasteurization to effectively control *Legionella*[56].

2.3.5. Point-of-use (POU) filtration

Point-of-use water filtration could be used to physically remove pathogenic bacteria such as *Legionella spp.*, *Pseudomonas aeruginosa*, and mycobacteria *spp.*, and fungi. Filters with a pore size of 0.1-0.2 micron are commonly used to remove contaminants from water[52]. The previously described disinfection methods (heat-and-flush, oxidizing substances and UV) efficiently reduce *Legionella* concentration from water distribution systems, however, complete *Legionella* elimination cannot be claimed. In some high-risk areas where an individual requires higher standards of care or in known contaminated taps,

POU filters could be used to eliminate pathogenic bacteria from water. It also is better tolerated by customers and patients than other disinfection methods. Several studies have proven the efficacy of these filters to eliminate *Legionella* from contaminated water in different hospitals[74] [68] and high-risk areas such as transplant units[75]. Filters could be installed at the inlet of the water systems or at tap-points. Some disadvantages of point-of-use filters are that filters are a potential source of contamination, suitable only for small drinking water systems and filters require to be changed frequently (2-4 weeks)[59].

2.4.Legionella uniqueness

2.4.1. Legionella Unique Growth/Nutrients Requirement

Culture-able *Legionella* species grow on Buffered Charcoal Yeast Extract (BCYE) with L-Cysteine and Iron salt such as ferric pyrophosphate, ferric chloride, ferric nitrate, and ferrous sulfate. The only issue with the latter three compounds is media transparency. BCYE media consists of 5 grams of Yeast Extract which is enough to provide nutrients including amino acids, as carbon and nitrogen source, required to support *Legionella* growth. The inability of *Legionella* to grow in common laboratory media such as Luria broth (LB) and Tryptic Soy Broth (TSB) may be due to the lack of strong antioxidants in these media since *Legionella* cannot tolerate very low levels of hydrogen peroxide and superoxide radicals[76]. Bacteria culture media have ascorbate and several phenolic compounds and autoxidation compounds generate superoxide radicals catalyzed by light. The culture media is even more prone to photochemical oxidation when autoclaved rather than being filter-sterilized[76]. The presence of L-cysteine in media, which is known to be unstable and is rapidly oxidized in media to L-Cystine, is known to form thiol radicals in media and these formed radicals are propagated at the presence of H_2O_2, and to a less

degree molecular oxygen(O_2) or another L-Cysteine molecule. It is believed that radical chain reaction may be formed and propagated in media in the presence of L-cysteine absence of strong antioxidants such as charcoal and α-Ketoglutaric acid.

Since *Legionella* is very sensitive to oxidative stress, the media used to grow *Legionella* should include strong antioxidants such as Powder Activated Charcoal (PAC), α-Ketoglutaric acid, Sodium pyruvate, Glutamate, Sodium Selenite and/or graphite, to some extent[77]. Additional ingredients added to media such as proteose peptone, beef extract, refabricated horse blood and liver extract enhances *Legionella* growth even in the absence of activated charcoal as a strong peroxides decomposer[78]. The reason of this enhancement could be one or more of the following, (1) these ingredients have some antioxidant activity or easily oxidized compounds such as pyruvate and α-Ketoglutaric acid, (2) the additional ingredients increase the buffer capacity which stabilizes the pH during growth, (3) the additional ingredients supply *Legionella* with easily accessible nutrients such as iron and (4) the additional ingredients may increase the broth turbidity and therefore minimize the photochemical oxidation.

2.4.2. *Legionella* oxidative stress "Cysteine-Iron-H_2O_2 paradox"

Reactive species such as reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS) are normally produced during cell function as a response to various stimuli. *Legionella* intolerance toward general oxidative stress *in-vitro* has led researchers to adopt strong antioxidants in *Legionella* cell culture media such as activated charcoal and α-Ketoglutaric acid. Several studies also have shown that cysteine and cystine play a role in bacteria sensitivity, in general, toward oxidative stress[79 80]. L-cysteine consumption during *Legionella* growth is only about 0.1 mM out of 2.3 mM

supplied (~0.04%). L-cysteine in Buffered Yeast Extract Broth (BYEB) is rapidly oxidized to L-cystine in the presence of iron salt (ferric pyrophosphate) which as a result could be reduced to ferrous iron (Fe^{2+})[80 81]. The conversion reaction is reversible and the equilibrium is pH dependent (pKa = 8 for the thiol/disulfide) and therefore, L-cystine can be used alternatively and would support the growth of *Legionella* especially on pH between 6.2 and 6.5 using a chemically defined medium (CDM) as previously described[82 83]. The addition of 0.4 gram of L-cysteine at pH of 6.9 results in steady state concentration of L-cysteine of 0.2-0.5 mM (>0.1 mM) which can support *Legionella* growth[84]. We don't know if the reduced ferrous iron (Fe^{2+}) could promote the Fenton reaction where ferrous iron (Fe^{2+}) reacts with H_2O_2 to form hydroxyl radicals and hydroxyl group:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \quad \text{(The Fenton reaction)}$$

This possible reaction may explain the high sensitivity of *Legionella* species grown *in-vitro* toward hydrogen peroxide, H_2O_2. *Legionella* species shows sensitivity to relatively low concentration of H_2O_2, about 26.5 μM[76]. Another study found that high levels of intercellular cysteine in *E. coli* drive Fenton reaction and promote oxidative DNA damage. It was concluded that L-Cysteine sensitized bacterial cells, such as *E. coli*, toward H_2O_2[80]. *In-vitro*, *Legionella* sensitivity toward H_2O_2 could be because of the high levels of supplied L-cysteine. The suggested driving mechanism behind that is cysteine. Cysteine, as a reducing agent, reduces ferric iron with an exponential speed which permits free iron (Fe^{2+}) which has a strong catalytic power to generate hydroxyl radicals ($^{\bullet}OH$) inside and outside the cell. In the presence of H_2O_2, Iron and Cysteine/L-cysteine, the highly reactive hydroxyl radical group would accumulate in the cell which makes cells more vulnerable to oxidative DNA damage. This damage occurs in three steps[80]:

(1) $RS\text{-}H + Fe^{3+} \rightarrow RS{=}SR + Fe^{2+}$

(2) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$ (The Fenton reaction)

(3) ${}^{\bullet}OH + DNA \rightarrow H_2O + damage$

In *Legionella* media, (RS-H) represents L-Cysteine, (RS=SR) represents L-Cystine, Fe^{3+} represents ferric pyrophosphate and Fe^{2+} represents: ferrous pyrophosphate.

*2.5.*Transformed *Legionella* Cumate-Inducible Expression System

2.5.1. Cumate-Induicable Expression Systems

Inducible gene expression systems are a powerful tool to study physiology and effects of different conditional expressions of a specific gene in the system. Most of these expressions systems rely on a transcriptional repressor that controls, turn off, the expression of the gene of interest by binding on specific DNA sequence, the operator site, in the promoter region in the absence of the inducer. These transcriptional repressors are either expressed from the chromosomal DNA such as lacI repressor naturally exist on some bacteria, or it could be added to the plasmid along with the gene of interest and selective marker gene or antibody-resistance gene. Despite the simple concept of a plasmid, identification of high inducible promoters, control elements and the different induction conditions for a specific organism is often a hard task. There are some factors that make difficult to use a particular system in an organism other than its original such as the inducer transport through the cell, specificity of the RNA polymerase and the requirements of cofactor for full promoter activity. These obstacles can be overcome by engineering artificial inducible systems by placing highly constitutive promoter of an organism of interest with operator site and a repressor. In this study, a Cumate-inducible expression system that has LacZ gene was used. Cumate-Inducible systems use CymR gene from

Psudmenous ptudia as a repressor that recognizes and binds to the following sequence ACAAACAGAC-N6-GTCTGTTTGT[85]. The two region CymR recognizes are inverse complementary to each other as shown in red in Figure 1.

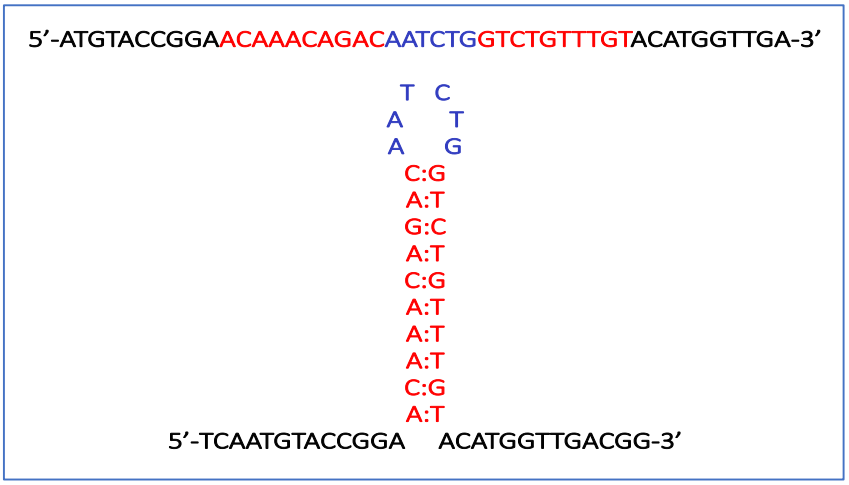

Figure 1: The sequence of CymR DNA-binding site on the promoter–operator region

The inducer is cumate, a non-toxic inexpensive cell-permeable organic substance. Cumate interacts with CymR and changes its conformation and will no longer bind to the operator sequence. The cumate-inducible plasmid, pQF-lacZ, was a gift from Julia Vorholt (Addgene plasmid # 48094)[86]. The plasmid has CymR gene, Tetracycline resistance gene, lacZ gene, origin of replication (ori) sequence, Cuo sequence and other features for transformation by conjunction as shown in Figure 2.

