ABSTRACT

EFFECT OF PH ON CADMIUM TOXICITY AND
ASSOCIATED GENE EXPRESSION IN *ESCHERICHIA COLI*

By Craig R. Worden

Metals can exert toxic effects on microorganisms. It has been widely reported that lowering environmental pH reduces cadmium toxicity in bacteria. Understanding mechanisms by which pH mediates cadmium toxicity would be useful for minimizing cadmium toxicity in the environment and for gaining insight into the interactions between organic and inorganic components of life. We confirmed that cadmium was less toxic to *Escherichia coli* at pH 5 than at pH 7 in M9 minimal salts medium through growth curve analysis. To investigate cellular mechanisms by which lowering pH decreases cadmium toxicity, we used DNA microarrays to characterize global gene expression patterns in *E. coli* in response to cadmium exposure at moderately acidic (5) and neutral (7) values of pH. Increased expression of several stress response genes including *hdeA*, *otsA*, and *yjbJ* at pH 5 after only 5 minutes was observed and suggests that acidic pH more rapidly induces genes that confer cadmium resistance. Genes involved in transport were more highly expressed at pH 5 than at pH 7 in the presence of cadmium. Of the genes that showed an interaction between pH and cadmium effects, 46% encoded hypothetical proteins. Geochemical modeling software predicted that concentrations of both monovalent hydroxylated cadmium ($CdOH^+$), previously implicated in the effect of pH on cadmium toxicity, and cadmium hydroxide chloride (CdOHCl) increased with pH; however, concentrations of both cadmium species were at least two orders of magnitude lower than concentrations of divalent ionic cadmium (Cd^{2+}). Our data both demonstrate that transcriptional responses of *E. coli* to cadmium are affected by pH as well as provide insight into mechanisms by which pH mediates cadmium toxicity.

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by

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CHAPTER I

INTRODUCTION

1.0. Biological functions of heavy metals

Life depends on interactions between organic and inorganic components. The organic building blocks of life consist of nucleic acids, proteins, carbohydrates, and lipids; however, many of these organic compounds require inorganic metals to function properly. Metals that have a biological function, such as copper, nickel, magnesium, manganese, and zinc, are deemed essential metals (Ji & Silver, 1995). These metals commonly serve as cofactors for proteinaceous enzymes, but they may also be used to stabilize proteins and bacterial cell walls (Hughes & Poole, 1989; Poole & Gadd, 1989; Ji & Silver, 1995). Enzymatic function is reliant on specific 3-dimensional structure. A change in a single amino acid may alter the shape of an active site, leading to inactivation of the enzyme. Metal cofactors also act by affecting folding patterns of enzymes. A positively-charged metal ion may have specific interactions with negatively-charged amino acids, such as aspartic and glutamic acid residues. The interaction between metal ions and amino acids is sufficient to alter the entire structure, and therefore function, of an enzyme.

Heavy metals with no known biological functions, such as aluminum, arsenic, lead, and mercury are non-essential metals (Bruins *et al.*, 2000). These metals are toxic because they can irreversibly bind to enzymes that require metal cofactors. Toxic metals readily bind to sulfhydryl groups of proteins (Nies, 1999). In fact, toxic metals interact so strongly with the sulfhydryl groups that the structure may be permanently distorted. These metals can have an even broader effect when they inhibit chaperone-assisted

refolding of denatured proteins (Sharma *et al.*, 2008). The non-essential metals are also involved in the formation of reactive oxygen species that impose oxidative stress on microorganisms (Kachur *et al.*, 1998). The results of heavy metal exposure have also been shown to result in single-strand breakage in DNA (Mitra & Bernstein, 1978). The presence of single-stranded DNA can be seen during stressful conditions, specifically during the mismatch repair process; however, metals have been shown to inhibit mismatch repair (Jin *et al.*, 2003). Therefore, non-essential metals can inactivate enzymes directly by binding to active sites in place of the necessary cofactors, or indirectly by maintaining mutations in the DNA sequence that are not repaired.

Cadmium is an example of a heavy metal that can produce toxic effects both directly and indirectly. Cadmium was once thought to be a non-essential toxic metal, but it was rather recently discovered that it could serve as an enzyme cofactor in the marine diatom *Thalassiosira weissflogii* (Lane & Morel, 2000). The diatom uses cadmium as a cofactor for carbonic anhydrase, an enzyme that catalyzes the reversible hydration of carbon dioxide to form bicarbonate and is used in acquiring inorganic carbon. Zinc is used in place of cadmium in other organisms, but *T. weissflogii* evolved under the zinc-limiting conditions of the ocean. Cadmium is not toxic to the diatom because the metal-binding site of enzyme is stable even in the absence of the metal, a unique adaptation to this carbonic anhydrase. Cadmium carbonic anhydrase can easily use cadmium or zinc at the active site (Xu *et al.*, 2008).

All metals, regardless of whether they are essential or non-essential, can exhibit toxic effects at high concentrations (Silver, 1996). Excess concentrations of essential

metals can lead to non-specific binding, which can alter the structure and inactivate vital enzymes. Because controlling metal concentrations is vital to maintaining homeostatic conditions within cells, evolution has bestowed upon organisms a number of ways to regulate concentrations of essential metals and to resist the toxicity of non-essential metals (Silver, 1992; Rouch *et al.*, 1995a).

1.1. Microbial response to metals

There are a number of different mechanisms by which microorganisms resist metal toxicity. There are five postulated mechanisms that microbes use for mediating metal toxicity. These mechanisms include: 1) formation of a permeability barrier, 2) active transport, 3) sequestration, 4) enzymatic detoxification, and 5) reduction in sensitivity (Bruins *et al.*, 2000). Microbes may use one or more of these mechanisms to exclude non-essential metals and regulate concentrations of essential metals.

Some microbes are able to decrease the permeability of their membranes to prevent toxic metals from entering. If the toxic metals are not able to physically enter the cell, they will not be able to affect vital metal-sensitive structures, such as proteins. One way to prevent heavy metals from entering is by decreasing the production of membrane channel proteins (Rouch *et al.*, 1995a). It is also possible for the metal-binding sites in the membrane and periplasm to be saturated with non-toxic metals (Mergeay, 1991). A third possibility is the formation of an extracellular polysaccharide coat, which binds and prevents metals from reaching the surface of the cell (Scott & Palmer, 1990).

In microbes without a permeability barrier, or when the barrier fails, a mechanism must be in place to export metals from the cytoplasm. These active transport systems involve energy-dependent, membrane-bound, efflux pumps that can be encoded by either chromosomal- or plasmid-borne genes. Active transport is the most-well studied metal resistance mechanism, so there are numerous different examples. Some of these include the *ars* operon for exporting arsenic from *E. coli*, the *cad* system for exporting cadmium from *Staphylococcus aureus*, and the *cop* operon for removing excess copper from *Enterococcus hirae* (Tsutomu & Kokayashi, 1998; Smith & Novick, 1972; Silver & Phung, 1996).

Microbes that lack a specific active transport system for removing toxic metals may be able to sequester heavy metals either inside or outside of the cell. Intracellular sequestration occurs when cytoplasmic molecules are produced in response to metal stress. The sequestering molecules bind the free metal ions, preventing them from interacting with vital cell structures. The two most common molecules used for intracellular sequestration are metallothioneins and cysteine-rich proteins (Rouch *et al.*, 1995a; Silver & Phung, 1996). Examples of molecules used for extracellular sequestration are glutathione and ionic phosphate, which can cause metals to form insoluble complexes (Murata *et al.*, 1985; McEntee *et al.*, 1986).

Some metals can be converted to a less toxic form through enzyme detoxification. The most well-known example of this mechanism is the mercury resistance system, which occurs in *S. aureus, Bacillus sp., E. coli, Pseudomonas aeruginosa, Serratia marcescens,* and *Thiobacillus ferrooxidans*. The *mer* operon in these bacteria includes

two different metal resistance mechanisms. MerA follows an enzyme detoxification method because it encodes a mercury reductase, which can convert the divalent mercury cation into elemental mercury. Elemental mercury is more stable and less toxic than the divalent cation. Other genes in the operon encode membrane proteins that are involved in the active transport of elemental mercury out of the cell (Misra, 1992; Wireman *et al.*, 1997; Rasmussen *et al.*, 2008).

Metals also create selection pressure for microbes with cell structures that are less sensitive to metals. For example, mutations may occur that alter metal-binding sites of proteins without rendering the enzyme inactive. Another method for preventing metal toxicity is to produce excess amounts of the target so that there is an insufficient amount of metal to bind to all of the cellular molecules (Bruins *et al.*, 2000).

Even with all of these mechanisms in place for metal resistance, microbes are still susceptible to heavy metals at high concentrations. In the environment, heavy metal toxicity affects many important bacterial processes, including litter decomposition, methanogenesis, acidogenesis, nitrogen transformation, and biodegradation of organics (Baath, 1989).

1.2. Effect of heavy metals on bioremediation

Many different heavy metal and organic pollutants, located at hazardous waste sites throughout the world, are endangering humans and wildlife (Fierens *et al.*, 2003; Järup, 2003). Excavation and incineration are two methods for eliminating organic wastes from these sites, but these methods have proven to be inefficient and costly (Lehr

et al., 2001). Bioremediation, which is the process of using microorganisms to break down organic pollutants, has been considered an attractive alternative (Balba *et al.*, 1998). One potential drawback to using bioremediation is that forty percent of the sites on the U.S. Environmental Protection Agency's (EPA) National Priority List (NPL) are co-contaminated with both organics and heavy metals. Metals affect organic pollutant degradation at these sites (Sandrin & Maier, 2003). Although the heavy metals show toxic effects on bacteria that could be used for bioremediation, it is not completely understood how the metals affect the degradation of organic pollutants.

Numerous studies have investigated effects of heavy metals on biodegradation. Studies examining biodegradation often utilize bacteria that can use organic contaminants as sole carbon sources (Springael *et al.*, 1993; Sandrin & Maier, 2002). Growth (i.e., changes in biomass over time), which is an indicator of biodegradation in these studies, can be measured by optical density, protein content, oxygen consumption, or the amount of target organic compound that is present over time (Madsen, 1997; Sandrin & Maier, 2003). Most studies have shown that heavy metals inhibit biodegradation of various aromatic and aliphatic hydrocarbons (Amor *et al.*, 2001; Kong, 1998; Pardue *et al.*, 1996; Riis *et al.*, 2002). One possible issue with these studies is that the metal may bind to the organic compound, preventing entry of the carbon source into the cell (White & Knowles, 2000). This result would not provide an accurate indication of how metal toxicity affects biodegradation because some of the carbon source is not available for degradation.

Not all studies have shown a connection between heavy metals and inhibition of biodegradation. Some studies have shown that heavy metals do not affect the

degradation of organic compounds. For example, Baldrian *et al.* (2000) showed that cadmium and mercury did not inhibit the ability of the fungus *Pleurotus ostreatus* to degrade polycyclic aromatic hydrocarbons. It is possible that the metals were being sequestered by a component of the soil medium used in these studies. Assuming the metal was being sequestered, it was not available to the organisms, thus potentially explaining why biodegradation was not affected.

There are even a few studies that have shown increased biodegradation in response to heavy metals (e.g., Said & Lewis, 1991; Kuo & Genthner, 1996). In most cases, there is a dose-dependent relationship between metal concentration and biodegradation. Said & Lewis (1991), however, observed that an aerobic consortium of microbes degraded 2,4-dichlorophenoxyacetic acid methyl ester (2,4-DME) at a faster rate when exposed to 100 μM cadmium than when exposed to 10 μM cadmium. These results could be due to selective pressures faced by the microbial consortium at higher metal concentrations. Perhaps, only the most resistant microbes survived when exposed to higher metal concentrations. These microbes no longer had to compete with other microbes for the carbon source. Another possible explanation is that metal resistance mechanisms may be more rapidly induced in response to higher metal concentrations.

There is a wide range of reported inhibitory metal concentrations in the literature. For example, the lowest zinc concentration required to reduce biodegradation ranges from 0.006 mg/L to 736 mg/L (Sandrin & Maier, 2003). This is likely due to the lack of standardized experimental conditions. Varying carbon sources, environmental conditions, and organisms of interest among studies likely account for the wide range of inhibitory

concentrations reported in the literature. Some studies were performed using a single strain of bacteria, while others used an indigenous community of microbes. Some organisms were grown in soil or sediment microcosms, while others were grown in minimal salts media. Environmental conditions affect metal toxicity by altering metal speciation and bioavailability.

2.0. Metal speciation

The total metal concentration in a system has been the most commonly-employed indicator of metal inhibition of biodegradation. Clearly, other factors are also involved, considering the large disparities between minimum inhibitory concentrations among studies. A better indicator of metal toxicity is the concentration of the most bioavailable form, or species, of the metal (Sandrin & Hoffman, 2007). The most bioavailable species are those that will presumably associate with enzymes and other active sites to initiate biological responses. Under most conditions, the most bioavailable form of a metal is considered to be the free, ionic, solution-phase species (Traina & Laperche, 1999; Behra *et al.*, 2002). Despite the free cation usually being the most abundant species, many other species are present, such as metal phosphates, carbonates, hydroxides, and sulfides, depending on the physiochemical conditions (e.g., pH, redox potential, and ionic strength) of the medium. Metals can also form hydroxo-complexes or complexes with various other organic ligands (Hughes & Poole, 1991). It is also possible for most of the metal to speciate into insoluble precipitates, such as metal phosphates.

Different metal species vary in their biological reactivity (Hughes & Poole, 1991; Behra *et al.*, 2002). For example, the free ionic form of a metal may act by replacing a cofactor for a vital enzyme. Hydroxylated metal ions have been suggested to bind to the cell surface and alter the net charge of the cell to reduce its viability (Ivanov *et al.*, 1997). Because different species may have different effects on biological processes, some species may be more toxic than others. There is a paucity of information in the literature regarding the relative toxicity of different metal species.

2.1. Measurement of bioavailable metal species

The total metal concentration in a solution can be determined easily using methods such as atomic absorption spectroscopy (AAS); however, not all species of a metal will be bioavailable, and much of the original concentration may have speciated into insoluble precipitates. Therefore, the concentration of some bioavailable species may be extremely low, perhaps even within or below the nanomolar range (Hughes & Poole, 1991). Ion selective electrodes are useful for measuring the bioavailable concentration of a metal because they only measure the free, ionic species, which is often most prevalent (Buck & Lindner, 2001).

Immunoassays are another technique that can be used to detect solution phase metal concentrations. Immunoassays utilize antibodies that recognize metal-bound chelating agents, such as EDTA (Blake *et al.*, 1996). Bioreporters have also been used to determine solution phase metal concentrations (Daunert *et al.*, 2000; Rensing & Maier, 2003). These are cells that contain a reporter gene, such as *lacZ*, that is attached to a

promoter region capable of being regulated by a heavy metal. Bioreporters for detecting mercury have been created using the *lacZ* system (Rouch *et al.*, 1995b) and the *lux* system, which will emit light in response to mercury exposure (Corbisier *et al.*, 1999). Immunoassays and bioreporters are both advantageous because they can be used to accurately measure the bioavailable metal concentration in complex media and soil systems; however, they cannot be used to determine the concentration of single species in the solution (i.e., $[MOH^+]$). Also, the total concentration of bioavailable metal reported by the biosensors can vary depending on the metal resistance mechanisms of the specific bioreporter cell (Rensing & Maier, 2003). The rates of uptake, efflux, and complexation affect the amount of metal that is detected by the biosensor.

There are other possible methods for separating or determining different metal species in solution, although they may not be as accurate as the techniques that measure total solution-phase concentrations. One way to determine which ligands are involved in forming metal species uses ligand-field (d-d) spectroscopy. The electrons from the d-orbitals of metals are excited at different wavelengths in different metal-ligand complexes (Hughes & Poole, 1989). Ion chromatography can also be utilized to separate species based on charge. Recently, dynamic speciation sensors that take into account the kinetic properties of different species have been employed. These sensors take into account diffusion and speciation reactions of species such as metal hydroxides, carbonates, and sulphates, as well as metal complexes with organic ligands to determine bioavailability of these species (Van Leeuwen *et al.*, 2005).

It is extremely difficult to directly measure the concentration of various metal species in solution, except for the free, ionic species. Therefore, it is useful to employ geochemical modeling software, such as MINEQL+ (Environmental Research Software, Hallowell, ME) or MINTEQA2 (Allison *et al.*, 1991), to predict metal speciation patterns as a function of ionic strength and pH (Pardue *et al.*, 1996). These programs take into account equilibrium constants for each ion in solution and accurately calculate the concentration of any metal species under specified conditions. The accuracy of programs such as MINEQL+ has been verified experimentally. For example, Sandrin & Maier (2002) used a cadmium ion-selective electrode to determine the concentration of divalent cadmium ion in a minimal salts medium over the pH range from 4 to 7. The experimental concentrations were comparable to those predicted by the modeling software. These programs do not take into account all organic ligands present in complex media, so they are more comparable to experimental situations in minimal media. In complex media, it is difficult to calculate the concentrations of all components because the composition of complex ingredients (e.g., yeast extract, beef extract) differs slightly in every batch. It is important to carefully control the composition of the media because it can have significant effects on metal speciation.

2.2. Effect of medium composition on metal speciation and toxicity

Many different chemical components in a medium may interact with metals. Results of many studies of metal toxicity are not comparable due to the wide range of media used. Some varieties of complex biological media contain metal-binding

components, such as yeast or beef extract, peptone, and amino acids (Hughes & Poole, 1991). These complex ingredients may differ slightly in the exact chemical composition among batches, so it is difficult to accurately and consistently determine metal speciation patterns in these types of media. Assessing the role of metal speciation on toxicity in these systems is complicated because most of the total metal can bind to components of the yeast or beef extract instead of vital cellular components (Ramamoorthy & Kushner, 1975).

Other studies use soil or sediment samples for a more accurate indication of microbial activity in natural environments. In these samples, organic matter and clay particles play a role in metal toxicity (Sandrin & Maier, 2003). Both organic material and clay particles in soil can bind metals and reduce their bioavailability. For example, Pardue *et al.* (1996) demonstrated that much less solution-phase cadmium was required to inhibit trichloroaniline (TCA) dechlorination in a mineral-based soil than in soil containing a higher concentration of organic matter. Other studies have shown that adding clay minerals to a medium mitigates toxicity. Clay minerals, such as kaolinite, montmorillonite, bentonite, and vermiculite, can bind to metals to decrease the amount that is bioavailable (Babich & Stotzky, 1977; Kamel, 1986).

Because metals can bind with components of complex media and soil to form species that are less bioavailable than the free, ionic species, it is important to use a chemically defined medium for correctly describing metal speciation in a system (Hughes & Poole, 1989; Twiss *et al.*, 2001). The most straightforward determination of metal speciation can be accomplished using a minimal medium. Minimal media usually

include a variety of mineral salts and a single carbon source. In experiments investigating effects of metal toxicity on biodegradation, the carbon source in the minimal medium is the organic pollutant to be degraded (Springael *et al.*, 1993; Amor *et al.*, 2001; Sandrin & Maier, 2002). To obtain a better representation of the natural environment, a defined amount of soil could be mixed into a minimal medium that contained a pollutant as a carbon source (Said & Lewis, 1991; Maslin & Maier, 2000). Even in relatively simple types of media, such as minimal media, buffers are present at higher concentrations than other components and can affect metal speciation (Hughes & Poole, 1991).

Many different buffers have been used to study metal toxicity. Phosphate buffers are commonly used in minimal media (Birch & Brandl, 1996; Amor *et al.*, 2001; De *et al.*, 2006; Adoki, 2007). Phosphate ions readily form insoluble precipitates with many metals, decreasing the bioavailability of the metal in the process. Some metals are less sensitive to phosphate precipitation. For example, concentrations of the free cobalt ion (Co^{2+}) show no significant decrease with up to 15 mM phosphate present. In contrast, when 3 mM phosphate is present, both nickel and cadmium free ion concentrations decline to 78% and 34% of the concentration when no phosphate is present (Fig. 1a, page 19: Sandrin & Hoffman, 2007). Phosphate is so efficient at sequestering metals in some cases that it has been used in some studies to decrease concentrations of free metal ions in solution. For example, White & Knowles (2000) added phosphate to their minimal medium to decrease the concentration of free cobalt and zinc ions that formed recalcitrant complexes with the organic compound nitrilotriacetic acid.

Phosphate buffers that form precipitates with metals can cause a number of problems in metal toxicity studies. For example, precipitates may be mistaken for cell biomass if the turbidity of the culture is being used as an indicator for growth (Hughes & Poole, 1991). One potential solution to this problem is decreasing the pH of the solution to dissolve metal-phosphate precipitates (White & Knowles, 2000); however, most metal-phosphates remain insoluble at neutral to moderately acidic pH values. Another method for circumventing this problem is using glycerophosphate instead of inorganic phosphate (Malakul *et al.*, 1998). Glycerophosphate will not form insoluble precipitates with metals, but some metal ions may still bind to the compound, and it creates a confounding variable in biodegradation studies by acting as an additional carbon source. A final possibility for addressing the problem of phosphate precipitates is to decrease the total phosphate concentration, although this alternative may compromise the buffering system (Sandrin & Hoffman, 2007). Changing the phosphate concentration can affect metal toxicity. Korkeala & Pekkanen (1978) observed that lowering the phosphate buffer concentration from 0.2 M to 0.05 M increased the minimum inhibitory concentration (MIC) of cadmium for *Micrococcus luteus* and *Bacillus subtilis* grown on Plate Count Agar. Curiously, the same decrease in phosphate concentration showed the opposite effect for *Streptococcus bovis* throughout the pH range from 6 to 8. The authors speculated that the variation in sensitivity to phosphates among these organisms could be explained by differences in the composition and structure of the cell walls. Since phosphate buffers lead to precipitation issues when examining metal speciation and toxicity, it is important to consider buffers that do not display such a strong affinity for metals.

Metals react less strongly with zwitterionic buffers than phosphate buffers (Good *et al.*, 1966). Zwitterions are ions that have both a positive and negative charge. Some examples of zwitterions buffers are HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), MES (morpholinoethanesulfonic acid), MOPS (3-(N-morpholino)propanesulfonic acid), PIPES (1,4-piperazinebis(ethanesulfonic acid)), and TES (N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid). These buffers have limited interactions with metals, so they do not complicate metal toxicity studies. A weakness of these buffers is that the studies should be conducted under the optimal pH range from 6 to 7.5 (Mash *et al.*, 2003). Tris-base (2-amino-2-(hydroxymethyl)-1,3-propanediol) is another buffer that has been used in many studies, but studies have shown that Tris-buffer is capable of binding some metals to some extent (Hughes & Poole, 1991; Twiss *et al.*, 2001).

Although buffer components are usually available at the highest concentrations in a medium, metals can also bind to inorganic ligands in solution. Ligands such as Cl^-, OH^-, and SO_4^{2-} can form soluble complexes with many metals (Reed & Nonavinakere, 1992). These newly formed metal species remain in solution, but are considered to be less bioavailable than the free, ionic species under most conditions (Traina & Laperche, 1999). Even though other soluble metal species are considered less bioavailable, they may still play previously unstudied roles in causing metal toxicity.

2.3. Effect of pH on metal speciation and toxicity

Medium affects metal speciation and toxicity. At lower pH, the free, ionic species of metals are at their highest concentrations, so metals are more bioavailable under these conditions (Sandrin & Hoffman, 2007). At acidic pH, more protons are available to saturate metal-binding sites (Hughes & Poole, 1991). For example, metals are less likely to form insoluble precipitates with phosphates when the pH of the system is lowered because much of the phosphate has been protonated. Under basic conditions, metal ions can replace protons to form other species, such as hydroxo-metal complexes. Some of the hydroxo-metal complexes are soluble, such as those formed with cadmium, nickel, and zinc, while those formed with chromium and iron are insoluble.

Many studies show that pH mediates metal toxicity (e.g., Korkeala & Pekkanen, 1978; Babich *et al.*, 1985, Franklin *et al.*, 2000). In some cases, increasing pH reduces metal toxicity. Babich & Stotzky (1982 and 1983) found that increasing pH reduces the toxicity of nickel to a variety of different organisms, including bacteria (*Serratia marcescens*), filamentous fungi (*Arthrobotrys conoides*, *Penicillium vermiculatum*, *Rhizopus stolonifer*), and a type of yeast (*Cryptococcus terreus*). Under moderately basic conditions (pH 8.5), much of the nickel may not be bioavailable because it forms complexes with various ligands. It is also possible that the nickel was less toxic at a higher pH because some organisms may prefer basic environments to neutral or acidic environments. Aluminum has also been shown to be more toxic to *E. coli* at pH 5.4 than at pH 6.8 (Guida *et al.*, 1991).

More commonly, metal toxicity increases with pH. Increasing pH has been shown to increase the toxicity of zinc, copper, and uranium to certain algae species (Hargreaves & Whitton, 1976; Franklin *et al.*, 2000) and of cadmium to various bacteria (*B. subtilis, E. coli, M. luteus, S. bovis*), actinomycetes (*Micromonospora chalcea, Nocardia corallina, Streptomyces flavovirens*), and fungi (*Saccharomyces cerevisiae, Schizosaccharomyces octosporus*) (Korkeala & Pekkanen, 1978; Babich & Stotzky, 1977). Metal toxicity may increase at higher pH values because cells may be able to take up or adsorb more of the metal ions under these conditions (Rudd *et al*., 1983; Sandrin & Maier, 2002). Under more acidic conditions, metals compete with protons for binding sites on the cell surface. Also, various functional groups associated with the membrane would be protonated under acidic conditions, reducing the electrostatic attraction between the metal cations and the membrane. A third possibility is that metals are removed from the cell more efficiently under acidic conditions by efflux pumps that are driven by the proton motive force (Sandrin & Maier, 2002).

Another possible explanation for increased toxicity at a higher pH is the formation of species that are more toxic, such as the hydroxo-metal species (Babich & Stotzky, 1985; Collins & Stotzky, 1992; Ivanov *et al*., 1997). Since most studies that have implicated hydroxo-metal species were carried out in complex media, the concentration of these species was not accurately determined. Sandrin & Maier (2002) used a minimal salts medium that allowed them to use MINEQL+ to predict the concentration of monohydroxylated cadmium from pH 4 to 7. The concentration of monohydroxylated cadmium increased with pH until reaching a peak at pH 5.9, and then the concentration

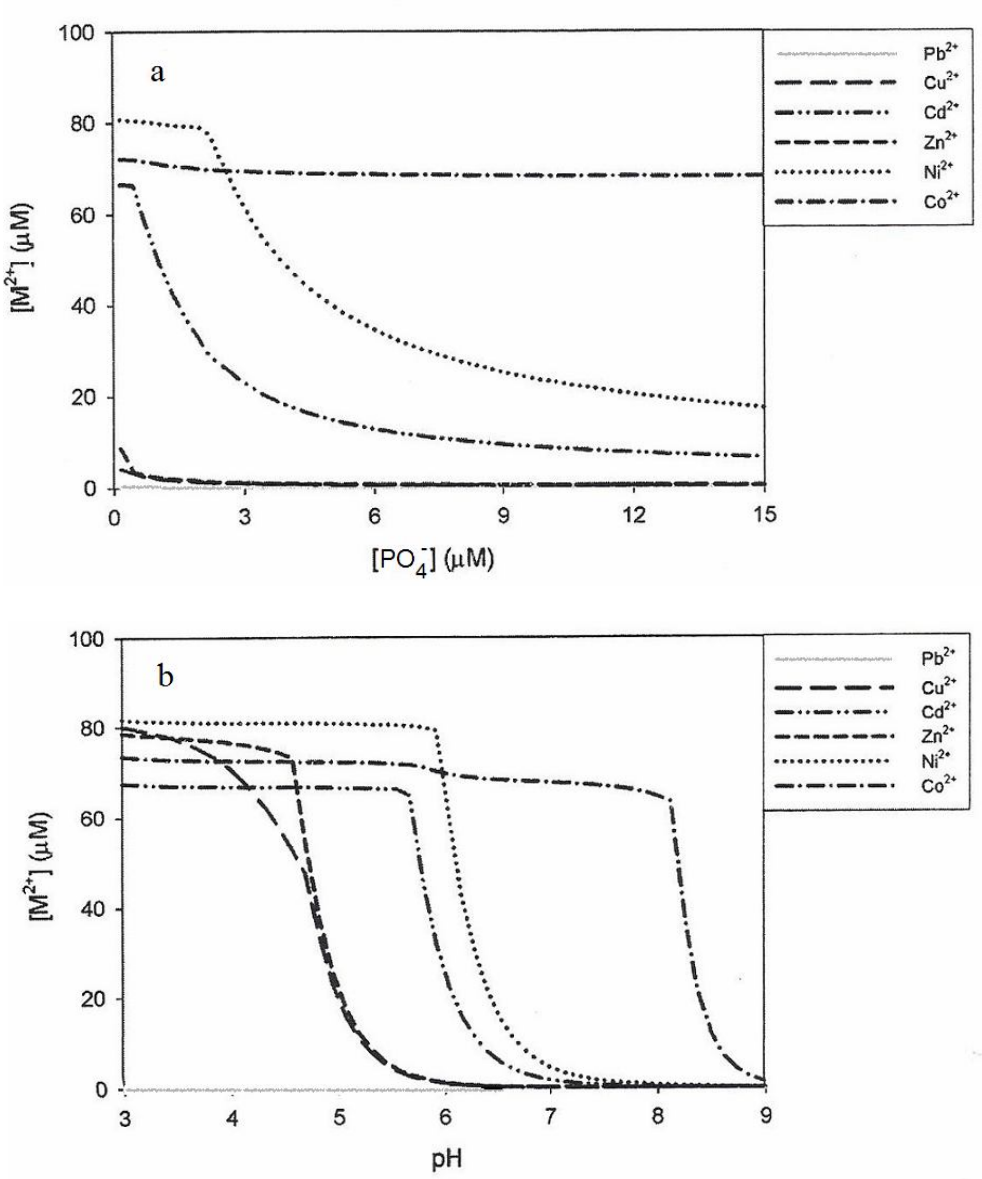
slowly decreased until pH 7. Although the concentration of divalent cadmium ion simultaneously decreased at higher pH values, those concentrations remained at least three orders of magnitude higher than monohydroxylated cadmium concentrations.

pH-dependent metal speciation patterns differ among metals. For example, concentrations of the free, ionic species of copper and zinc in minimal media rapidly decline at pH values higher than 5, while the free, ionic form of cobalt remains prevalent until the pH value is higher than 8 (Fig. 1b, page 19: Sandrin & Hoffman, 2007). The effect of pH on hydroxo-metal species also varies among metals. In a study that investigated pH-dependent chemical speciation in seawater, Zirino & Yamamoto (1972) found that monohydroxylated zinc levels reach a peak at pH 8, while monohydroxylated cadmium levels increase from pH 8 to 9.