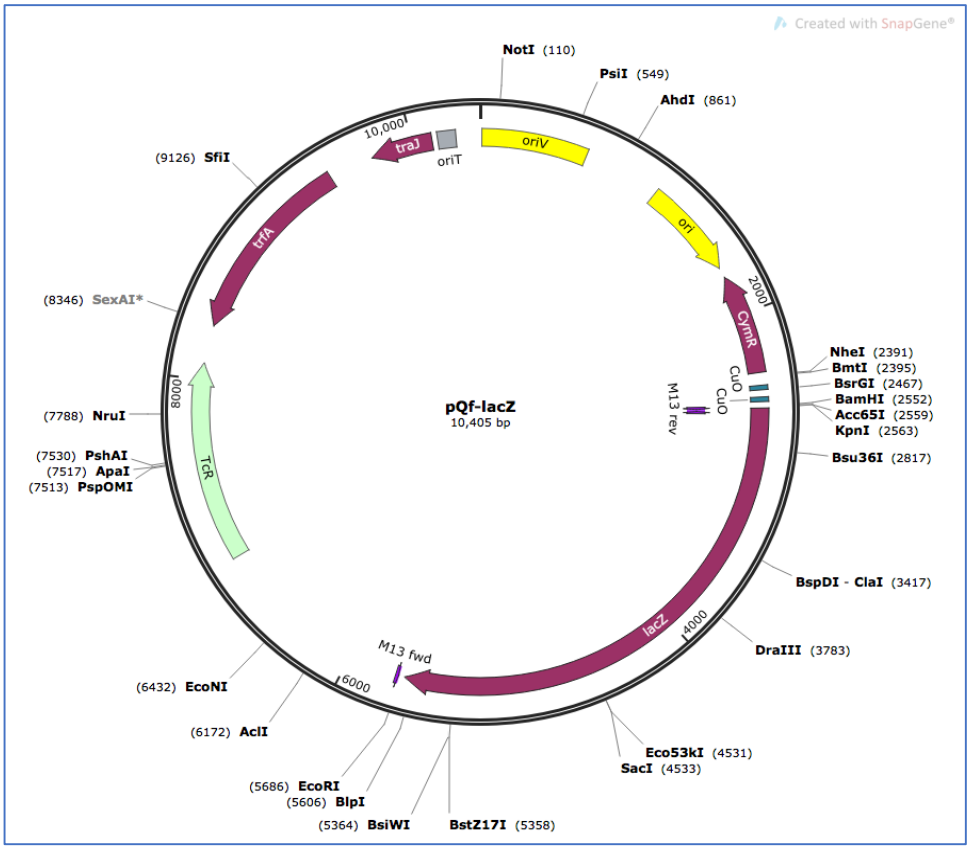

Figure 2: pQf-lacZ map

lacZ gene that codes for β-galactosidase protein can be used as a selective marker and can be used to determine the promoter strength. β-galactosidase is a protein that converts lactose, and similar-lactose products, into glucose. It is commonly used in molecular biology with X-gal and ONPG which are soluble colorimetric substances and in the presence of β-galactosidase, they are hydrolyzed into galactose and colored substance. X-gal is converted into 5,5'-dibromo-4,4'-dichloro-indigo- 2, an insoluble blue compound. and is usually used in blue-white screening. ONPG is utilized by β-galactosidase into galactose and ortho-nitrophenol, soluble yellow substance, and is used usually to measure the enzyme activity.

CHAPTER 3

MATERIALS AND METHODS

3.1. Legionella spp. culturing from Environmental Samples.

As a part of the Regional Water Quality project at Arizona State University, monthly water samples were collected from surface water sources across central Arizona. These samples are assayed for bacteria including *E. coli, Mycobacterium and Legionella*. Samples are collected from 20 different surface water locations in Arizona State, USA shown in Figure 3.

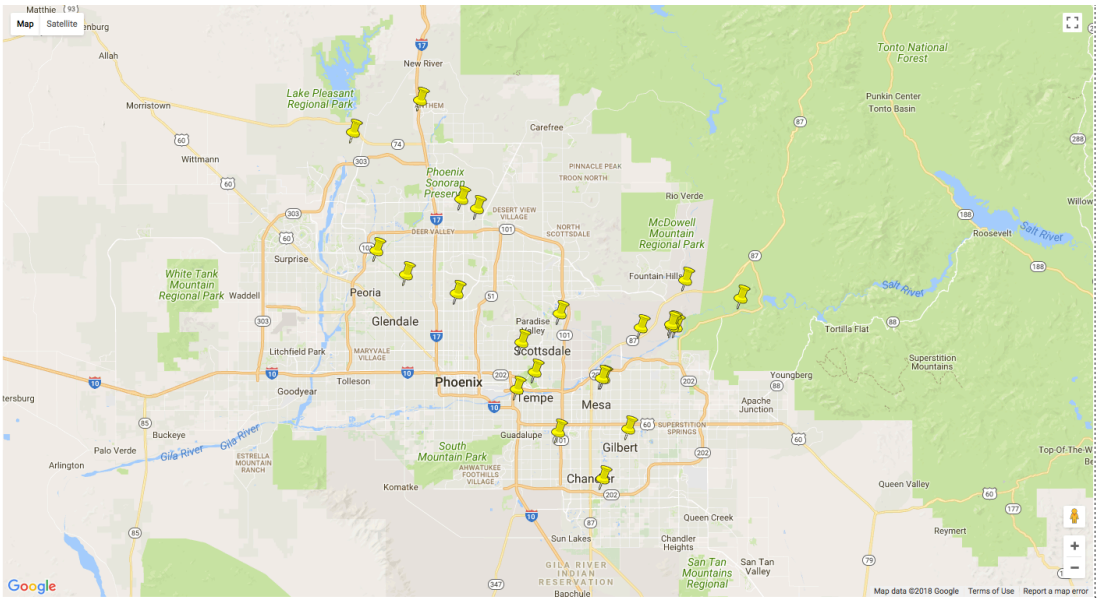

Figure 3: Samples collection locations map

Samples for *Legionella* assay are kindly collected and provided by Andrew Buell, a Ph.D. student at ASU. All samples are transported to the laboratory in insulated coolers to protect samples from heat and light. Samples are immediately stored at 4 °C upon arrival and assayed .100 ml samples are filter-concentrated by pouring the samples into a sterile 47-mm filter funnel assembly containing a 0.45um filter (EMD Millipore). A vacuum source and flask are used to operate the apparatus properly. When the sample passes

through the filter, the filter is removed aseptically from the holder with sterile filter forceps, laid (upside/down) on buffered charcoal yeast extract (BCYE) agar medium (NEOGEN #7728A) with antibiotics (described in detail in section 2.2.) and incubated at 37 °C overnight to allow *Legionella* contact with the nutrients. The filters are then removed and samples are re-incubated for 7 to 10 days. *Legionella* colonies are counted based on the morphological and phenotypic characteristics of the bacterium, and reported as CFU/100 ml. No further confirmation analysis of *Legionella* colonies such as PCR, IFA or EIA were performed.

3.2. Legionella Culture Media

All the environmental samples are assayed using BCYE agar with supplements and antibiotics. ACES (N-(2-Acetamido)-2-aminoethanesulfonic acid with pKa of 6.8) is added to BCYE to maintain pH of the media at pH of 6.9 and to provide the required osmolality. Sodium Chloride and Sodium Hydroxide are found to inhibit *Legionella* growth and therefore Potassium Chloride and Potassium Hydroxide are used to adjust pH and osmolality[87]. Vancomycin, polymixin B and cycloheximide are added to increase the selectivity of the media and to suppress other microorganisms, especially eukaryotic cells and Gram-positive bacteria[88]. All supplements and antibiotics include vancomycin and polymixin B, cycloheximide and L-cysteine are carefully and aseptically added after cooling down the media to 45-50 °C to prevent heat inactivation of the reagents. Quality of the prepared media is occasionally tested using *L. pneumophila* ATCC 33153. Culture plates are placed in the incubator (Forma Scientific#3111) at 37 °C up to 10 days.

*3.3.*PQF-lacZ plasmid

The plasmid is received as transformed bacteria (Top10 *E. coli*) in stab culture format. The bacteria strain is streaked upon arrival on LB agar plate with Tetracycline, Cumate and X-gal with final concentrations of 10 mg/L, 100 µM and 40 mg/L respectively. A single colony was isolated and grown on 10 ml of TSB with Tetracycline, and the plasmid was purified using GeneJET Plasmid Miniprep Kit (ThermoFisher: K0502). NanoDrop™ UV-Vis Spectrophotometer (ThermoFisher) is used to assess the purity and the concentration of extracted and purified plasmid.

*3.4.*Chemical Transformation of *L. pneumophila* with pQF-lacZ.

In order to transform pQF-LacZ plasmid into *Legionella pneumophila*, we tried a chemical transformation method taking advantage of the fact that *Legionella* is known to survive in relative hot-water systems where temperatures are below 66 ^{O}C[28]. The heat shock temperature was elevated from 42 °C, which is commonly used in *E. coli* and other bacteria, up to 62 °C. 5 different heat shock temperatures were used, 48, 52, 58, 60 and 62 °C. To make chemically competent *Legionella*, the bacterium was grown in BCYE for 72 hours and harvested with NPH_2O. OD600 of cells is adjusted to 0.5 and subjected to centrifugation and resuspension twice with NPH_2O. Pellet is then suspended in TSS buffer which contains (5g PEG 8000, 0.3 g $MgCl_2*6H_2O$, 2.5 ml DMSO and the volume adjusted to 50 ml using LB). The buffer is filter sterilized using 0.45 µm Nylon membrane. Cells are then stored at –20 °C till use.

Another attempt to chemically transform *Legionella* with pQF-lacZ was done using iTOP technology, A small-molecule-based method that serves as an efficient method for the transduction of native proteins and other macromolecules into primary mammalian

cells[89]. This method depends on NaCl-mediated hyper-osmolality and non-detergent Sulfobetaine-201 (NDSB-201) to intracellularly deliver macromolecules. This technique is recently discovered, and it was successfully used to deliver native proteins and small RNA molecules such as CRISPR application where they deliver recombinant Cas9 protein and its short guide RNA into primary cells directly instead of transforming a plasmid that carries Cas9 gene. Since iTOP functions through micropinocytosis, we decided to try transforming pQF-lacZ using this technique with some optimization. High bacteria concentration of approximately $10^{10\text{-}11}$ cell/ml was used to achieve transformation using this technique. Since NaCl might inhibit *Legionella*, we decided to use KCl along with NaCl to increase osmolality and causes hypertonicity. The concentration of NDSB-201 used for transformation was 25 μM while different concentrations of NaCl and KCl were used as shown in Table 1.

Table 1: Different temperatures, NaCl and KCl concentrations used in iTOP transformation

Sample ID	Osmolality (mOsm/L)	NaCl (g/L)	KCl (g/L)	Temperature (°C)	NDSB-201 (mM)
800 Na	800	23.4	0	RT	25
800 Na+K	800	11.7	15.9	37	25
800 KCl	800	0	31.8	37	25
400 NaCl	400	11.7	0	42	25
400 KCl	400	0	15.9	42	25
200 KCl	200	5.85	0	42	25

The osmolality was achieved by centrifuging and washing the cells two times with NPH_2O and then suspending the pellet using solution with 25mM NDSB-201 with 400 mM NaCl or KCl. Dilutions were made using 25 mM NDSB-201solution. Around 0.1 μg

plasmid was added into 50 µl of cells and incubated for 15 minutes at the given temperatures. After incubation, tubes volume is adjusted into 500 µl using LB broth without NaCl to reduce the osmolality and incubated for 6 hours. The concentration of cells after volume adjustment is still relatively high, around $10^{9\text{-}10}$. Volume is then adjusted to 10 ml and antibody (Tetracycline) is added and incubated overnight at 37 °C to select for transformants. Isolated transformed cell colonies are then obtained by streaking cells on BCYE + Tetracycline.