The pH of a medium also impacts the formation of metal-phosphate precipitates. For example, cadmium concentrations rapidly decline as both phosphate concentration and pH increase. Sandrin & Hoffman (2007) determined that when no phosphate is present in a commonly used mineral salts medium, the concentration of divalent ionic cadmium remains relatively constant until an abrupt fall above pH 8 (Fig. 1c, page 20). When 15 mM inorganic phosphate is added to the medium, divalent cadmium ion concentrations rapidly decline at pH values above 6.

Maintaining a constant pH during an experiment is crucial because of the dependency of metal speciation on pH. Buffers are required to prevent large deviations in pH throughout an experiment. Studies examining the effect of metal toxicity on biodegradation usually use a buffer that has a neutral to moderately acidic operational pH

range (Said & Lewis, 1991; Amor *et al.*, 2001; Sandrin & Maier, 2003). The operational pH range is determined by the pKa of the buffer, which is the pH at which half of the weak acid used for buffering is protonated. When the pH is beyond the operational range of a buffer, even small additions of acid, such as the excretion of acidic metabolic end products, may drastically change the pH and alter metal speciation patterns.



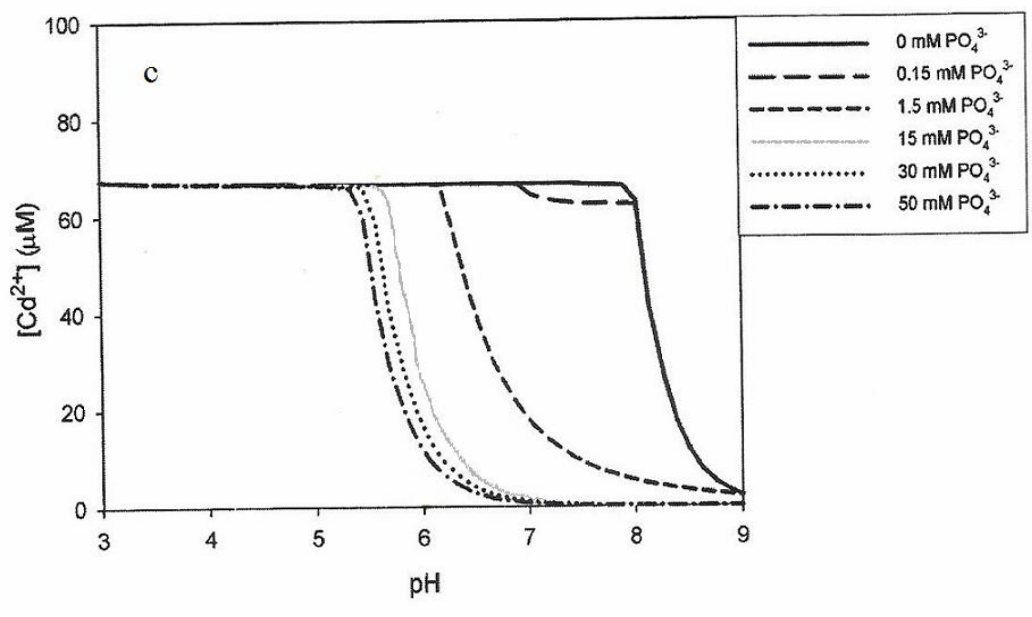


Figure 1 (Sandrin & Hoffman, 2007)**:** Effect of phosphate concentration (a), pH (b), and the interactions of phosphate and pH (c) on solution-phase, ionic metal concentrations ($[M^{2+}]$) as predicted by MINEQL+ (Environmental Research Software, Hallowell, ME) in Bushnell-Haas medium (Difco, Sparks, MD) amended with 100 µM total lead, copper, cadmium, zinc, nickel, or cobalt. The pH of the medium in (a) was set at 6.5.

3.0. Microarrays as tools to assess global gene expression in response to metals

As of June 2008, the complete genomes of 746 organisms have been sequenced (National Center for Biotechnology Information, 2008); therefore, it is necessary to apply functional analysis to entire genomes. Microarrays are one possible application for investigating organisms on a genomic scale. Microarrays are chips containing many tiny spots of DNA, protein, or other biological molecules. The remainder of this literature review focuses on DNA microarrays. The DNA fragments can be oligonucleotides that have been synthesized directly to the surface by means of photolithography using either pre-made masks (Fodor *et al.*, 1991) or micromirrors (Singh-Gasson *et al.*, 1999). The nucleotide fragments can also be complementary DNA molecules that have been amplified by polymerase chain reaction (PCR). The PCR products are then spotted onto the chip using robotic techniques. The chips are usually made of glass or silicon because these materials can provide stable attachment through reactive amine, aldehyde, or epoxide groups, low background signal, and homogenous surface chemistry (Stears *et al.*, 2003).

Microarrays have many different applications. Some of these include the comparison of genome content in different types of cells or from different strains of an organism (Moran *et al.*, 2004), identifying single base differences among different groups (Hacia *et al.*, 1999), and resequencing a portion of a genome to locate mutations (Syvanen, 1999). One of the most utilized applications of microarrays is observing global gene expression patterns in cells exposed to various treatments or conditions. Microarrays can be utilized to compare gene expression between different tissue types,

between normal and diseased organisms, or between a treated and control organism. For example, a better picture of how metal toxicity affects gene expression on a global scale can be obtained using microarrays. Regardless of the application, microarrays are useful because they can examine many different genes in parallel.

Before microarrays were produced on a large scale, the expression of specific genes could be determined using colony hybridization (Grunstein & Hogness, 1975) or Southern blotting (Southern, 1975). In these techniques, either entire colonies or isolated DNA fragments are attached to a substrate. A known gene or intergenic sequence can then be used as a labeled probe to determine which samples were expressing the gene of interest. It would be costly and time-consuming to perform these tests to determine the expression of every gene.

Microarrays use the same principle of hybridization as Southern blotting and colony hybridization. Hybridization involves the annealing of single-stranded nucleic acids from different sources based on complementary base pairing. The short DNA fragments on the surface of the array can hybridize to single-stranded cDNA that is a product of the reverse transcribed mRNA isolated from treated cells. Similar to Southern blotting, the isolated cDNA is labeled with a probe. Fluorescent dyes, such as cyanine 3 (Cy3) and cyanine 5 (Cy5), are commonly used in microarray experiments (Stears *et al.*, 2003). The fluorescence of the hybridized samples is then scanned by lasers to produce images from two channels. The automated data gathering process is relatively quick. The entire process is summarized in Figure 2 (Lukac, 2006).

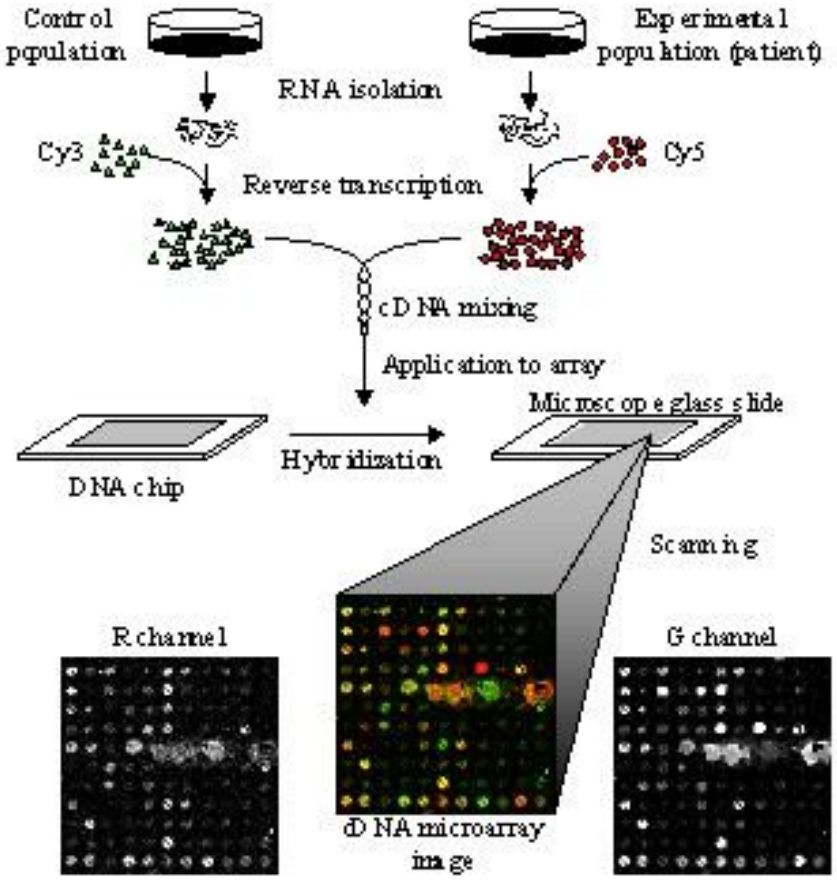


Figure 2: Summary of microarray procedures. Briefly, mRNA is isolated from control and experimental populations, reverse transcribed into cDNA, and labeled with fluorescent probes. The labeled cDNA from each sample are mixed and hybridized to the array, which can then be scanned using different lasers for each dye to produce intensity values for each sample (Lukac, 2006).

Microarrays have other advantages over previous techniques. Besides being able to test every gene in an organism at the same time, the process is also miniaturized and automated (Stears *et al.*, 2003). Microarrays also produce accurate results compared to other techniques. Yue *et al.* (2001) used ten replicates to demonstrate that there is only

12% variation in non-differentiated genes, while there is a maximum of 25% variation in differentially expressed genes. Real-time PCR (qPCR) is used in most studies to validate microarray results. This validation technique quantifies cDNA during the initial replication cycles to produce an accurate indication of gene expression. Biological and technical variability in experimental protocols can have significant effects on both microarray and qPCR results. Correlations between microarray and qPCR studies have ranged from -0.48 to 0.94 (Beckman *et al.*, 2004; Etienne *et al.*, 2004). Lower correlations were usually seen for genes that showed less than a 2-fold change in expression (Etienne *et al.*, 2004). Genes with low spot intensities, which were defined as intensities lower than that of the highest negative control intensity, also showed lower correlations (Beckman *et al.*, 2004). Morey *et al.* (2006) found that genes that had a fold change of at least 1.4 and a p-value of less than 0.0001 produced correlations of at least 0.80. Both microarrays and qPCR have benefits and limitations for analyzing gene expression patterns, so validation with qPCR is useful for genes showing significantly different changes in expression.

It is important to normalize the data from all arrays to account for variation among individual arrays. One source of variation has been reported to be the different labeling efficiency of the two dyes (Stears *et al.*, 2000). Normalization creates a median of zero for the distribution of the log-ratios of dye intensities for all genes. There may also be spatial effects of dye bias (dyes binding to one area of the array more efficiently than to other areas), so regional normalization techniques, such as print-tip lowess, may be necessary (Quackenbush, 2002). Another data analysis issue is created by the large

number of genes being tested. Analysis of variance (ANOVA) tests are run on each gene to determine if there are significant differences in gene expression across treatments. When thousands of individual statistical tests are carried out, several tests will yield false positives. False positives result from tests that yield a significant p-value, but they do not show true differences between expression levels among different treatments. Benjamini & Hochberg (1995) generated equations to produce a false positive discovery rate, which indicates the expected proportion of false positives among the results that are deemed statistically significant.

With different statistical techniques, as well as variation in experimental procedures, it is difficult to compare microarray results from different experiments. One way for standardizing microarray experiments is by using the Minimum Information About a Microarray Experiment (MIAME) checklist (Brazma *et al*., 2001). According to the checklist, all published microarray studies should include information about the experimental design as a whole, the array design, sample preparations, hybridization protocols, measurements, and normalization controls. Gene expression data are only useful if the environmental conditions and data analysis methods are specified.

3.1. Effects of metal toxicity on genome-wide expression patterns

Despite the lack of standardization in microarray experimentation, there have still been many useful studies that have elucidated the effects of metal toxicity on global gene expression for a variety of organisms. For example, studies have examined the genome-wide response of *E. coli* to zinc (Lee *et al.*, 2005), cadmium (Wang & Crowley, 2005),

cobalt, and nickel (Brocklehurst & Morby, 2000). Yeast microarrays have looked at global effects of cadmium (Momose & Iwahashi, 2001), and human microarrays have been used to determine global gene expression patterns in response to antimony, arsenic, cadmium, chromium, mercury, and nickel (Kawata *et al.*, 2007). The results of these studies have indicated that metals generally suppress genes involved in protein synthesis and increase the expression of genes involved in various stress responses and efflux systems. Periplasmic metal-binding proteins, such as ZraP for zinc (Lee *et al.*, 2005), YodA for cadmium (Puškárová *et al.*, 2002), and CusF for copper and silver (Franke *et al.*, 2003) have also been produced in response to metals.