*3.5.*Electrical Transformation of pQF-lacZ into *Legionella*

Electroporation is the preferred method used for transformation a plasmid into *Legionella*. High transformation efficiency can be achieved using this method which makes it perfect for screening purposes as well as basic transformation. Electrical component *Legionella* was prepared by grown the bacterium on BCYE and harvested after 72 hours by washing them out of the plate using nano-pure water. Cells concentration was adjusted to 2 OD600, and were subjected to centrifugation and resuspension twice with nano-pure water with 10% Glycerol. Cells concertation was condensed to achieve a concentration of $10^{10\text{-}11}$ and were stored at -80 °C till use. *Legionella* transformation procedure was done using high electric field strength (22 kV/cm) with a pulse time of 5 ms and high cell density around $10^{10\text{-}11}$ using Eppendorf Electroporator 2510. The electroporation conditions were adapted from previous studies aimed to optimize *Legionella* transformation using electroporation[90 91]. 90 µL of ($10^{10\text{-}11}$ CFU/mL) cells were added with 2 µL of pQf-lacZ plasmid (22 ng/µL) into 0.1 cm sterile Cuvette 5 minute before electroporation. After the pulse, cells were immediately and gently transferred into 4 ml of chilled *Legionella* Broth, and incubated at 37 °C with shaking. Cells recovery is measured after 1 hour and 4 hours

and three different samples are taken each time with different tetracycline concentrations, 0, 10 and 25 μg/ml. Samples are diluted to measure the transformation efficiency.

3.6.L. pneumophila confirmation using PCR.

PCR reaction is occasionally carried to confirm transformed *Legionella* colonies using LEG225 (5 -AAGATTAGCCTGCGTCCGAT-3) and LEG858 (5-GTCAACTTATCGCGTTTGCT-3) primers which are specific for the bacterial 16S rRNA gene and result in approximately 654 base pairs DNA band[92]. PCR cycles are as follow, one cycle of 95°C for 5 min followed by 30 cycles of 95°C for 10 s, 64°C for 1 min, and 72°C for 1 min and a final extension cycle of 72°C for 5 min was performed. before adding PCR reagents mixture, A small portion (> 10^4 cells) of a suspected colony is suspended in 10 μL of ddH2O and incubated at 95 °C for 5 min instead of DNA purification to reduce DNases activity in *Legionella* before the addition of primers and other reaction reagents. For each PCR reaction containing, 10 μL of Bacterial suspension in H_2O, 1 μM primers LEG-858 and LEG255 (around 1.25 μL) and 12.5 of 2x PCR ready-mix (Fermentas). PCR results are conducted to suspected colonies along with known *Legionella* colonies as a control. PCR products are confirmed by Gel Electrophoresis made with SYBR™ Green (ThermoFisher) to visualize double-stranded DNA bands under UV.

3.7.H_2S detection in Transformed *Legionella*

When *Legionella* with pQF-lacZ plasmid was incubated, the colonies that grew in the middle of the plate were darker than the ones growing near edges, and there was a foul smell in the incubator. Since the colonies near the perimeter have a whiter appearance, it was assumed that a toxic gas was produced by the bacterium which could be from

volatilization of indole (x-gal byproduct) or could be something else such as hydrogen sulfide H_2S. The indole could be the cause of that odor, however, since the indoxyl glycoside, the insoluble color, was not detected, we assumed that H_2S could be the cause of that odor. To investigate whether it is metabolically possible for *Legionella* to convert the sulfur from L-cysteine into H_2S. We searched *L. pneumophila* genome using KEGG (Kyoto Encyclopedia of Genes and Genomes) database[93], and found several pathways that could utilize L-cysteine and produce H_2S.

One possible pathway to produce H_2S byproduct from L-cysteine is by utilizing cysteines to produce pyruvate. L-cysteine can be directly converted to pyruvate by L-cysteine desulfhydrase enzyme (EC:4.4.1.28)[93]. Another way to form pyruvate from cysteine is from D-cysteine by D-cysteine desulfhydrase (EC:4.4.1.15). D-cysteine is derived from L-cysteine by L-amino acid racemase (EC 5.1.1.100)[93]. Conversion of L-cysteine and D-cysteine into pyruvate would result in the formation of H_2S and NH_3 as byproducts, as shown in Figure 4.

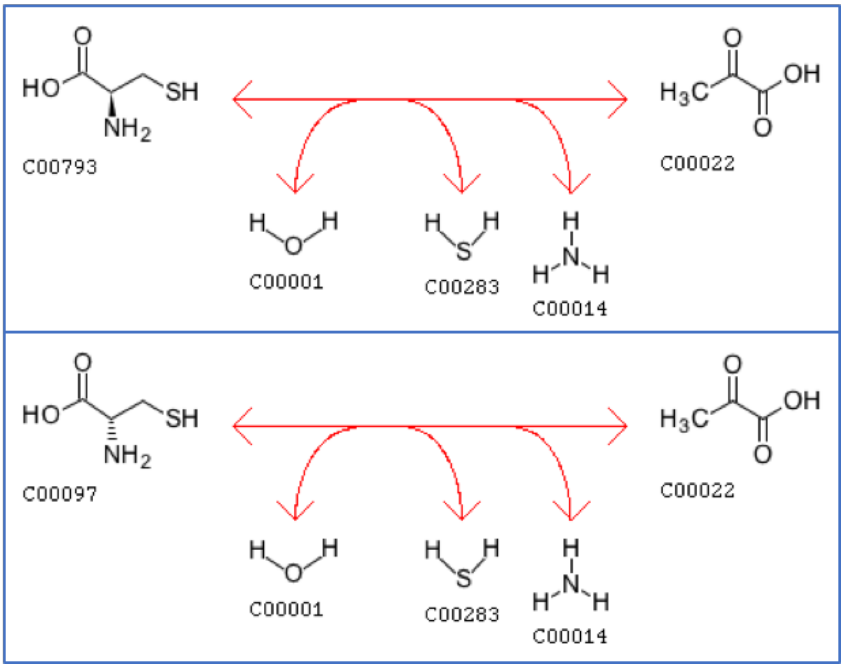

Figure 4: The conversion of D-cysteine and L-cysteine into pyruvate, H_2S and NH_3

Legionella genome also may have Cysteine lyase enzyme (EC 4.4.1.10) which is known to decompose L-Cysteine to L-Cysteate and results in the formation of hydrogen sulfide in one step reaction[93]. The chemical reaction catalyzed by cysteine lyase is shown in Figure 5.

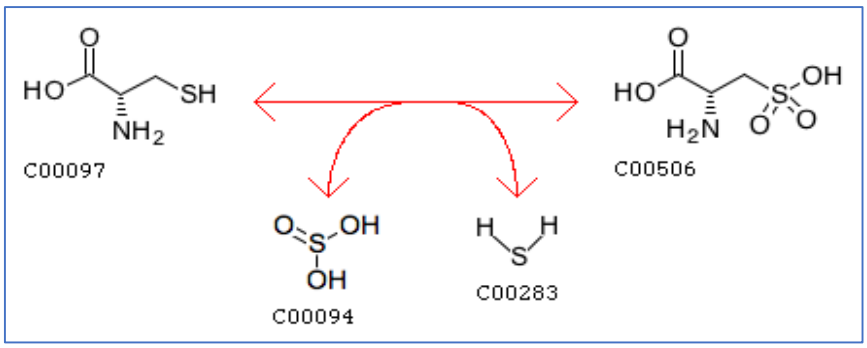

Figure 5: The conversion of L-Cysteine into L-cysteate by cysteine lyase enzyme

To test our hypothesis, the bacterium was regrown in BCYE with L-cysteine. Lead acetate paper was attached to the inner surface of the plate's lid. In the presence of H_2S, it

will react with Lead acetate and form lead sulfide (PbS, a black solid). If PsS washed with H_2O_2, it would be reduced to lead sulfate ($PbSO_4$, a white solid) as explained in the following reaction.

$$PbS + 4H_2O_2 \rightarrow PbSO_4 + 2H_2O$$

The plates were sealed with parafilm to contain any H_2S inside the plate. Accumulation of H_2S will ultimately kill *Legionella*, however, sealing Petri dishes is important to validate negative results.

3.8. Cysteine and Iron Role in *Legionella* Peroxide Sensitivity

The transformed *Legionella* is somehow able to grow on media lacking L-Cysteine, additional Iron salt, α-Ketoglutaric acid and charcoal. The mutant *Legionella* grows in regular laboratory media such as TSA, TSB and LB. The first question we wanted to answer was that if these unique *Legionella* growth requirements, L-Cysteine, Iron salt and strong antioxidants, are related and if the antioxidants are added to *Legionella* media because of adding L-Cysteine or Iron salt. There are two or more possible interoperations why *Legionella* grown *in-vitro* is very sensitive hydrogen peroxide and requires strong antioxidants to support the growth. L-cysteine which is known to rapidly undergo non-enzymatic autoxidation to L-Cystine may form thiol radicals in elevated O_2 levels. These thiol radicals have a very high affinity to react with H_2O_2 and to less extent with O_2 or another cysteine. These reactions will generate other free radicals in the media and probably inside the cell also which would result in propagation of the oxidative process. Another possible phenomenon is that the addition of Iron salt would enhance the Fenton reaction inside the cell where the reduced iron (Fe^{+2}) would react with H_2O_2 and form hydroxyl radicals. To test these two hypotheses, we designed two experiments to show

the importance and the role of α-Ketoglutaric acid for growth stimulation of *Legionella* in the presence of L-Cysteine and/or Iron salt such as ferric pyrophosphate. Three different conditions were tested with and without antioxidant addition, L-Cysteine alone, Iron alone and L-Cysteine with Iron. Agar media with these conditions was used to see if the different nutrients conditions would affect the sensitivity of *Legionella* toward H_2O_2 using a method similar to diffusion disk method. Reagents concentration used with TSA agar is as follow L-Cysteine 0.4 g/L, ferric pyrophosphate 0.25 g/L, α-Ketoglutaric acid 1 g/L. To measure the inhabitation zone caused by the addition of 100 µL of 3% H_2O_2, 100 µL of *Legionella* with pQF-lacZ (OD600= 0.5) was spread on each plate. Autoclaved membrane filters were then placed at the center of each plate, and H_2O_2 was added into the center of the membrane filter. After 30 minutes, plates were inverted and incubated overnight. The other assay used to determine the effects of adding L-cysteine and ferric pyrophosphate into the media was done using TSB broth with similar conditions including a control which is only TSB. The goal of this assay is to see the effect of these different conditions with or without antioxidants on the growth rate of the bacteria.

The second experiment goal is to examine the growth rate difference resulted from addition L-cysteine, iron salt and or α-Ketoglutaric acid. The reagents concentration is similar the previous experiment. 1/100 volume (0.2 ml) of pre-incubated transformed bacteria (OD600=0.56) is transferred to 20 ml of TSB broth with different concentrations of the reagents. Readings were taken hourly using Nanodrop by aseptically transferring 0.5 ml from each sample into 1.5 ml Eppendorf tubes and immediately put on ice to stop growth. The experiment consists of 8 samples, 4 samples with α-Ketoglutaric acid and 4

similar samples without α-Ketoglutaric acid. The 4 kinds of broths are TSB control, TSB with L-cysteine, TSB with iron and TSB with L-cysteine and iron.

Another experiment was conducted using alternative thiols compounds to L-cysteine such as reduced glutathione (GSH), N-acetylcysteine (NAC). The goal of this experiment is to investigate if these sulfur compounds could potentially utilize molecular O_2 and generate peroxides in cell culture. In this experiment, Sodium pyruvate is used as antioxidant instead of α-Ketoglutaric acid. The molar concentrations of GSH, NAC and L-cysteine used in this assay were similar, 2.5 mM. Sodium pyruvate final concentration was 1 mM. The assay was carried on using BioTek Synergy H1 Hybrid Multi-Mode Reader using 24-well plate, and readings were taken every 15 minutes until all samples reached stationary phase. Biological samples were duplicated, and the plate was on continuous orbital shaking (205 cpm).

3.9. Peroxide Sensitivity of transformed and wild-type *Legionella*

To study whether the transformed *Legionella* strain can be used for disinfection studies, we designed an assay to determine whether the transformed strain has similar sensitivity to oxidation compared to the wild-type or not. We think this is important because there is a possibility that high intercellular L-cysteine in *Legionella* grown *in-vitro* may result in underestimation of *Legionella* resistance to disinfectants. The resistance of *L. pneumophila* grown in amoebae is more resistant to high temperatures, pH, disinfectants and osmolality changes from *L. pneumophila* grown in BCYE[94 95]. It is well known that growing cells in culture is different in many ways from cells growing *in-vivo*. Vulnerability to damage, increase in mutations rate, and the antioxidant demands are usually predicted from cells grown *in-vitro*. Some reagents in cell culture impose

oxidative stress which, under certain circumstances, might lead to a misleading conclusion[96].

In This experiment, we harvested wild-type *Legionella* and transformed *Legionella* from BCYE agar. The cells were washed 2 times with Nano-pure water, and OD600 was adjusted to 1. Cells were then diluted to achieve a proximate concentration of 10^3, 10^4 and 10^5 CFU/ml. Plates counting taken before addition of H_2O_2 was considered as time-zero (t=0 min). Samples are plated 10 and 30 minutes after the addition H_2O_2 to achieve a final concentration of 1 mM. H_2O_2 was not neutralized after 10 or 30 minutes, and it was assumed plating the samples on BCYE which consists of activated charcoal will quickly decompose H_2O_2 and neutralize the sample.

In future studies, we are planning to design an experiment to test whether cysteine in media has an effect on the sensitivity of *Legionella* toward disinfectants such H_2O_2 and chlorine. The result of the assay would give us an idea if *Legionella* sensitivity toward disinfectants was previously underestimated because of the high level of intracellular L-cysteine pool resulted from the obligatory L-Cysteine addition to the media.

3.10. β-Galactosidase Activity assay for mutant *Legionella.*

LacZ gene is used in this study as a reporter gene to distinguish between mutant *Legionella* that carries pQF-lacZ plasmid from other bacteria located in the simulated environmental systems. The lacZ gene expression is tightly repressed by CymR, and by the addition of cumate, it interferes with CymR which will result in the CymR dissociation from its DNA binding site. Since the plasmid should have a very strong promoter, the expression of lacZ is expected shortly after cumate addition, less than 10 minutes in the model organisms. However, β-Galactosidase activity was not detected form colonies

grown in agar with X-gal and cumate. The expression of the reporter gene seems to be very low, and this could be from the specificity of the promoter to the model organisms, or it could be because of CymR overexpression in *Legionella*. CymR is global cysteine regulator in many different bacteria, and it might be overexpressed in *Legionella* as a result of unknown factors.

β-Galactosidase activity was determined using the chromogenic substrate ONPG (Biogold) using the method of Miller[97]. Transformed *Legionella* was grown fresh from stock overnight. Next day, the culture was diluted (1/100) by volume and was grown till OD600 of 0.4-0.6. Expression was then induced by adding 100 μM cumate and it was grown with shaking for 3 hours. After 3-hour induction, cells were incubated on ice for 15 minutes to stop any reaction, β-Galactosidase activity assay was made using Roth Lab protocol from UC-Davis[98]. The time of reaction was increased to be 2.5 hours to allow low concentration detection of β-Galactosidase. 4 samples with different cell concentration were used, 0.5, 0.25, 0.125 and 0 ml of cells were assayed. The wavelength readings of OD_{420}, OD_{550} and OD_{600} were taken using Nanodrop for each sample. The β-Galactosidase activity was measured in Miller unit using the following equation.

$$Miller\ Unit = \frac{OD_{420}(1.75 \times OD_{550})}{T \times V \times OD_{600}}$$

Where T is the reaction time and V is the cell volume used.

CHAPTER 4

RESULTS AND DISCUSSION

As noted in the introduction section, the thesis work included a study of *Legionella* occurrence in surface water and the construction of a genetically modified cell. In this section, the occurrence data is presented first (section 4.1) and then the results and discussion of the study of constructing the recombinant *Legionella* cell (section 4.2-4.7).

4.1. *Legionella* seasonality in Arizona Surface Waters

Legionella occurrence and concentrations in central Arizona source water including reservoirs and rivers were monitored on a monthly basis program starting January 2017. The monthly samples collected from different locations in the central region of Arizona were assayed using BCYE containing antibiotics to select for presumptive *Legionella spp.* The number of *Legionella* colonies for each location in 2017 is shown in Table 2

Table 2: Number of CFU/100ml of presumptive *Legionella* for each month from different locations

Sample ID	*Jan (17)*	*Feb*	*March*	*April*	*May*	*Jun*	*July*	*Aug*	*Sep*	*Oct*	*Nov*	*Dec*	*Feb (18)*	*March*
R3	**27**	**20**	**7**	**6**	**5**	**11**	**11**	**9**		**12**	**5**	**7**	**19**	**23**
R11	**32**			**1**	**5**	**8**	**11**	**9**	**3**	**17**		**17**	**15**	**14**
R12	**25**	**7**		**4**	**3**	**5**	**9**	**10**	**9**	**19**	**4**	**5**		**14**
R13	**43**	**22**		**4**		**10**	**10**	**10**	**8**	**13**	**3**	**6**	**13**	**25**
R25	**38**	**18**	**11**	**5**	**2**	**8**	**12**	**7**	**12**	**20**	**8**	**16**	**23**	**18**
CENT	**44**	**21**	**9**	**2**	**3**	**6**	**8**	**8**	**7**	**20**	**10**	**10**	**16**	**9**
HTC	**42**	**29**	**11**	**5**	**5**	**7**	**10**	**3**	**8**	**18**	**3**	**11**	**17**	**8**
HOC	**30**	**23**	**9**	**4**	**3**	**5**	**6**	**8**	**9**	**24**	**7**	**7**	**12**	**16**
PIMA	**28**		**7**	**3**	**2**	**12**	**11**	**11**	**9**	**19**	**4**	**9**	**24**	**10**
56TH	**40**	**26**	**5**	**4**	**1**	**6**	**8**	**7**	**7**	**16**	**4**	**7**	**14**	**22**
MOC	**45**	**24**	**9**	**11**		**5**	**7**	**5**	**1**	**26**		**8**	**28**	**14**
SOCA			**4**	**1**	**6**	**6**	**8**	**17**	**6**	**14**	**5**		**16**	**13**
HWY87	**50**	**22**		**3**	**3**	**9**	**10**	**10**	**8**			**10**	**14**	**19**

- ***Samples couldn't be collected in January 2018.***

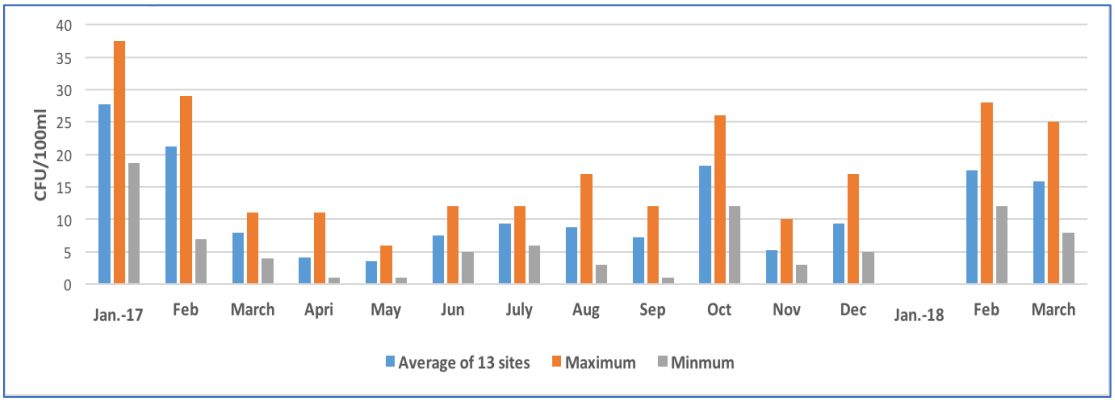

Figure 6: *Legionella* concentration in Arizona surface water

The highest number of *Legionella* colonies were detected in January and February with gradual monthly decrease reaching its lowest in May before it started to gradually increase again (Figure 6). A variety of factors could be responsible for variance observed in *Legionella* concentration from winter to summer. It is known that microbial activities in aquatic systems increase with the increase of ambient temperature; however, *Legionella* concentration in environmental surface water may inversely correlate with the total microbial concentrations. *Legionella* growth in the environment is host-dependent. In summertime, the surface water temperatures are optimal for the growth of wide range of heterotrophic bacteria which may compromise the sensitivity of *Legionella* assay. The high number of bacteria are known to suppress the growth of *Legionella* in media which would result in underestimated its concentration. *Legionella spp.* is also found naturally in soil; therefore, rainfall events may also increase *Legionella* numbers in surface waters.

Pure cultures of known strains of *Legionella pneumophila Knoxville-I* were grown using non-selective BCYE media with only L-Cysteine. While BCYE without L-Cysteine is used as a negative control where wild-type *L. pneumophila* strains do not grow.