Gene expression can be a useful indicator for characterizing the mechanisms by which organisms adapt to changes in their environment. Gene expression patterns provide clues as to how environmental conditions affect the genome, alter protein synthesis, and guide external responses of the organism. Conversely, experiments that look at stressor-specific expression patterns could eventually lead to a fast method for the screening of suspected toxicants in the environment (Lettieri, 2006).

4.0. Present study: E. coli – A model organism

E. coli has been extensively used as a model organism to study various aspects of microbial genetics, molecular biology, and biotechnology (Blattner, 1983). *E. coli* is a good model organism because it has a short generation time, it is readily accessible, it can be easily manipulated, and its genome has been extensively studied. When the sequence of the *E. coli* genome was completed, only 1827 of the 4288 predicted protein-coding

regions had been characterized (Blattner *et al.*, 1997). Many of the genes (38%) had no attributed function at the time of sequencing. One approach to characterizing FUN (function unknown) genes has been to expose bacteria to novel conditions. Microarrays experiments involving *E. coli* have been conducted to determine global gene expression patterns in response to novel conditions. For example, 117 genes with unknown function showed significant changes in expression after exposure of cadmium to *E. coli* (Wang & Crowley, 2005).

4.1. Present study: Cadmium toxicity

Cadmium is one of many toxic heavy metals found in the environment. Cadmium enters the environment as by-products from mining, smelting, and other industrial processes. Cadmium is used in stabilizers of plastics, pigments, alloys, anti-corrosion agents, and nickel-cadmium batteries. Cadmium can also enter the environment through natural causes, such as volcanic activity and forest fires (Filipič *et al.*, 2006).

Cadmium affects many different kinds of organisms, ranging from microbes to humans. Human exposure to cadmium mainly occurs through cigarette smoking, but exposure can also occur through contaminated food, water, or air (Järup, 2003). Cadmium is a known carcinogen to mammals (Bertin & Averbeck, 2006). Cancer occurs in these exposed mammals because their DNA is mutated by reactive oxygen species and DNA repair mechanisms are inhibited. Cadmium exposure can also lead to a number of other health problems in humans, including kidney damage, osteoporosis, anemia,

chronic rhinitis, and eosinophilia, which is an increase in white blood cells (Filipič *et al.*, 2006).

The mode of cadmium toxicity has been characterized in a number of organisms, and many genes affected by cadmium toxicity have been elucidated. In yeast, cadmium toxicity appears to be mediated by the overproduction of glutathiones and sulfur-containing amino acids that bind free, ionic cadmium. Genes for glutathione synthesis (*GSH1*) and for enzymes involved in sulfur amino acid synthesis (*MET14* and *MET17*) were upregulated in response to cadmium. Other stress response genes, such as heat shock proteins (*HSP26* and *HSP12*) were also upregulated (Momose & Iwahashi, 2001). The heat shock proteins function as chaperones to refold proteins damaged by cadmium-induced stress. Since cadmium is involved in the production of reactive oxygen species, genes encoding antioxidant defense enzymes, such as superoxide dismutase (*SOD*), are also upregulated in response to cadmium (Brenann & Schiestl, 1996).

There have been numerous studies investigating the effects of cadmium on *E. coli*. Initial studies used two-dimensional polyacrylamide gel electrophoresis to determine which proteins were being expressed in response to cadmium exposure (Ferianc *et al.*, 1998). Many of the proteins observed in this study were products of genes involved in the SOS global stress response, oxidative stress response, and heat shock stress response. Khazaeli & Mitra (1981) also discovered increased expression of proteins, likely metallothioneins, which could bind free-ionic cadmium. For these reasons, *E. coli* is able to accommodate to low levels of cadmium exposure after an extended lag phase (Mitra, 1984).

Wang & Crowley (2005) used microarray analysis to characterize the global gene expression responses of *E. coli* to cadmium exposure. As in other studies, many stress response genes were upregulated, such as *clpB*, which prevents protein aggregation (Houry, 2001). There was also a shift to anaerobic metabolism, noted by the repression of *pdhR*, *sdhCDAB*, and *sucABCD*, which are all involved in aerobic metabolism (Cronan & LaPorte, 1996). Differential expression of 117 genes with unknown functions was also observed. Previous research has investigated some of the novel cadmium-induced proteins in *E. coli*. Puškárová *et al.* (2002) examined *yodA*, a gene that encodes a periplasmic metal-binding protein. This gene showed basal expression during exponential growth, but showed a 50-fold increase in expression after exposure to cadmium. Increased expression was not seen in response to copper, cobalt, or nickel.

Some organisms have become resistant to cadmium toxicity. *Stenotrophomonas* sp. CD02 was isolated from a site heavily contaminated with cadmium (Chien *et al.*, 2007). This strain was able to survive in Luria Bertani medium (a nutrient-rich medium with many potential metal-binding components) with a cadmium ion concentration of up to 4 mM. It could also remove up to 80% of the solution-phase ions after reaching stationary phase.

4.2. Present study: Genes affected by pH differences

Another factor that affects microbial viability is pH (Small *et al.*, 1994). Microarray analyses have been carried out to elucidate the response of *E. coli* to pH (Tucker *et al.*, 2002). Two genes that were upregulated were *gadA* and *gadB*, which code

for glutamate decarboxylase. Glutamate decarboxylase catalyzes the decarboxylation of arginine and glutamate to consume excess protons that enter the cell in acidic environments (Gale & Epps, 1942). Some other patterns of pH regulation include the repression of flagellar (*flg*) and chemotaxis (*cheA*) genes under basic conditions and the induction of oxidative stress genes (*ahpC*) and genes encoding periplasmic proteins (*oppA*) under acidic conditions (Maurer *et al*., 2005).

Cadmium toxicity has been shown to be mitigated by pH (Hargreaves & Whitton, 1976; Babich *et al*., 1985; Sandrin & Maier, 2002); however, there is still debate about the mechanism of pH-mediated cadmium toxicity. To gain insight into effects of different toxic cadmium species, it is necessary to examine the interaction between pH and cadmium toxicity on a genome-wide level. Global genome analysis on the effects of pH-induced cadmium speciation could identify genes that are involved in mitigating cadmium toxicity in the environment for bioremediation purposes.

4.3. Present study: Objectives

The objectives of this thesis were to:

1. determine whether transcriptional responses of *E. coli* cadmium toxicity are similar at pH 7 and pH 5,

Null hypothesis: Moderately acidic pH (5) does not induce expression of genes that confer resistance to cadmium.

2. identify genes in *E. coli* that may be regulated by pH-mediated cadmium speciation in order to determine whether species other than Cd^{2+} play roles in cadmium toxicity.

Null hypotheses: There are no differences in the genes being expressed in response to cadmium toxicity at different pH values. Cadmium species other than Cd^{2+} do not affect gene expression in *E. coli*.

CHAPTER II

EFFECT OF PH ON CADMIUM TOXICITY AND ASSOCIATED GENE EXPRESSION IN *ESCHERICHIA COLI*

INTRODUCTION

Life depends on interactions between organic and inorganic components. Many of the organic components (i.e., nucleic acids, proteins, carbohydrates, and lipids) require inorganic metals to function properly. Essential metals commonly serve as cofactors for enzymes (Hughes & Poole, 1989). Metals with no known biological function are toxic to organisms because they can irreversibly bind to enzymes that require metal cofactors (Nies, 1999). All metals, regardless of whether they are essential or non-essential, can exhibit toxic effects at high concentrations (Silver, 1996). Excess concentrations of essential metals can lead to non-specific binding to enzymes, which can alter their structure, thus inactivating them. In the environment, excess metals can inhibit a variety of microbially-mediated processes, including biodegradation of organic compounds and pollutants.

Bioremediation is considered relatively cost-effective and efficient compared to conventional methods for the remediation of hazardous waste sites contaminated with organic pollutants (i.e., excavation, incineration, pump and treat) (Lehr *et al.*, 2001); however, forty percent of the hazardous waste sites on the U.S. EPA National Priority List (NPL) are contaminated with both metal and organic pollutants (Sandrin *et al.*, 2000). Metal toxicity has been shown to inhibit the biodegradation of organics in systems co-contaminated with organics and metals, and may thus compromise the effectiveness of

bioremediation of metal and organic co-contaminated environments (Said & Lewis, 1991; Kuo & Genthner, 1996; Amor *et al.*, 2001; Sandrin & Maier, 2003).

Cadmium is the second most common metal pollutant in U.S. EPA Superfund sites (Enger & Smith, 2002). Cadmium is toxic, and until recently, had no known biological function. Lane & Morel (2000) discovered an exception in a marine diatom that utilizes cadmium in place of zinc as a cofactor for carbonic anhydrase. Cadmium toxicity is in part attributable to its ability to substitute for essential cations within physiologically important enzymes (Nies, 1999). Cadmium also plays roles in the generation of reactive oxygen species (Goering *et al.*, 1994) and the inhibition of DNA replication (Mitra *et al.*, 1975) and mismatch repair (Jin *et al.*, 2003). Several stress response systems, including the heat shock, cold shock, oxidative stress, and SOS systems, have been shown to be upregulated in response to cadmium exposure using two-dimensional gel electrophoresis (VanBogelen *et al.*, 1987; Ferianc *et al.*, 1998). Microarray studies (i.e., Brocklehurst & Morby, 2000; Wang & Crowley, 2005) have confirmed many of these results and observed the overall response of bacteria to cadmium exposure on a genomic level. In addition to the upregulation of several genes involved in stress response systems, Wang & Crowley (2005) have shown that cadmium exposure also results in a shift toward anaerobic metabolism and energy conservation.

Many different techniques have been proposed for mitigating the toxicity of cadmium and other metals in co-contaminated systems. These strategies for enhancing biodegradation of organics in co-contaminated systems include using metal resistant bacteria (Springael *et al.*, 1993), treatment additives (Jonioh *et al.*, 1999), clay minerals

(Babich & Stotzky, 1977), and chelating agents (Malakul *et al.*, 1998). The manipulation of physiochemical factors (i.e., divalent cation concentrations, pH) has also been explored as an approach to diminish metal toxicity (Hughes & Poole, 1991). The effect of pH on metal toxicity is the most well-studied of these physiochemical factors.

Lowering environmental pH has been widely reported to reduce metal toxicity in a variety of organisms (Hargreaves & Whitton, 1976; Korkeala & Pekkanenen, 1978; Babich *et al*., 1985; Franklin *et al*., 2000; Sandrin & Maier, 2002). The mechanism by which pH influences cadmium toxicity may involve cadmium uptake or the generation of different cadmium species that vary in toxicity. Divalent ionic cadmium (Cd^{2+}) has been reported to be the most bioavailable species at moderately acidic to neutral values of pH (Sandrin & Maier, 2002); however, monovalent hydroxylated cadmium ($CdOH^{+}$) has been implicated in increased toxicity at pH 7 (Babich & Stotzky, 1977; Ivanov *et al.*, 1997). Other cadmium species also vary in concentration with pH (e.g., $CdPO4^{-}$ and CdOHCl), but their roles in toxicity have not been investigated.

The reduction of metal accumulation by bacteria has also been shown at acidic pH (Rudd *et al.*, 1983; Sandrin & Maier, 2002). The mechanism by which metal accumulation is reduced is not well-known. One possibility is that there is increased competition between metal and hydrogen ions at the cell surface when hydrogen ion concentrations increase at lower pH. Lowering pH may also increase metal efflux out of the cell, especially by transporters driven by the proton motive force.

To better understand the cellular mechanisms by which lowering pH decreases cadmium toxicity, we used DNA microarrays to characterize global gene expression

patterns in *E. coli* in response to cadmium exposure in moderately acidic (5) and neutral (7) values of pH. The specific objectives of this study were to: 1) determine whether transcriptional responses of *E. coli* to cadmium are similar at pH 7 and pH 5 and 2) identify genes in *E. coli* that may be regulated by pH-mediated cadmium speciation in order to determine whether species other than Cd^{2+} play roles in cadmium toxicity.

METHODS

Bacteria, media, and reagents. An overnight culture of *E. coli* K-12 (MG1655) was started by inoculating a single colony from a Luria-Bertani (LB) plate into 50 mL of M9 minimal medium. The M9 medium was prepared according to Sambrook & Russell (2001) and supplemented with 0.4% (w:v) glucose. The culture was grown on a rotary shaker (200 rpm) at 37 °C. The overnight culture was diluted with phosphate-buffered saline (PBS) to standardize the optical density at OD_{600} = 0.8.

Measurement of effect of pH on cadmium toxicity. To quantify the effects of pH on cadmium toxicity, triplicate 125 mL Nalgene® flasks containing 50 mL of sterile M9 medium were supplemented with 0, 5, or 50 μM cadmium added as $CdCl_2$ and adjusted to pH 7 or pH 5 using concentrated HCl. The flasks were stored at 4 °C for 24 hours before growth curves were initiated. Cells (0.5 mL) from the diluted overnight culture were added to the triplicate flasks, which were placed on a rotary shaker (200 rpm) at 37 °C. Culture turbidity (OD_{600}) was determined at one-hour intervals and used to construct growth curves. Differences in length of the lag phase, yield, and growth rate among

treatments were determined by a one-way ANOVA, followed by Tukey post hoc tests. Differences were deemed significant for comparisons in which $p \leq 0.05$.

Growth conditions for microarray experiments. Two overnight cultures of *E. coli* K-12 were started in M9 medium as described above. A 250 mL Erlenmeyer flask containing 100 mL of M9 medium was inoculated with 0.5 mL for each of the two overnight cultures, each of which was considered a biological replicate. The cultures were grown on a rotary shaker (200 rpm) at 37 °C until the contents of the flask reached an OD_{600} of 0.3 (mid-log phase of growth). Each culture was divided into 25 mL aliquots, transferred to four 50 mL conical tubes (Corning), and centrifuged at 2540 x g for 12 minutes. The supernatant was decanted, and the cells were resuspended in 25 mL of M9 medium at pH 7 or pH 5 in the presence or absence of 5.4 μM (1 μg/mL) total cadmium, added as $CdCl_2$. The cultures were incubated at 25 °C for either 5 or 15 minutes with manual rotations of the flasks once per minute to resuspend the cells. After the appropriate amount of time, 15 mL of RNAProtect^{TM} Bacteria Reagent (Qiagen) was added to each culture to immediately halt all metabolic processes. The solutions were vortexed, incubated at 25 °C for 5 minutes, and centrifuged for 12 minutes at 3750 x g. RNA was extracted from the cell pellets immediately following centrifugation.