The techniques that can be used to enhance assay selectivity are: heat treatment and acid treatment. These techniques may become necessary for water samples with high microbial activity to reduce non-*Legionella* bacteria. Incubation of environmental and clinical *Legionella* samples at 50 °C for 30 minutes dramatically reduces non-*Legionella* bacteria from concentrated water samples by a factor of 39.8-7900 whereas *Legionella* concentration is reduced by a factor of 1.05-1.44[99]. Acid pretreatment also improves the detection of *Legionella spp.* from environmental water samples. The 0.2 M HCl-KCl buffer (pH 2.2) is usually used to eliminate non-*Legionella* (heterotopic) bacteria colonies formation using the plates culture method. For high alkaline samples, The 0.2 M acid-phosphate acid (0.2 M phosphoric acid and potassium dihydrogen phosphate (pH 2.2)), can be used alternatively because of its high buffer capacity[100].

4.2.Transformation of *Legionella*

4.2.1. Transformation by Heat Shock

The pQF-lacZ plasmid (Addgene # 48094) is 10,405 base pairs which is used for expression of lacZ gene (β-Galactosidase) using highly regulated expression system that uses PQ5 promoter and Cumate, 4-Isopropylbenzoic acid, for induction. Additional to LacZ, the plasmid also has CymR gene as lacZ gene expression repressor and Tetracycline resistant gene TcR as a selective marker.

To the best of our knowledge, *L. pneumophila* cannot be transformed by chemical methods used in *E. coli*. In early studies, plasmid transfer into the bacterium was mediated by conjugation which limited the application of this method to specific plasmids that carry gene transfer function[101]. Heat shock using 42 °C for 1 minute is a common method for transforming *E. coli* and some other model organisms, however *Legionella* cannot be transformed using the same criteria. Since *Legionella* can stand temperature as high as 62 °C for short period of time, we used heat shock temperatures (48, 52, 58, 60 and 62 °C) to transform *Legionella* with pQF-lacZ plasmid. The plasmid was added into *Legionella* component cells and was subjected to heat shock for 1 minute. As shown in Figure 7, the survival of *Legionella* was not severely affected by the temperatures used in the heat shock experiments, however, no transformation was observed. The plates on the right side of Figure 7 were used as a survival/negative control to validate any negative result while the plates in the left and in the middle of the picture are selective for transformants.

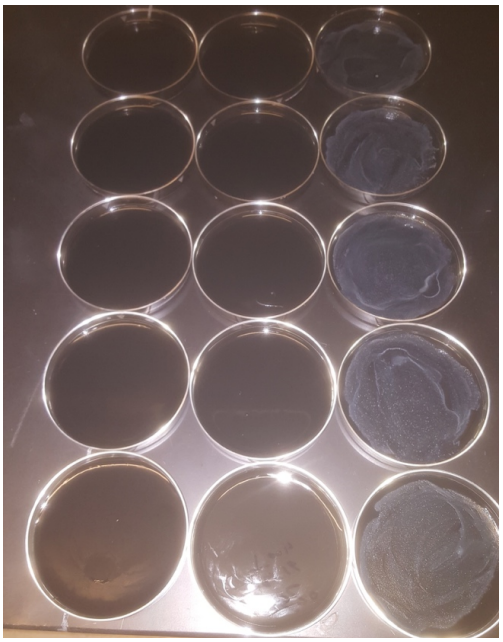

Figure 7: The result of *Legionella* transformation using elevated heat shock

4.2.2. Transformation of *Legionella* using Osmolality and NDSB 201

Legionella transformation was performed using hypertonicity gradient across the bacterial cell membrane and data are presented in Table 3. The concept of hypertonicity and NDSB-201 seems to be promising for bacterial transformation, only if we can optimize the various conditions such as osmolality and NDSB-201 concentrations, cells density, cells recovery and incubation time and temperatures. The results shown in Table 3 confirm that transformation of plasmid into *Legionella* is possible using this method.

Table 3: *Legionella* transformation using NDSB-201 and different osmolality concentrations

Sample ID	Osmolality (mOsm/L)	NaCl (g/L)	KCl (g/L)	Transformation?
800 Na	800	23.4	0	Yes
800 Na+K	800	11.7	15.9	Yes
800 KCl	800	0	31.8	Yes
400 NaCl	400	11.7	0	Yes
400 KCl	400	0	15.9	No
200 KCl	200	5.85	0	No

Transformed *Legionella* was confirmed using PCR and gel electrophoresis. The main drawbacks of using this method for transformation are the low transformation efficiency and post-transformation low recovery of cells after subjecting to high salinity. Reproducibility also could be another issue associated with this method. We do believe that osmolality and NDSB 201 method has the potential to be optimized in future to become an alternative tool for delivering DNA, RNA and native proteins into cells. Future studies may involve compounds that promote cell restoration such as pyruvate and glutamate

Revitalization after sub-lethal injury is critical, as it is assumed that cell would be more receptive to transformation if they have better capability to handle physio-chemical assault without getting killed. Therefore, increasing the prospectus of cell transformation with a foreign DNA and increasing the recovery of cells would increase the efficiency of transformation.

4.3. Transformation of *Legionella* using high-voltage Electroporator

Transformation of pQf-lacZ using electroporation was achieved with relatively high transformation efficiency. Several colonies were chosen and were confirmed by PCR primers targeting a specific region of 16S ribosomal DNA. The number of CFU for 1 and 4-hour incubation time is shown in Table 4.

Table 4: Number of CFU for 1 and 4-hour incubation time with different Tetracycline concentration

Incubation time(hr)	25 µg/ml	10 µg/ml	0 µg/ml
1 (No dilution)	51	82	106
4 (No dilution)	TNTC	TNTC	TNTC
4 (1/10)	193	526	504
4 (1/100)	24	58	70

Legionella usually grows after at least 48 hours of incubation; however, plates were incubated at 37 ^{o}C, and checked for colonies after 30 hours. Surprisingly, *Legionella* colonies were detected after 30 hours. Plates with no antibody were used as negative control, however, the number of colonies grown after 30 hours in plates with no antibody

were similar to the number of colonies grown in selective media (Figure 8) suggesting that these colonies are transformants. That means *Legionella* transformed with pQF-lacZ has higher growth rate than wild-type. Cumate, as an inducer, and X-gal, β-Galactosidase substrate, were used to screen transformants, blue/white screening. However, blue color colonies were not detected even with the 10-fold increase in cumate concentration.

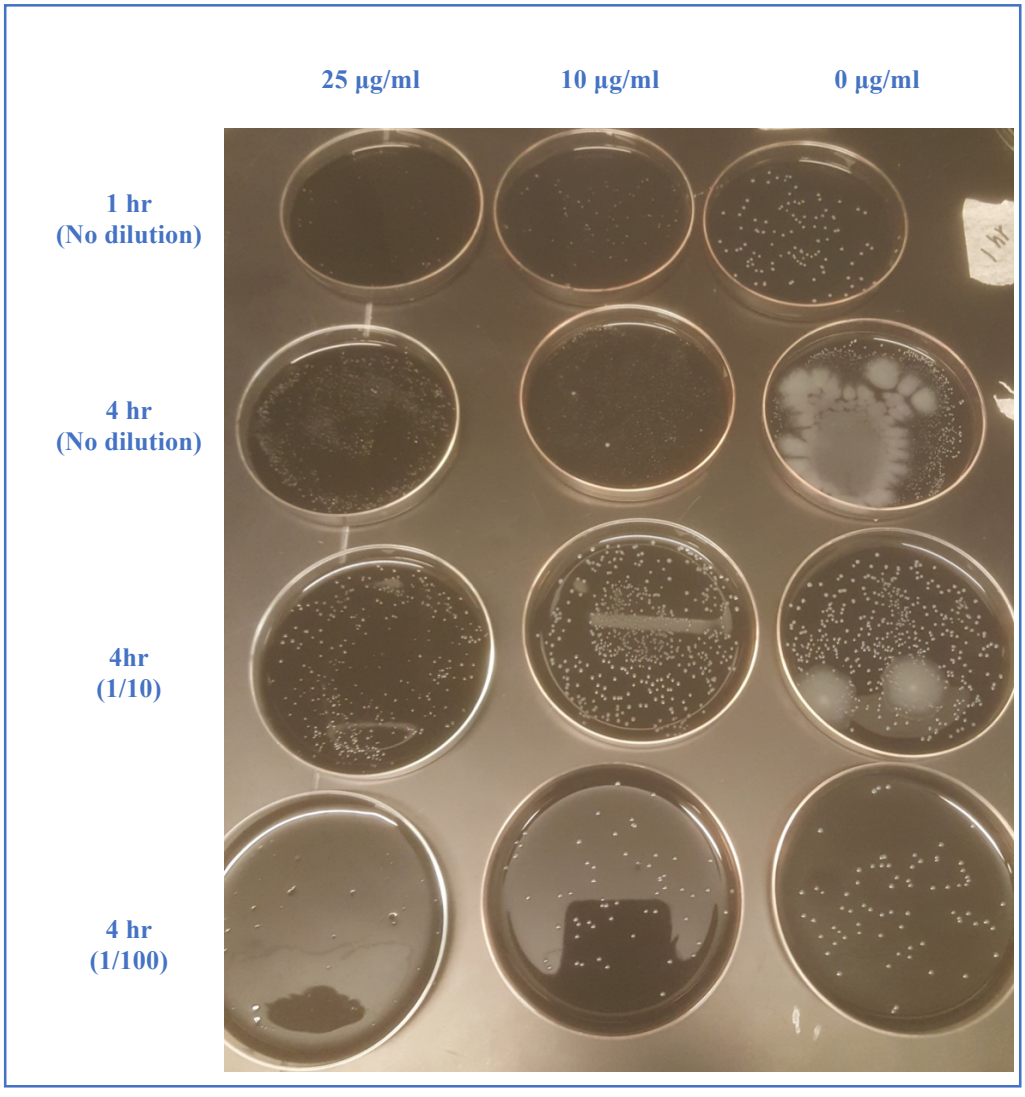

Figure 8: Results of *Legionella* transformation by electroporation with 1 and 4 hours' incubation time

Plates with no tetracycline were re-incubated for additional 2 days and a monolayer of wild-type *Legionella* were obtained (Figure 9). Transformed *Legionella* colonies are surrounded by a no-growth zone which could be inhabitation zone or nutrient-depleted zone.