RNA extraction and microarray procedures. RNA was extracted and purified using a Masterpure RNA purification kit (Epicentre Technologies). The quantity and quality of the RNA samples were determined spectrophotometrically. Preparation of the cDNA,

labeling with Cy3 and Cy5, and the successive hybridizations were accomplished using a 3DNA Array 900MPX kit following the manufacturer's protocols (Genisphere) with the following modifications. 3DNA reverse transcriptase enzyme (Genisphere # RT300320) was added to 1 µg of RNA and 2 µL of a random primer (1 µg/µL). The final cDNA hybridization mix contained 58 µL (20 µL cDNA with the attached capture sequence for binding fluorescent labels, 2 µL locked nucleic acid (LNA) dT Blocker, 7 µL nuclease free water, and 29 µL 2X enhanced cDNA hybridization buffer). The cDNA mix was added to a cDNA microarray (Operon version 1.0 oligonucleotides were spotted by the Microarray and Proteomics Facility at the University of Alberta). The array was incubated for 13 hours in a dark, humidified chamber at 67 °C. Fifty ml of the flourophore mix (2.5 µL Cy3 capture reagent, 2.5 µL Cy 5 capture reagent, 20 µL nuclease free water, and 25 µL 2x SDS-based hybridization buffer) were added to the cDNA on the array. After the fluorophore hybridization mix was added, the array was incubated for four hours in a dark, humidified chamber at 68 °C. All post-hybridization washes of the array were carried out for 20 minutes each, and the array was dried by centrifuging at 180 x g for two minutes in a 50 mL conical tube containing a kimwipe placed in the bottom. The arrays were scanned with a Versarray ChipReader (BioRad) with laser power at 75%, photomultiplier tube (PMT) sensitivity at 800 V, and detector gain at 1.

Data analysis. The data analysis and reporting were performed according to MIAME standards (Brazma *et al.*, 2001), and the GEO accession number for the microarray data

reported in this paper is (in progress). Each array directly compared transcription at pH 5 and pH 7 for a given cadmium treatment (0 or 5.4 μM cadmium) and exposure time. Two biological replicates, in the form of dye swaps, were used for each of the following treatments: 5 minutes with cadmium exposure, 5 minutes without cadmium exposure, 15 minutes with cadmium exposure, and 15 minutes without cadmium exposure. The treatment consisting of 5 minutes of pH exposure without cadmium was considered the 0 cadmium exposure time. Spot intensities and locations were determined using TIGR Spotfinder, Version 3.1.1 (Saeed *et al.*, 2003). All subsequent analyses were performed using the ma-anova package (Wu, 2007) in the open-source statistical software package, R (www.r-project.org), Version 2.4.1 (R Development Core Team, 2006). The data were normalized using the regional lowess method (Quackenbush, 2002). Genes were represented in triplicate on each array, and median expression values were determined for each gene. ANOVA and F3 tests (Cui *et al.* 2005) were performed separately for each time point to identify genes with a significant pH:cadmium interaction (FDR-adjusted $p \leq 0.05$).

Clustering and gene ontology. Of the 151 genes that exhibited a pH:cadmium interaction at one or both time points, 91 were chosen that showed an increase in expression at either pH 5 or pH 7 when cadmium was present, but showed less than 50% of the higher with-cadmium expression value for both pH values in the absence of cadmium (Table S1). Heatmaps that compared the expression of these genes after 0, 5, and 15 minutes of exposure to cadmium were constructed using the Stats package (R

Developmental Core Team, 2006) in R. The 0 time point represents the difference in expression between the pH 5 and pH 7 treatments after five minutes of exposure in the absence of cadmium. The clustering was calculated based on Euclidean distance using the average linkage method. Gene products were identified using EcoGene, an internet database (http://ecogene.org/) of *E. coli* gene sequences and functions. The ontology of each gene was determined using the MultiFun grouping (http://biocyc.org/ ECOLI/class-tree?object=Genes).

RESULTS

Cadmium is less toxic to *E. coli* under moderately acidic conditions

The effects of cadmium exposure on the growth kinetics of *E. coli* were compared in neutral (pH 7) (Fig. 1a) and moderately acidic (pH 5) (Fig. 1b) M9 media. The duration of the lag phase, cell yield, and growth rate during log phase for each treatment were determined (Table 1). There were no differences in any of the three variables when comparing the two pH treatments without cadmium; however, the lag phase for all pH 5 treatments was similar to that of *E. coli* grown at pH 7 in the presence of 5 μM cadmium. Cadmium exposure did not affect the durations of the lag phase at pH 5 (Table 1; Fig. 1b). In contrast, the lag phase increased with cadmium concentration at neutral pH (Table 1; Fig. 1a). The lengths of the lag phases at pH 5 and pH 7 in the absence of cadmium were not different. Taken together, these results suggest that moderately acidic conditions mitigated the cadmium toxicity observed under neutral conditions, as has been reported

previously (Babich & Stotzky, 1977; Korkeala & Pekkanen, 1978; Sandrin & Maier, 2002). Neither yield nor growth rate were affected by cadmium exposure or pH.

pH affects gene expression in *E. coli* exposed to cadmium

Duplicate microarray experiments were conducted comparing gene expression at pH 5 and pH 7 with different exposures to cadmium (0 and 5.4 μM). For each time point (5 and 15 minutes), microarrays evaluating the effects of pH alone were compared to microarrays evaluating the interactive effects between pH and cadmium exposure. The two sets of microarray results were compared to determine which genes were only differentially expressed between pH 5 and pH 7 when cadmium was present. An interaction between cadmium effects and pH effects was observed for 151 genes at 5 minutes, 15 minutes, or both time points. Specifically, an interaction was observed for 77 genes only after 5 minutes of cadmium exposure, for 40 genes only after 15 minutes of exposure, and for 34 genes at both time points. Of the 151 genes that were differentially expressed in response to pH only in the presence of cadmium, 91 were selected for further analysis because they showed relatively little expression at either pH in the absence of cadmium, but were highly expressed at either pH 5 or pH 7 in the presence of cadmium. We hypothesized that these genes may be involved in the mitigation of cadmium toxicity at acidic pH. The 91 genes of interest were subjected to cluster analysis based on similarity of their expression over time. This resulted in 89 of the genes being placed into five distinct clusters, designated I-V (Fig. 2). Table 2 lists the

genes in the order they appear on the heatmap and provides gene products and ontology terms.

Cluster I (Table 2) included 11 genes (12% of total) at a Euclidean distance of 5.4. These genes shifted to higher levels of expression at pH 5 in the presence of cadmium. This group consisted of genes that encoded transporters, reductases, hypothetical proteins, and a prophage. Five (45%) were hypothetical proteins with no known functions. Of those with known functions, two (33%) were involved in cell structure, two (33%) were involved in transport, and three (50%) were involved in metabolism.

Cluster II (Table 2) included 26 genes (29% of total) at a Euclidean distance of 3.7. These genes were more highly expressed at pH 5, but only after 15 minutes of exposure to cadmium. Seven proteins encoded by these genes (27% of cluster II) were involved in information transfer specifically, DNA repair, transcription activation, RNA modification, translation, and post-translational modification. Four (15%) were involved in protection and adaptation to stress. An increase in the expression of five genes (19%) involved in metabolism and carbon utilization was also observed, suggesting that the cells were able to adapt and resume growth after 15 minutes of cadmium exposure at pH 5. Two oxidoreductases (8%), eight hypothetical genes (31%), and two prophage genes (8%) were more highly expressed in response to cadmium at pH 5 than pH 7 after 15 minutes of exposure.

Cluster III (Table 2) included 4 genes (4% of total) at a Euclidean distance of 6.2. These genes showed higher expression at pH 7 in response to cadmium after five minutes

of exposure. All four genes in this group were hypothetical, with one being a putative transporter and another a putative receptor.

Cluster IV (Table 2) included 22 genes (24% of total) at a Euclidean distance of 7.0. These genes showed higher expression at pH 5 after 0 and 15 minutes of cadmium exposure, but were more highly expressed at pH 7 after five minutes of exposure to cadmium. This cluster contained seven (32%) hypothetical genes, three genes involved in transport (14%), seven genes involved in metabolism and carbon utilization (32%), and four genes involved in transcriptional regulation (18%). Three genes involved in the biosynthesis of macromolecules (14%), and a metal cation transporter (*ygiD*) was induced.

Cluster V (Table 2) included 26 genes (29% of total) at a Euclidean distance of 8.5. These genes were highly expressed at pH 5 after 5 minutes of cadmium exposure, but exhibited greater expression at pH 7 after 15 minutes. This group included five genes (19%) involved in adaptation toward a variety of stresses, including pH (*hdeA*), osmotic pressure (*otsA*), and heat shock (*yciM*). This group contained eleven hypothetical genes (42%), four genes involved in carbon utilization (15%), one gene involved in transcriptional regulation (4%), and a prophage.

Effect of pH on cadmium speciation

The pH-dependent speciation of cadmium in M9 minimal medium was predicted using MINEQL+ geochemical modeling software (Environmental Research Software, Hallowell, ME). MINEQL+ predicted that the concentration of Cd^{2+} would decrease as

pH increased from 5 to 7 (Fig. 3a). MINEQL+ also predicted that much of the Cd^{2+} interacted with the phosphate in the buffer to form $Cd_3(PO_4)_2$, an insoluble precipitate. The concentration of $Cd_3(PO_4)_2$ in M9 medium was predicted to increase at pH values higher than 6.7, the same point at which Cd^{2+} concentrations began to decrease, suggesting that the pH-dependent loss of Cd^{2+} was due to formation of Cd_3PO_4 as has been shown previously (Sandrin & Maier, 2002).

Cadmium species that become more prevalent from pH 5 to pH 7 may be involved in increased cadmium toxicity observed at neutral pH (Babich & Stotzky, 1977; Ivanov *et al*., 1997). One species that has been implicated in cadmium toxicity is $CdOH^+$. The concentration of $CdOH^+$ increases from 1.08×10^{-5} μM at pH 5 to 4.75×10^{-4} μM at pH 7 (Fig. 3b). Even though $CdOH^+$ concentrations were predicted to increase while Cd^{2+} concentrations decreased, the concentration of Cd^{2+} at pH 7 (0.571 μM) remained more than three orders of magnitude greater than the concentration of $CdOH^+$.

Two other cadmium species were predicted to become more prevalent with increasing pH and may thus play roles in the mechanism by which pH mediates cadmium toxicity. Concentrations of $CdPO_4^-$ showed a similar pattern to $CdOH^+$; however, this species was listed as a dissolved solid by MINEQL+, indicating that it has the potential to precipitate. The concentration of aqueous CdOHCl also increased over the investigated pH range and was an order of magnitude larger than $CdOH^+$ concentrations across the entire range (Fig 3c). These two species have not previously been implicated in cadmium toxicity.

DISCUSSION

Cadmium has been widely reported to be less toxic in acidic environments than at neutral pH. It has previously been shown that *E. coli* is able to adapt to low cadmium concentrations and resume growth after a period of stasis (Mitra *et al.*, 1975; Mitra & Bernstein, 1977; Mitra 1984). The results of this study confirmed those findings by showing an increase in the duration of lag phase only at pH 7.

Stress response genes, including *yciM* (heat shock), *hdeA* (acid resistance), *blr* (drug resistance), *otsA* (osmotic pressure), and *yjbJ* (predicted stress response), were expressed under acidic conditions after 5 minutes of cadmium exposure, while 15 minutes were required for these genes to be expressed under neutral pH conditions. Thus, acidic pH may more rapidly induce expression of genes that confer cadmium resistance.

Genes coding for oxidoreductases may be involved in repairing damage caused by reactive oxygen species produced by cadmium (Ikediobi *et al.*, 2004). Such genes were more highly expressed at pH 5 than at pH 7 after either 5 minutes (*yjeS*, *ydgJ*) or after 15 minutes (*ydiS*, *ygfT*, *ybjN*) of cadmium exposure. After 15 minutes of cadmium exposure at pH 5, much of the cadmium-mediated cellular damage may be repaired, as genes involved in cellular metabolism (*celF*, *lyxK*, *potC*, *hemF*, *rfbB*, *agaI*) were more highly expressed than they were at pH 7 after 15 minutes of cadmium exposure.

Both ATP-driven (*ycfU*) and electrochemical potential-driven (*nupG*) transporters were expressed more highly at pH 5 than pH 7when cadmium was present. Both ATP-driven and proton motive force (pmf)-driven transporters have been identified for exporting metal ions from cells. For example, *zntA* has been shown to be an ATPase that

exports divalent cadmium, lead, and zinc ions from *E. coli* (Rensing *et al.*, 1997; Binet & Poole, 2000). Other transporters have been shown to remove metal ions through proton-cation antiporters, which utilize the proton motive force to export metal ions (Nies & Silver, 1995). It is possible that higher expression of transport-related genes at pH 5 leads to increases in transporter synthesis and cadmium removal from the cell. This may account, in part, for the cadmium resistance of *E. coli* in moderately acidic environments.

The formation of cadmium species other than Cd^{2+} may also play a role in increased cadmium toxicity at pH 7. As acidic environments become neutral, cadmium species such as $CdOH^{+}$ and CdOHCl increase in concentration. $CdOH^{+}$ has been speculated to be more toxic than the more abundant Cd^{2+} (Babich & Stotzky, 1985; Collins & Stotzky, 1992; Ivanov *et al.*, 1997). The proposed mechanism of $CdOH^{+}$ toxicity involves a decrease in the stability of the membrane by altering the charge. The effects of CdOHCl on bacteria, if any, have not been studied. It is possible that some of the hypothetical genes that exhibited increased expression at pH 7 (i.e., those in clusters III and V) were involved in increasing membrane stability to counter the effects of $CdOH^{+}$. The hypothetical genes may also be unique transporters specific for $CdOH^{+}$ or CdOHCl.

Many genes encoding hypothetical proteins exhibited a pH:cadmium interaction (i.e., they exhibited differential expression in response to pH only when cadmium was present). Of the 91 genes that showed increased expression at either pH 5 or pH 7 in the presence of cadmium, 42 (46 %) encoded hypothetical or predicted proteins. Some of these proteins, especially those in Cluster I (i.e., ECs2202, Z1987, and ECs4337), may

have novel functions involved in mitigating cadmium toxicity because they are only expressed at pH 5 in the presence of cadmium and are expressed at only low levels at both pH values in the absence of cadmium. Furthermore, these three genes are highly expressed after both 5 and 15 minutes of cadmium exposure. Basic local alignment search tool (BLAST) searches (Altschul *et al.*, 1997) revealed homology of these three gene products to hypothetical proteins in other bacteria, and the product of ECs4337 showed 80% identity to a hypothetical lipoprotein in *Enterobacter* sp. 638. Lipoproteins are hydrophobic compounds located in cellular membranes (Hayashi & Wu, 1990). It is possible that the ECs4337 lipoprotein binds to cadmium at the membrane, preventing it from associating with physiologically important enzymes as has been suggested previously for the role of *cutF* in copper protection in *E. coli* (Gupta *et al.*, 1995). Another possibility is that the membrane becomes less negatively charged by incorporating ECs4337 lipoproteins, reducing electrostatic attractions between the metal and the cell surface.

Wang & Crowley (2005) did not examine pH when investigating genome-wide effects of cadmium on *E. coli*. They observed which genes were upregulated or downregulated in response to cadmium at pH 7.2. Some of the same genes that were downregulated in that study in response to cadmium exposure at pH 7.2 also showed lower expression at pH 7 than at pH 5 in response to cadmium (i.e., *efp, sucC, trs5*, *mutL*, *potC*) in the present study. Most of these genes are involved in translation and metabolism, so they were downregulated under toxic conditions, such as when cadmium was present at pH 7.2. However, we found that these same genes were more highly

expressed at pH 5 than at pH 7 in the presence of cadmium. One stress response gene (*yfiD*) was previously shown to be upregulated in response to cadmium at pH 7.2, but was more highly expressed at pH 5 than at pH 7 in the presence of cadmium in the present study. Although *yfiD* is an acid-inducible gene, it was only expressed at pH 5 in the presence of cadmium.

More in-depth functional and quantitative analyses of some of the hypothetical genes that were highly expressed at pH 5 only in the presence of cadmium are warranted. Understanding the functions of these genes may further elucidate mechanisms by which pH mitigates the toxicity of cadmium to *E. coli*. This knowledge could then be applied to other microorganisms involved in environmentally and economically important applications including bioremediation.

Acknowledgements

The authors would like to thank the Genome Consortium for Active Teaching (GCAT) for access to the microarrays at reduced cost. We are also grateful to Dr. Xiangqin Cui for her insight concerning statistical analysis of microarray data and to Dr. Dan Nettleton for providing useful information about cluster analysis using R. This material is based on work supported by the National Science Foundation under Grant Nos. 0452825 and 0321545. Any opinions, findings, conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

Figure Captions

Figure 1

Example of gene with Type I interaction of pH and cadmium (*ydeB*) with increased expression at only pH 5 in the presence of cadmium (a). Example of gene with Type II interaction (*yciM*) where expression increased only at pH 7 in the presence of cadmium (b).

Figure 2

Growth of *E. coli* K-12 in M9 medium containing 0 (■), 5 (●), or 50 (▲) μM total cadmium at pH 7 (a) and pH 5 (b). Points represent the mean OD_{600} in triplicate flasks.

Figure 3

Cluster analysis and heatmap comparing the expression of 91 genes in response to cadmium in *E. coli* at pH 5 and pH 7. Each column represents one time point, ranging from 0 (no Cd) to 15 minutes of exposure, from left to right. Each row represents one gene. Red indicates higher expression at pH 5, green indicates higher expression at pH 7, and black indicates no difference in expression. The transcription profiles were divided into five clusters (I-V) based on hierarchical clustering using Euclidean distance and the average linkage method.

Figure 4

Effect of pH on speciation of cadmium (~5.4 μM) in M9 medium as predicted by MINEQL+ geochemical modeling software (Environmental Research Software, Hallowell, ME) (a). Effect of pH on [$CdOH^+$] (b) and [CdOHCl] (c) in M9 medium as predicted by MINEQL+ (note the difference in scale of the y-axes).

Table 1. Effect of cadmium on growth kinetics of *E. coli* in minimal salts medium at pH 5 and pH 7. Values of the same parameter (i.e., lag phase, yield, or growth rate) followed by the same letter are not significantly different at P=0.05 as measured by the Tukey test (n=3).

	pH 5			**pH 7**		
[$CdCl_2$] (μM)	0	5	50	0	5	50
Lag Phase (h)	7.33ab	10.0bc	10.0bc	3.33a	9.00b	13.3c
Yield* (OD_{600})	1.37a	1.39a	1.40a	2.19a	2.20a	0.969a
Log Phase Growth Rate† (OD_{600}/h)	0.178a	0.225a	0.223a	0.238a	0.253a	0.118a

*Yield represents the maximum OD_{600} observed over the 20 h course of the experiment.
† Growth rates were calculated using the relationship: $(OD_f-OD_i)/t$, where OD_f is the OD_{600} at the end of exponential growth, OD_i is the OD_{600} at the beginning of exponential growth, and t is the length (h) of the log phase.

Table 2. Genes involved in pH-mediated responses to cadmium in *E. coli*. The clusters represent genes that exhibit similar temporal expression patterns.

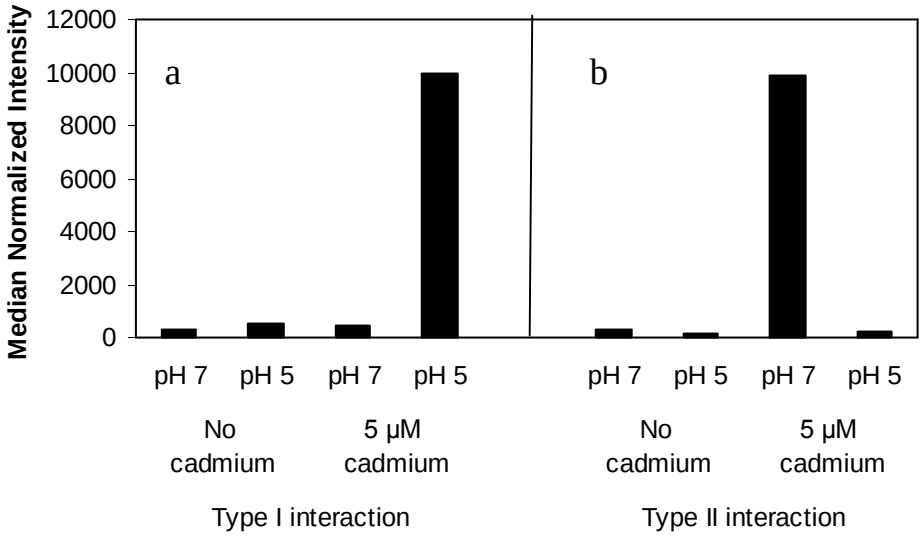
Cluster	Gene	pH*	Fold-change†	P value	Ontology‡	Gene Product
I	ECs2202	5	-1.07	4.50×10^{-2}		Hypothetical protein
	nohA	5	-0.858	3.49×10^{-5}		Qin prophage; predicted packaging protein
	ycfU	5	-0.489	1.95×10^{-2}	S:membrane, T:channel-type	Outer membrane-specific lipoprotein transporter subunit
	ECs2164	5	-1.48	1.09×10^{-6}		Putative minor tail protein
	yeaU	5	-1.37	3.76×10^{-3}	M	Malate dehydrogenase
	ECs1171	5	-1.94	0		Putative host-specificity protein
	yjeS	5	-1.66	2.07×10^{-2}		Fumarate reductase, membrane anchor subunit
	Z1987	5	-1.13	2.98×10^{-9}		Hypothetical protein
	nupG	5	-1.89	1.76×10^{-9}	M:nucleoside conversion, S:membrane, T:electrochemical potential-driven	Nucleoside transporter
	ECs4337	5	-2.19	1.19×10^{-11}		Hypothetical lipoprotein
	ydgJ	5	-3.95	6.31×10^{-7}	M:carbon utilization	Putative oxidoreductase
II	*efp*	5	-1.62	3.04×10^{-2}	I:translation	Protein chain elongation factor EF-P
	ycbJ	5	-2.14	2.21×10^{-2}		Conserved hypothetical protein
	ydiS	5	-1.8	1.97×10^{-2}	M: carbon utilization and electron carrier	Putative oxidoreductase
	yjbC	5	-2.06	7.30×10^{-5}	I:RNA modification	23S rRNA pseudouridine synthase
	celF	5	-2.34	8.95×10^{-5}	M:carbon utilization	Diacetylchitobiose-6-phosphate hydrolase
	yciI	5	-2.81	1.64×10^{-8}		Predicted enzyme
	yidI	5	-3.78	0		Predicted inner membrane protein
	yhfK	5	-3.67	1.40×10^{-6}	M	Conserved inner membrane protein
	ygfT	5	-3.3	6.44×10^{-12}		Predicted oxidoreductase
	ydjF	5	-3.44	9.53×10^{-3}	I:transcription	Predicted DNA-binding transcriptional regulator
	ECs5377	5	-3.32	2.70×10^{-5}		Conserved hypothetical protein
	ECs4659	5	-3.68	3.11×10^{-6}		Hypothetical protein
	ECs3594	5	-4.05	0	I:transcription, P:cell killing, R:activator	Transcriptional activator (MarR-family)
	ygjJ	5	-4.34	5.88×10^{-12}		Conserved protein
	ECs0825	5	-3.17	9.89×10^{-3}		Putative terminase large subunit
	cpxA	5	-3.43	0	I:post-translational, M:degradation of proteins, P:detoxification, R:two-component system, S:membrane	Histidine protein kinase, periplasmic stress sensor; inner membrane protein
	lyxK	5	-3.03	0	M:carbon utilization	Subunit of L-xylulose kinase
	yqaC	5	-3.09	1.30×10^{-3}		Conserved protein
	ydaA	5	-3.26	4.83×10^{-4}	A, P:radiation	Universal stress protein with a role in resistance to UV irradiation
	mutL	5	-3.12	4.88×10^{-3}	I:DNA repair	Methyl-directed mismatch repair
	pphA	5	-3.5	5.99×10^{-3}	A:Temperature extremes, I:post-translational	Serine/threonine-specific protein phosphatase 1, signals protein misfolding

	ECs5495	5	-2.17	3.51×10^{-5}		Hypothetical protein
	ECs1767	5	-2.47	3.70×10^{-7}		Qin prophage; predicted protein
	ECs0761	5	-2.61	4.93×10^{-3}		3-methylaspartate ammonia-lyase
	tfaS	5	-2.39	1.72×10^{-11}		CPS-53 (KpLE1) prophage; tail fiber assembly protein fragment
	ybjE	5	-2.24	8.46×10^{-3}		Predicted transporter
III	ECs1243	7	3.6	3.43×10^{-3}		Hypothetical protein
	ynfM	7	2.12	4.59×10^{-2}	S:membrane, T:electrochemical potential-driven	Putative transporter
	yhhM	7	0.882	2.47×10^{-10}		Putative receptor
	ECs0951	7	5.24	5.26×10^{-10}		Hypothetical protein
IV	ECs3855	5	-5.74	0		Putative enterotoxin
	yfiE	5	-5.81	0	I:transcription, R:activator/repressor	Predicted DNA-binding transcriptional regulator
	potC	5	-4.95	7.21×10^{-11}	M:carbon utilization, S:membrane, T:channel-type	Subunit of putrescine/spermidine ABC transporter
	yciR	5	-5.56	1.31×10^{-13}	A, I:transcription	Modulator of RNase II stability
	hemF	5	-5.54	9.01×10^{-13}	M:cofactors	Coproporphyrinogen oxidase, aerobic
	fliF	5	-5.37	0	C, M:biosynthesis of macromolecules, S:flagella	Flagellar basal-body MS-ring and collar protein
	yhbW	5	-6.94	3.11×10^{-6}		Predicted enzyme
	ygiD	5	-6.98	9.99×10^{-6}		Metal cation transporter
	ydeB	5	-4.56	4.60×10^{-2}	P:drug resistance, S:membrane, T	Inner membrane protein involved in multiple antibiotic resistance
	ycfJ	5	-3.49	2.78×10^{-6}		Predicted periplasmic protein
	ECs2914	5	-3.57	0		Putative type-I fimbrial protein
	ybjN	5	-3	2.66×10^{-3}		Predicted oxidoreductase, protein stabilizer
	yrdA	5	-3.71	1.88×10^{-6}		Conserved protein
	ECs1121	5	-4.02	0		Hypothetical protein
	rfbB	5	-3.06	3.52×10^{-3}	M:biosynthesis of macromolecules and cofactors, S:surface antigen	Subunit of dTDP-glucose 4,6-dehydratase
	yqgE	5	-3.87	4.77×10^{-8}		Predicted protein
	ybbX	5	-3.8	1.54×10^{-4}	M:carbon utilization	Subunit of allantoinase
	*molR_* B	5	-4.19	4.87×10^{-2}		DNA-binding transcriptional regulator subunit
	yfiD	5	-4.14	3.97×10^{-2}	A:pH, M:anaerobic respiration	Stress-induced alternate pyruvate formate-lyase subunit
	agaI	5	-4.03	4.68×10^{-9}	M	Galactosamine-6-phosphate isomerase
	cafA	5	-6.69	0	D, I:RNA modification and degradation	Subunit of ribonuclease G
	yehV	5	-5.88	0	I:transcription	DNA-binding transcriptional regulator
V	ECs3637	7	4.28	0		Hypothetical membrane protein
	yjbJ	7	4.08	1.56×10^{-4}		Predicted stress response protein
	yciM	7	5.62	3.04×10^{-2}		Putative heat shock protein
	yggG	7	4.51	1.74×10^{-2}		Putative metalloprotease lipoprotein, predicted peptidase)
	ECs3635	7	2.72	2.17×10^{-2}		Hypothetical membrane protein
	hdeA	7	3.19	4.47×10^{-2}	A:pH	Acid-resistance protein, possible chaperone
	ECs4187	7	1.7	9.89×10^{-3}		Hypothetical protein