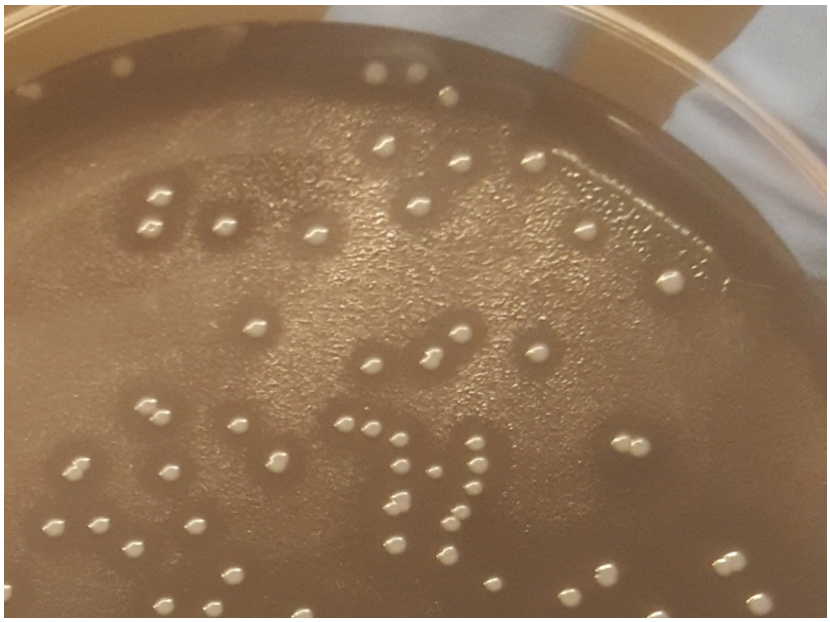

Figure 9: Wild-type *Legionella* monolayer surrounding transformed *Legionella* colonies

A 6-fold increase in transformation efficiency was achieved by increasing the incubation time from one hour to 4-hour. The transformation was calculated for 4-hour incubation vs.10 µg/ml tetracycline. The transformation efficiency (TE) is calculated using the following equation

$$TE = \frac{CFU}{\mu g\ DNA} \times \frac{total\ volume}{volume\ plated} \times Dilution$$

$$TE = \frac{58\ CFU}{0.044\ \mu g\ DNA} \times \frac{4000\ \mu l}{25\ \mu l} \times 10^2 = 2.1 \times 10^7 \frac{CFU}{\mu g\ DNA}$$

The transformation efficiency was high despite the relatively large size of the plasmid (10.4 kb), and the non-homogenous culture of the competent cells since they were harvested from agar media.

4.4. H_2S production by the transformed *Legionella*

The nutritional and metabolic features of the transformed *Legionella* was tested by growing on BCYE medium containing different supplements and it was to grow on with or with L-cysteine. Transformed *Legionella* was grown on BCYE with L-cysteine and tetracycline, which resulted in H_2S production. Additional experiments were performed to test whether the H_2S formation is as the result of L-cysteine degradation. Transformed *Legionella* was cultured using spread plate technique on plates with and without L-cysteine, and lead acetate papers were attached to the inner lid surface of petri dish as shown in Figure 10.

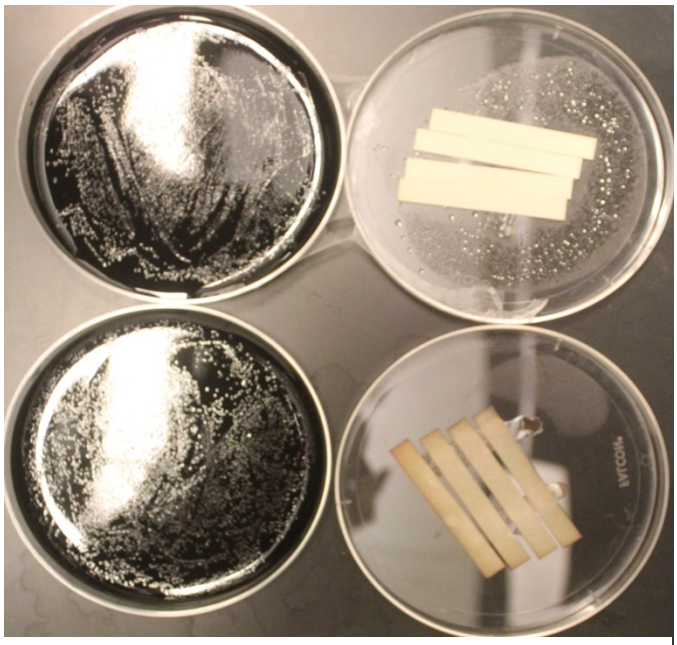

Figure 10: Transformed *Legionella* grown in BCYE ± L-cysteine

To verify that the black coloration in lead acetate paper incubated with transformed *Legionella* grown in BCYE+L-cysteine is due to H_2S, papers were washed with 3% H_2O_2 solution. The restoration of white color (Figure 11) is an indication that the black coloration is not volatile organic matter but indeed is a sulfur reduced compound.

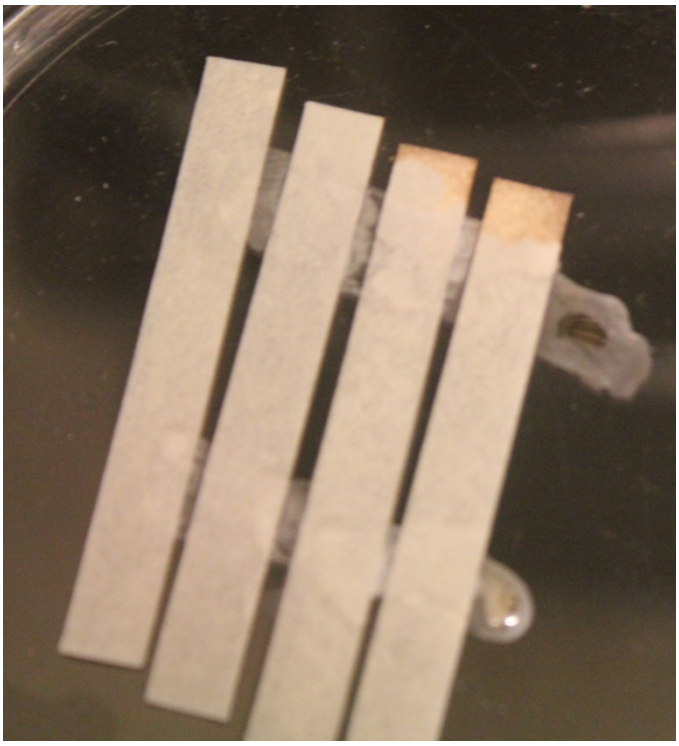

Figure 11: Lead acetate papers partially washed with H_2O_2 solution.

Lead acetate papers were washed with few drops of 3% H_2O_2 solution. A small part in the upper right corner of the papers were left dry to demonstrate the difference in color before and after. The H_2S production is detected in wild-type and transformed *Legionella* grown in BCYE with L-cysteine. Whereas, H_2S was not detected in the transformed *Legionella* grown on BCYE media without L-cysteine indicating that L-cysteine is the main source of H_2S production. It is assumed that CymR gene in the transformed plasmid plays a major role in *Legionella* L-cysteine dependency and in L-cysteine utilization. It is unknown if L-cysteine is being used as an easy carbon source or it is being recognized as a sulfur-containing toxic compound and converted to non-sulfur amino acids and carbohydrates. Future studies may aim to quantitate the production of H_2S and to detect if there is NH_3 formation to better understand the fate of high intracellular cysteine.

4.5.Cysteine and Iron Role in *Legionella* Peroxide Sensitivity

Legionella transformants capable of growth *in-vitro* without L-cysteine is a valuable tool to better understand the unique nutritional and growth requirements of *Legionella*. Further tests were performed to investigate whether these mandatory growth requirements make *Legionella* more vulnerable to oxidizing agents (disinfectants) which would result in underestimation of *Legionella* disinfection requirements. The difference in sensitivity of transformed *Legionella* grown in media with or without L-cysteine and iron salt was measured by using a modified disk diffusion test. Where diffusion disks were replaced with membrane filters and we used H_2O_2 as an oxidizing agent to measure the inhabitation zone caused by the intolerance of *Legionella* toward H_2O_2.The results of inhabitation zones caused by H_2O_2 for 6 different media are shown in Figure 12. The upper 3 media plates are with α-Ketoglutaric acid while the bottom ones are without any antioxidant. The two Petri dishes shown in the left in Figure 12 are with L-cysteine only, and the middle ones with iron only while the right ones have both L-cysteine and iron salt.

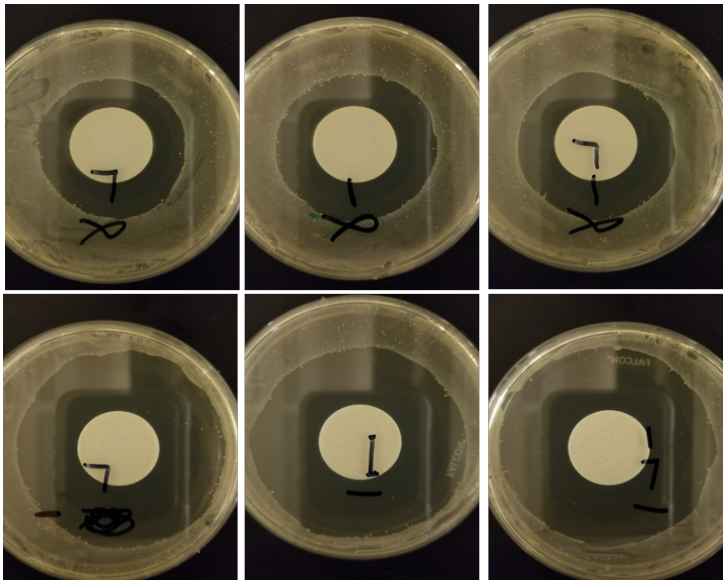

Figure 12: H_2O_2 Inhabitation zone of 6 kinds of media with or without L-cysteine, iron salt and α-Ketoglutaric acid

Even though we cannot make any conclusion about the role of cysteine or iron on the sensitivity of *Legionella* toward H_2O_2 from the previous experiment, we can clearly observe the effect of α-Ketoglutaric acid as an antioxidant to scavenge the peroxide. Since peroxidases and radicals' species naturally occur at low concentrations in biological reactions, we designed an experiment to investigate whether L-cysteine can enhance the formation of these radicals especially thiol radicals or whether iron can enhance the radicals' formation as a Fenton reagent. In this experiment, we assumed that radicals' formation would negatively affect the growth rate. Four types of TSB media were prepared including: TSB control, TSB with L-cysteine, TSB with iron and TSB with L-cysteine and iron. The growth rate of transformed *Legionella* in the4 different types of TSB is shown in Figure 13.