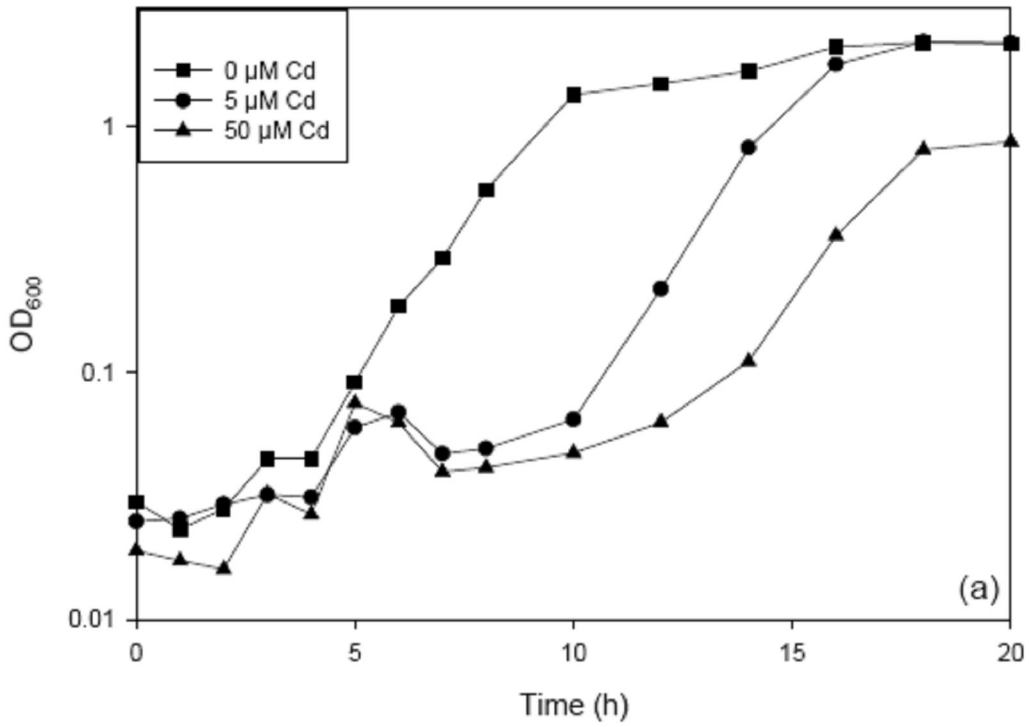
ECs0350	7	4	1.06×10^{-9}		Putative adhesion
otsA	7	4.35	1.02×10^{-2}	A:osmotic pressure, M:glucose	Trehalose-6-phosphate synthase
aphA	7	5.25	3.22×10^{-2}	M:nucleoside conversion	Subunit of acid phosphatase/phosphotransferase
ECs2213	5	-2.92	2.69×10^{-6}		Hypothetical protein
sucC	5	-3.05	1.55×10^{-2}	M:TCA cycle	Subunit of succinyl-CoA synthetase
ybeH	5	-1.95	3.52×10^{-3}		Predicted protein
flag	7	5.44	4.60×10^{-2}	C, S:flagella, M:biosynthesis of macromolecules	Assembly protein for flagellar basal-body periplasmic P ring
mhpR	5	-2.03	4.90×10^{-2}	I:transcription, M:carbon utilization, R:activator	DNA-binding transcriptional activator
mhpD	7	5.2	4.45×10^{-3}	M:carbon utilization	2-keto-4-pentenoate hydratase
ygiQ	5	-2.23	2.69×10^{-11}		Conserved protein
gpsA	7	2.71	1.74×10^{-2}	M:glycerol	Subunit of glycerol-3-phosphate-dehydrogenase
yraR	7	2.1	2.63×10^{-2}		Predicted nucleoside-diphosphate-sugar epimerase
ECs0813	7	3.36	9.53×10^{-3}		Serine/threonine protein phosphatase
blr	5	-1.59	2.96×10^{-2}	P:drug resistance	β-lactam resistance protein
ECs1068	7	4.31	1.79×10^{-9}		Hypothetical protein
btuE	7	3.95	1.30×10^{-2}		Predicted glutathione peroxidase
ydeR	5	-1.81	4.61×10^{-2}	S:pilus	Predicted fimbrial-like adhesin protein
trs5 _2	7	3.81	7.52×10^{-3}		DLP12 prophage; IS5 transposase and trans-activator
yeeI	7	3.54	4.89×10^{-3}	R	Titration factor

* The pH at which expression was higher when cadmium was present.
† Fold-change is given as the $\log_2$-ratio of the expression values at pH 7 and pH 5: $\log_2$(pH 7/pH 5).
‡A-adaptation, C-chemotaxis and motility, D-cell division, I-information transfer, M-membrane, P-protection, R-regulation, S-cell structure, T-transport

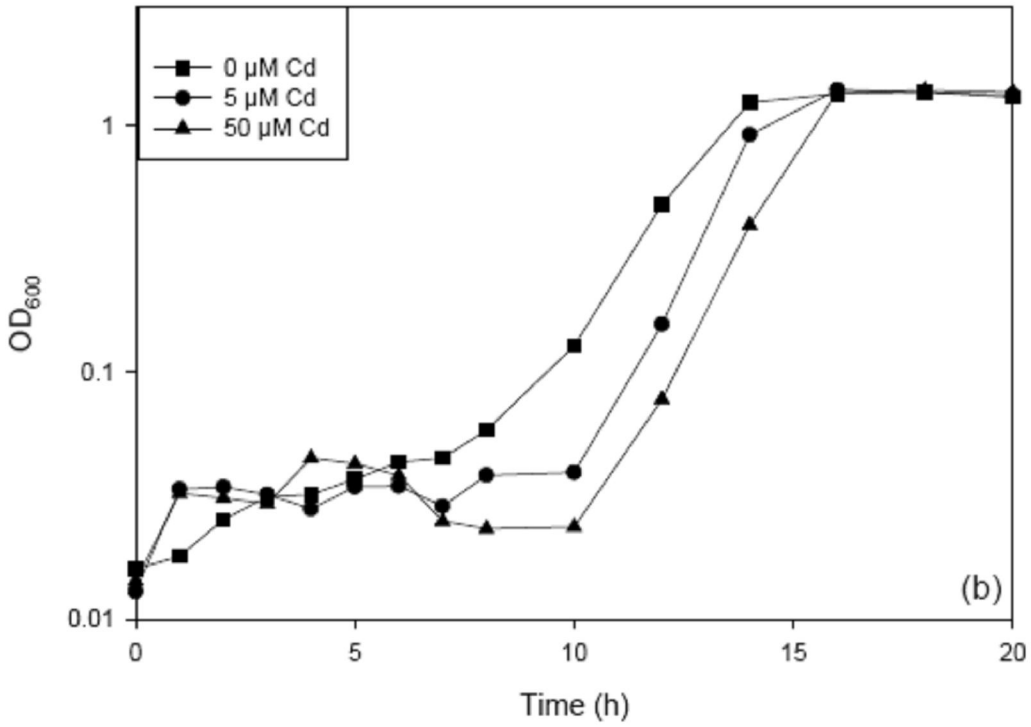
(Figure 1)


Median Normalized Intensity
12000
10000
8000
6000
4000
2000
0
a
b
pH 7
pH 5
pH 7
pH 5
pH 7
pH 5
pH 7
pH 5
No cadmium
5 µM cadmium
No cadmium
5 µM cadmium
Type I interaction
Type II interaction

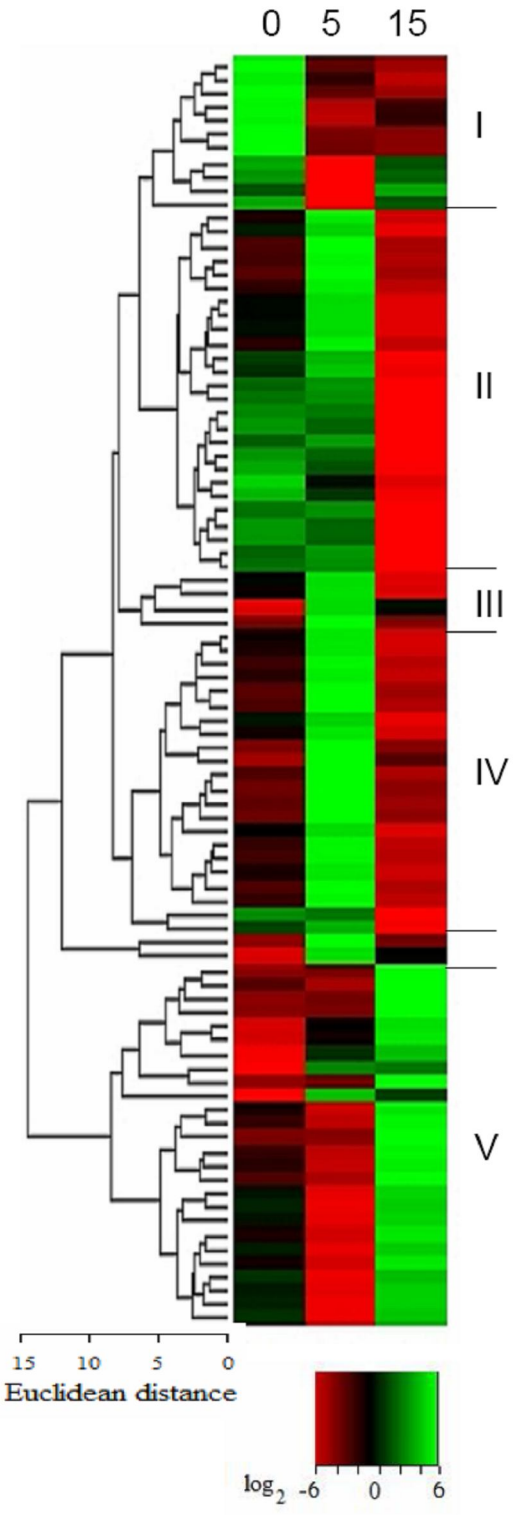
(Figure 2a)



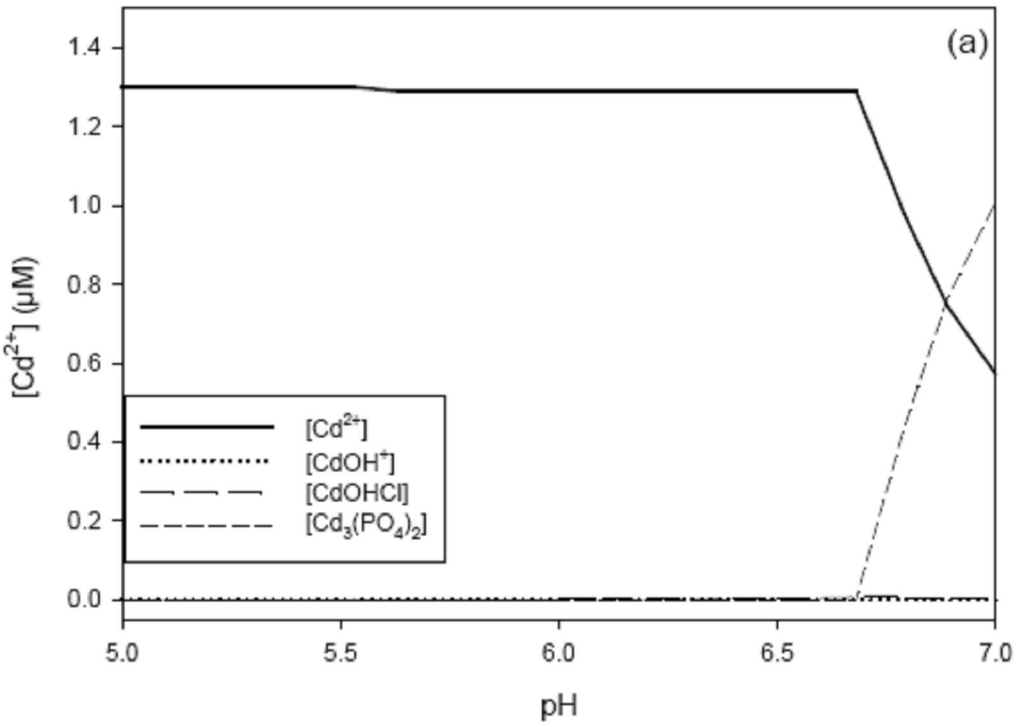
(Figure 2b)



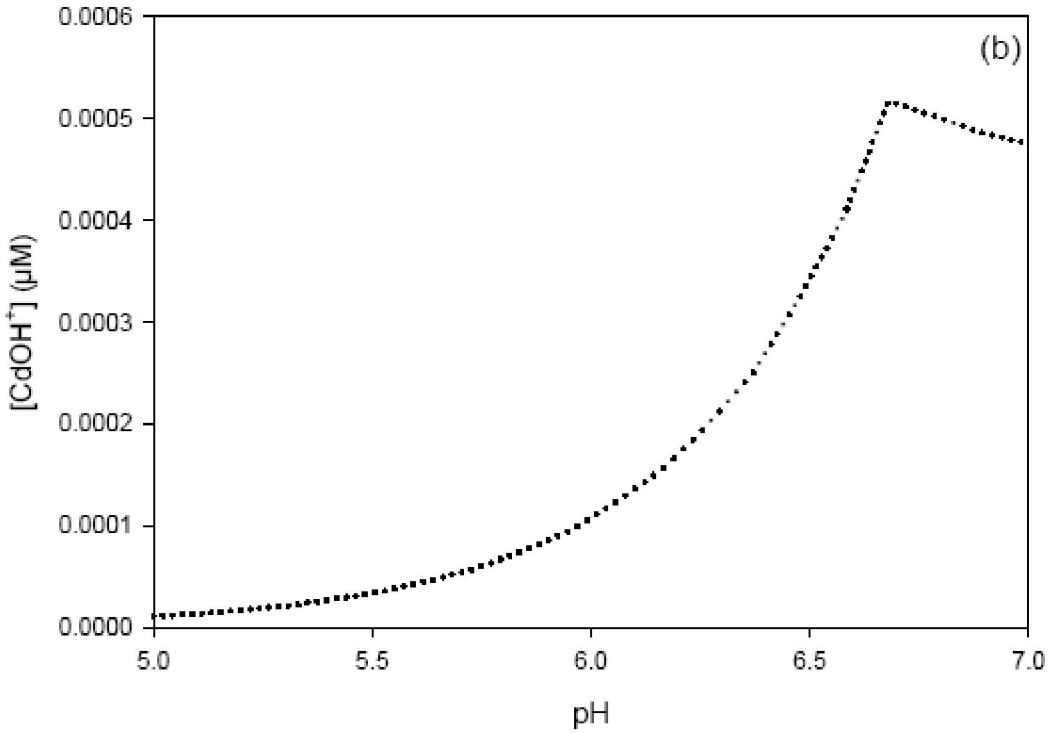
(Figure 3)