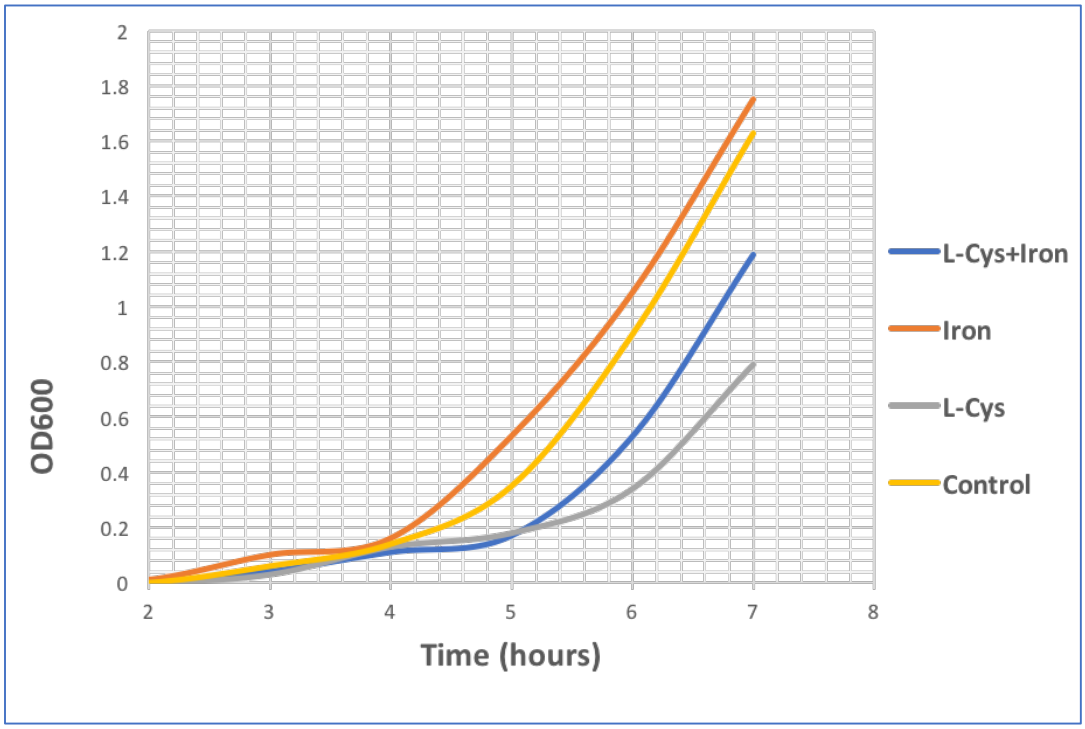

Figure 13: The growth rate of transformed *Legionella* grown in TSB only, with L-cysteine, with iron and with L-cysteine and iron

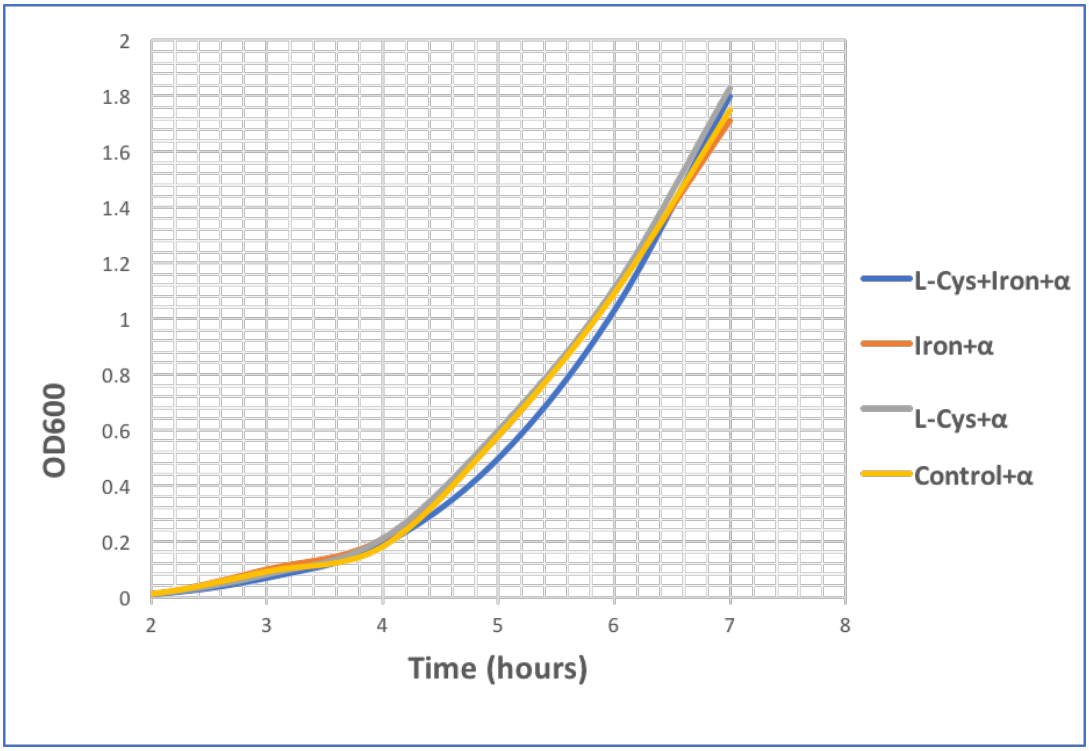

Figure 14: The growth rate of transformed *Legionella* grown in TSB and α-Ketoglutaric acid, with L-cysteine, with iron and with L-cysteine and iron

Figure 13 shows the rapid growth rate of transformed *Legionella* stimulated by iron. However, the growth rate of transformed *Legionella* in TSB with filter-sterilized ferrous pyrophosphate seems to be higher than the growth rate from autoclaved one (data not shown). It is believed that iron itself is not a limiting growth factor, however, it seems to play a role in electron transport or O_2 transport. On the other hand, the addition of L-cysteine alone without α-Ketoglutaric acid reduces the growth rate of transformed *Legionella*. The reason could be the production of toxic gas byproduct such as H_2S or it could be the formation of thiol radicals because of the rapid non-enzymatic autoxidation of cysteine into cystine. H_2S in media is also known to create anaerobic condition and α-Ketoglutaric acid might involve in transporting O_2 into the cell. However, the growth rate of transformed bacteria in TSB with L-cysteine and α-Ketoglutaric acid was similar to other conditions as shown in Figures 13 and 14.

The precise role of α-Ketoglutaric acid in obtaining normal growth rate in media with L-cysteine is unknown. Since L-cysteine is converted into H_2S in transformed *Legionella*, H_2S could have an impact on the bacterium oxidative stress. The role of H_2S in some biological reactions might be double-faced. Sometimes, it has some antioxidant properties and acts as a strong reducing agent since H_2S is the most reduced form (-2 oxidation state). On the other hand, autoxidation of H_2S generates free radicals especially in elevated molecular O_2 conditions that are usually encountered in cell culture[102]. However, as shown in Figure 14, α-Ketoglutaric acid eliminates the effect of cysteine and/or H_2S in transformed *Legionella* growth rate which indicates the generation of radicals in *Legionella* culture. Further experimental work is needed to quantify the production rate and aqueous concentration of H_2S to determine whether it reaches the

toxicity threshold in bacteria. Quantitative analysis of the production of succinate in α-Ketoglutaric acid-containing media is needed to investigate the formation of hydrogen peroxide. α-Ketoglutaric acid is added into BCYE and other *Legionella* culture media to inhibit superoxide production and to reduce cysteine oxidation rate[83]. The result suggests that L-cysteine could be a major source of radicals which could damage DNA and cell membrane, and this can be inhibited by addition of strong antioxidant such α-Ketoglutaric. The role of iron in growth rate is not affected by the addition of an antioxidant. Since *Legionella* is a host-dependent intercellular pathogen, iron concentration outside the cell could play signaling role imitating *in-vivo* conditions.

To investigate whether other thiol compounds behave like cysteine, transformed *Legionella* were grown in three different thiol compounds GSH, NAC and L-cysteine with and without sodium pyruvate, another ROS scavenger. The growth rate of *Legionella* with different thiol compounds with and without sodium pyruvate is shown in Figures 15 and 16.

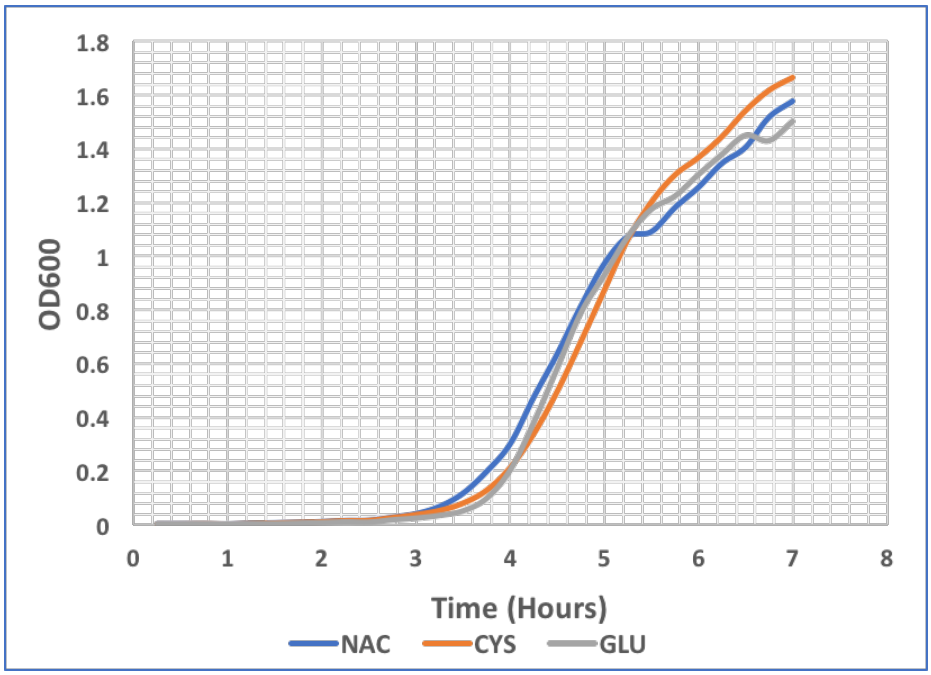

Figure 15: The growth rate of transformed *Legionella* grown in TSB with different thiol compounds without antioxidant

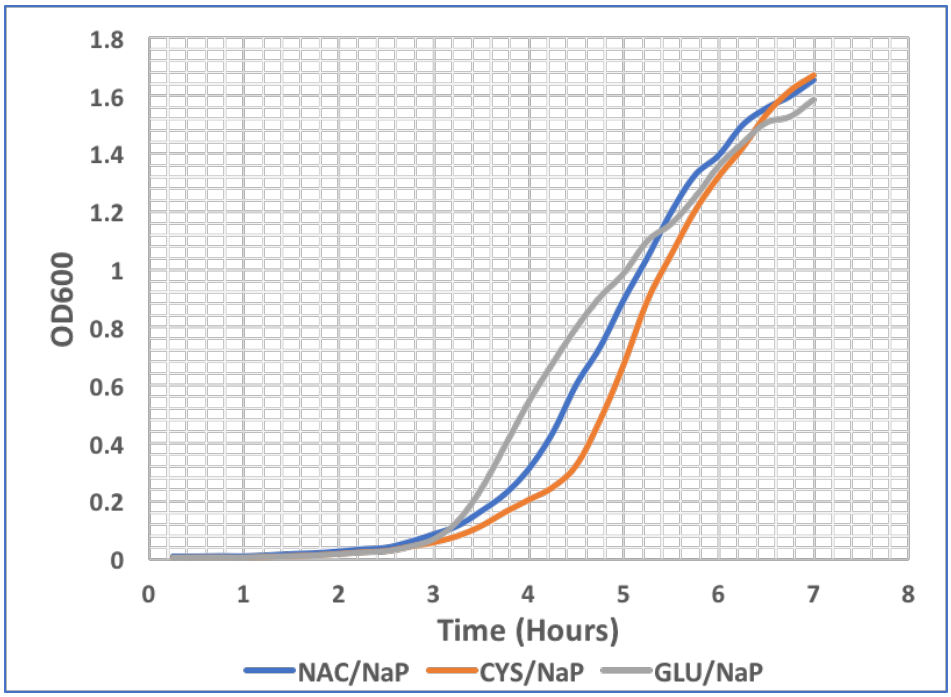

Figure 16: The growth rate of transformed *Legionella* grown in TSB with sodium pyruvate in different thiol compounds

The growth rate of transformed *Legionella* in the presence of NAC is slightly higher than the GSH and L-cysteine media in the absence of sodium pyruvate in media. NAC is more stable, and it has lower affinity to react with molecular O_2 compared to GSH and L-cysteine. GSH and L-cysteine in the presence of O_2 are associated with peroxides production[103]. The growth rate of transformed *Legionella* in the presence of NAC is not modulated by sodium pyruvate, however, in the presence of GSH, growth rate of transformed *Legionella* increases with the addition of sodium pyruvate while the growth rate in the presence of L-cysteine in media decreases when sodium pyruvate is added. Sodium pyruvate is an antioxidant and it is supposed to scavenge reactive species and enhance the growth. however, sodium pyruvate does not enhance the growth rate of transformed *Legionella* when grown in media with L-cysteine. The reason for the growth decline is unknown, but it could due to the conversion of L-cysteine in transformed *Legionella* into pyruvate, hydrogen sulfide and ammonia. Addition of sodium pyruvate may change the reaction equilibrium resulting in less L-cysteine degraded.

4.6.Peroxide Sensitivity of Transformed and wild-type *Legionella*

Though *Legionella* can survive in tap water for about 1 year without significant reduction[104], it is sensitive to very low concentrations of H_2O_2 as low as 26.5 μM[76]. Peroxide sensitivity comparison between transformed *Legionella* and wild-type *Legionella* was carried out to investigate transformed *Legionella* applicability in disinfection studies. Wild-type and transformed *Legionella* were grown under the same condition BCYE with L-cysteine. H_2O_2 was not neutralized before plating; we also assumed that H_2O_2 is momentarily neutralized by charcoal in BCYE media. The survival

rate of the wild-type and transformed *Legionella* strains in 1 mM H_2O_2 is shown the Figure 17.

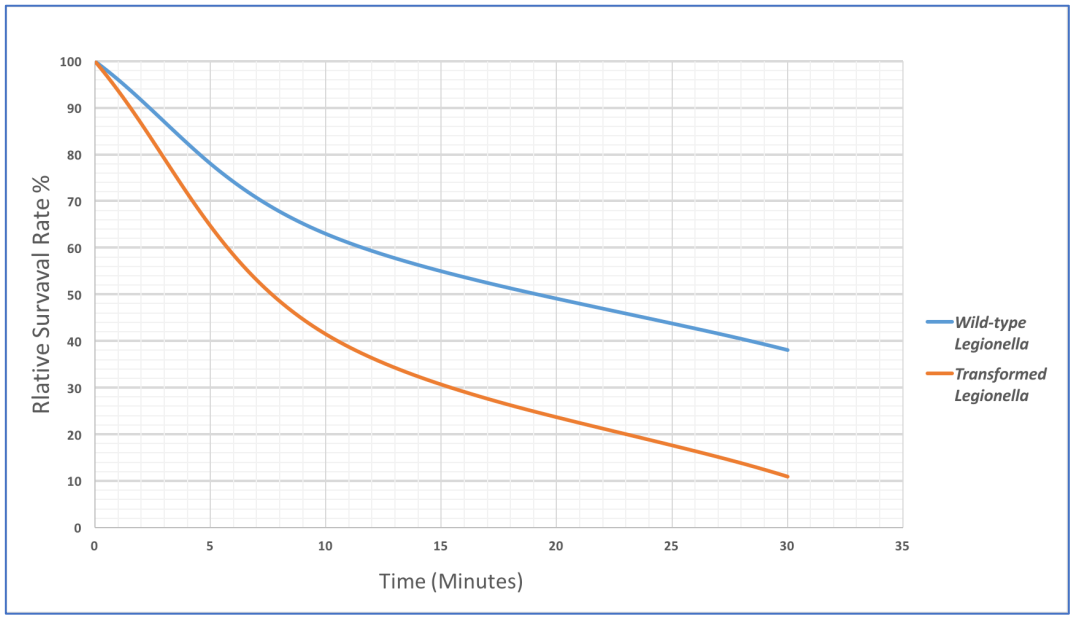

Figure 17: Relative Survival Rate of wild-type vs. transformed *Legionella* in 1 mM H2O2

The wild-type *Legionella* revealed greater resistance toward 1 mM compared to the transformed *Legionella*. This may be due to having high-copy-number of the plasmid which may have caused excessive metabolic stress on the growing *Legionella* cells, or it could be also because of increasing of L-cysteine uptake. Initially, we assumed CymR could play a role in reducing the oxidative stress in general, and we expected transformed *Legionella* would be suited for disinfection studies. However, the developed strain somehow is less stable and more sensitive toward oxidizing agents compared to wild-type cells. The main differences between Transformed and wild-type *Legionella* are summarized in Table 5.

Table 5: Comparison between wild-type and transformed *Legionella*

	Wild-type *Legionella*	**Transformed *Legionella***
Growth rate	3-5 days	1-2 days
Nutrients	Selective media (BCYE)	Non-selective Media (LB, TSB)
L-Cysteine	Required for growth, produces H_2S	Not required, produces H_2S
Iron Salt	Required for growth	Not required, enhance growth
Antioxidants	Required for growth	Not required, enhance growth
Survival rate	Very stable	Stable
Detection in systems	Difficult	Relatively easy
Antibiotics needed in Media	Polymyxin B, Vancomycin, Cycloheximide	Tetracycline

4.7.β-Galactosidase Activity Assay for Transformed *Legionella*

The goal of this assay is not only to measure the promoter strength or the induction profile, but it was used to validate that the undetected activity using x-gal was due to the low protein expression rather than the absence of the plasmid. The result shown in Table 6 is the average of duplicated samples of different cells volumes.

Table 6: β-Galactosidase Activity Assay results

Cell volume (ml)	420 nm	550 nm	650 nm	Miller unit
0.5	1.68	0.84	0.6	**3.89**
0.25	0.87	0.395	0.28	**14.19**
0.125	0.54	0.26	0.2	**18.89**

The average β-Galactosidase Activity is 12.32 Miller unit which is considered very low, lower than the un-induced expression on other organisms used in the previous studies[86]. Future studies should investigate the pQ5 promoter strength in *Legionella*, and should include quantitative expression profile for both lacZ and CymR genes to better understand the plasmid role in transformed *Legionella*. The transformation of *Legionella* in this study was confirmed using 3 different techniques, PCR targeting 16S rDNA, tetracycline resistance assay and β-Galactosidase assay.

CHAPTER 5

CONCLUSION

Detection of *Legionella* has many challenges including prolonged incubation time and the relatively short half-life time of antibiotics used in BCYE agar. Furthermore, identification using PCR or EIA is required for species and sub-species confirmation. However, *L. pneumophila* serogroup 1 is the only *Legionella* detected clinically which may be an underestimation of the overall *Legionella* cases from other serogroups of *L. pneumophila* and other *Legionella* species such as *Legionella longbeachae*.

Even though *Legionella* growth is limited to intercellular growth under environmental conditions and it only could cause illness if contaminated water/aerosols are inhaled. The number of identified and hospitalized cases is more than 6,000 cases in 2017 and the number is not expected to decrease[105]. Understanding the factors that affect *Legionella* growth and survival in surface water might lead us to better strategies for controlling the bacterium in the built environment. The current pathogen-control practices such as chlorine are inadequate to control *Legionella* in the environment as it uses amoeba as a host. Further studies in the association of *Legionella* with other bacteria in colonizing biofilms are required for better understanding of *Legionella* ecology. We believe that this will help us to design innovative applicable strategies to reduce the occurrence of *Legionella* in the built environment.

Legionella cannot grow in media without L-cysteine, Iron and strong antioxidants such as activated carbon and α-Ketoglutaric acid. The mutant *Legionella* constructed in this study using pQf-lacZ, is able to grow without any of these requirements. Perhaps, some of the supplements are required to encounter the side effects of the added reagents. For

example, we believe that α-Ketoglutaric acid is added to reduce cysteine oxidation rate, scavenge thiol radicals and inhibit superoxide production resulted from L-cysteine addition. It is assumed that in transformed *Legionella*, L-cysteine is degraded to pyruvate or non-sulfur amino acids, and H_2S was produced when transformed *Legionella* is grown in media with L-cysteine. The complex phenotypes in transformed *Legionella* are believed to be because of CymR gene used as a repressor in cumate-inducible systems. CymR is known to regulate (repress or activate) multiple genes in cysteine metabolism and oxidative respond genes.

Studies were performed to evaluate whether the transformed *Legionella* can be used to validate different engineering processes and other application. The genetically engineered *Legionella* constructed in this study has a higher growth rate than the wild-type strain: *Legionella pneumophila Knoxville-I*. Transformed *Legionella* has less stringent nutritional requirements than the wild-type strain: *Legionella pneumophila Knoxville-I* and presumably most other known strains. In addition, it also offers advantages in terms of simplicity, ease and time in engineering processes validation to study pathogen fate and transport in terrestrial and aquatic environments.

5.1. Future Studies

Future study should include examining the applicability of using transformed *Legionella* for validation of different disinfection and transport studies. Additional work is needed to investigate whether L-cysteine in media has an impact on the sensitivity of *Legionella* toward disinfectants such as H_2O_2 and chlorine.

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