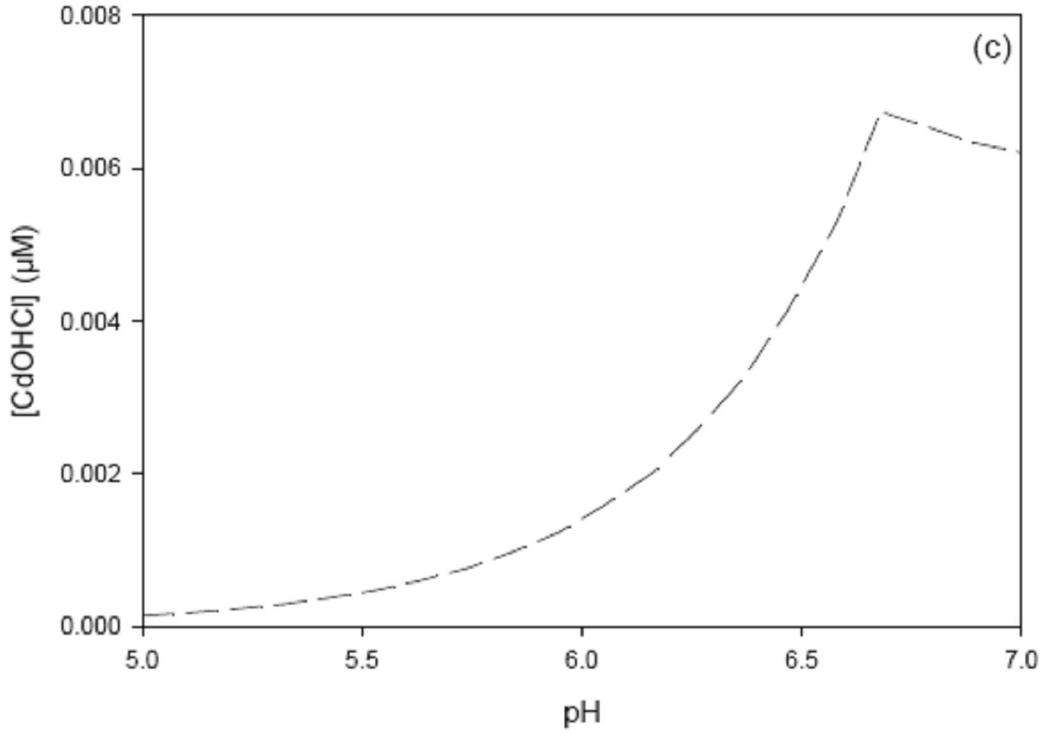
(Figure 4a)


(a)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
[Cd2+] (µM)
5.0
5.5
6.0
6.5
7.0
pH
[Cd2+]
[CdOH+]
[CdOHCl]
[Cd3(PO4)2]

(Figure 4b)

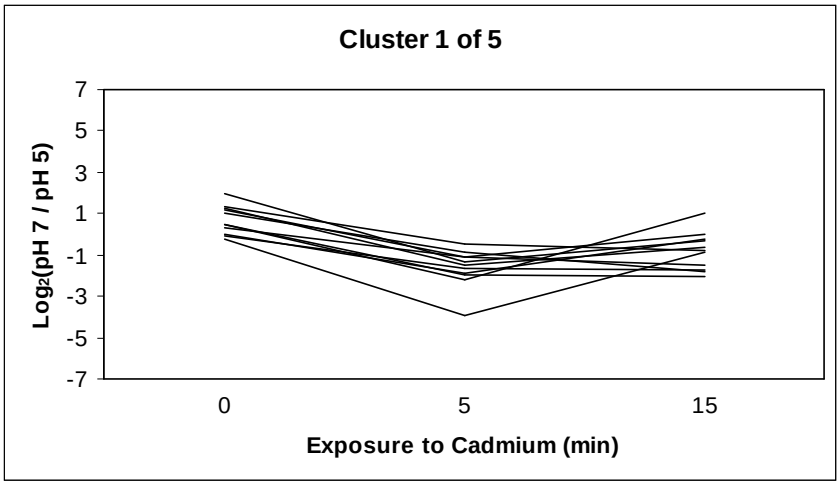

(b)
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
[CdOH+] (μM)
5.0
5.5
6.0
6.5
7.0
pH

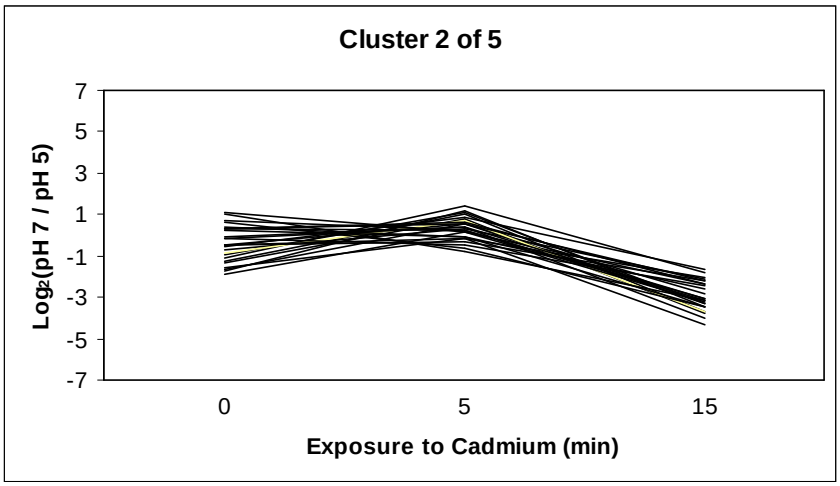
(Figure 4c)

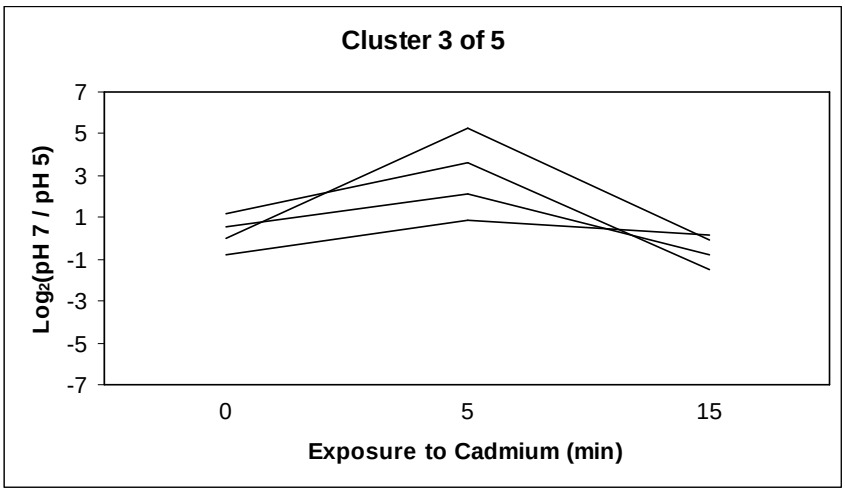


APPENDIX A

Temporal Expression Patterns


Cluster 1 of 5
Log2(pH 7 / pH 5)
7
5
3
1
-1
-3
-5
-7
0
5
15
Exposure to Cadmium (min)


Cluster 2 of 5
Log2(pH 7 / pH 5)
7
5
3
1
-1
-3
-5
-7
0
5
15
Exposure to Cadmium (min)


Cluster 3 of 5
Log2(pH 7 / pH 5)
7
5
3
1
-1
-3
-5
-7
0
5
15
Exposure to Cadmium (min)

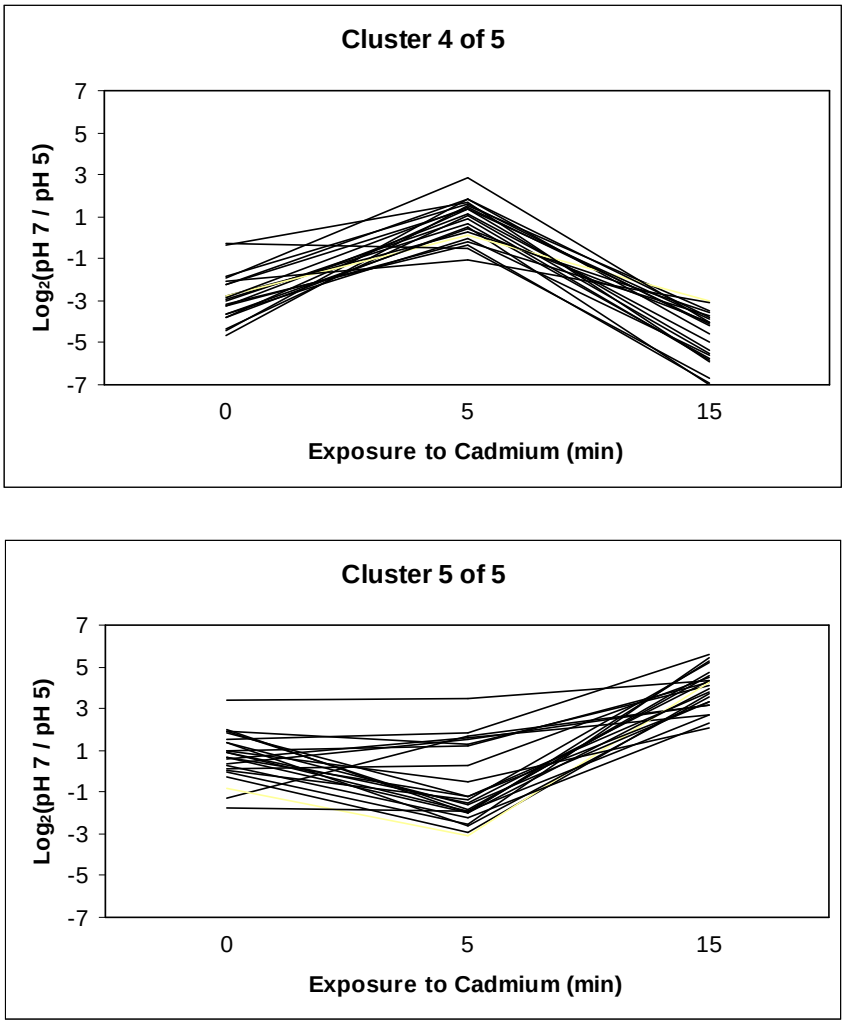


Figure A-1: Cluster analysis comparing temporal expression patterns of 91 genes in *E. coli* in response to 5 μM cadmium at pH 5 and pH 7. Clusters were determined using Euclidean distance and the average linkage method.

APPENDIX B

Cluster Analysis Comparing pH Responses

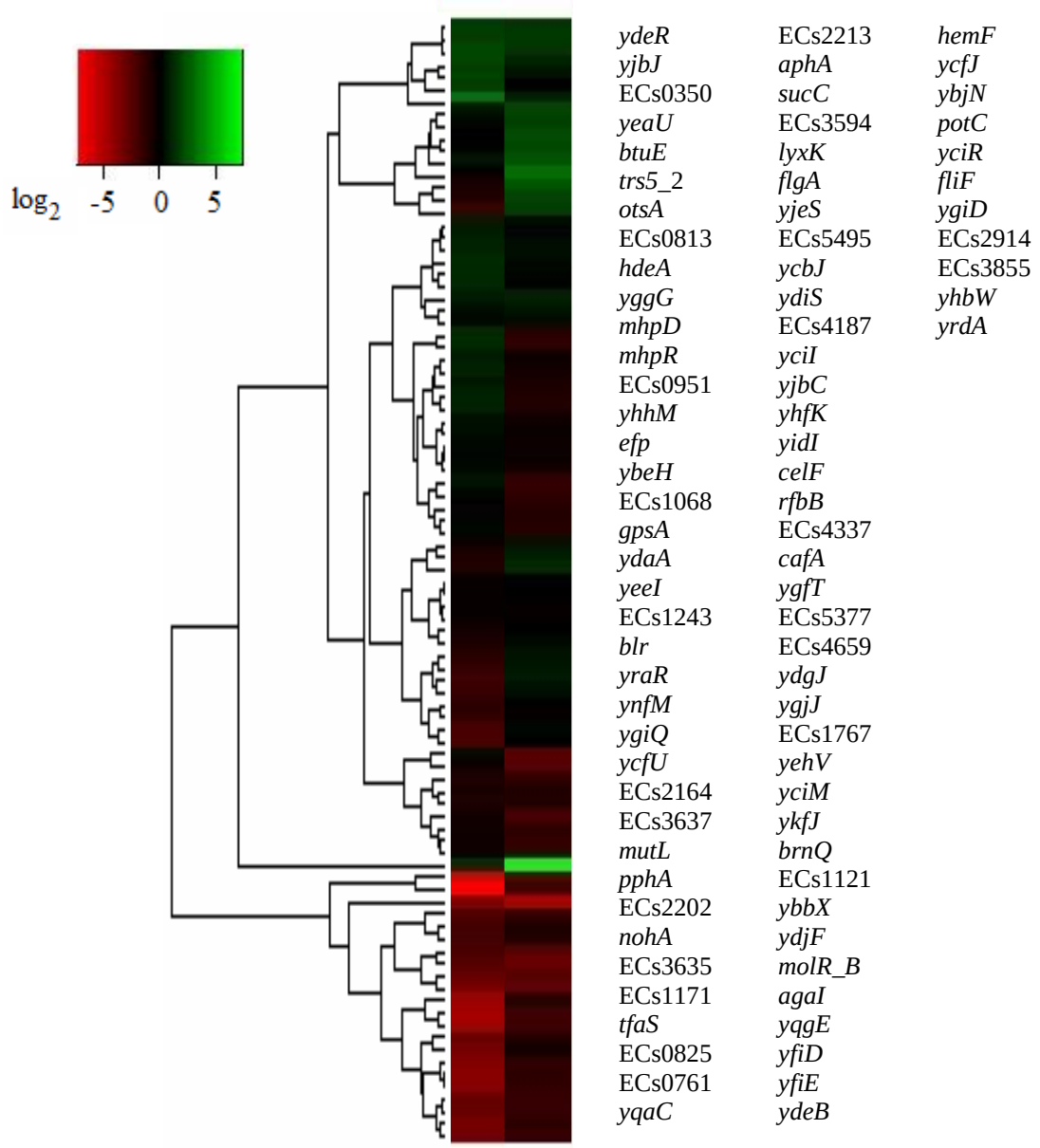


Figure B-1: Cluster analysis and heatmap comparing the expression of 91 genes in *E. coli* in response to pH. Each column represents one time point, ranging from 5 to 15 minutes of exposure, from left to right. Each row represents one gene. Red indicates higher expression at pH 5, green indicates higher expression at pH 7, and black indicates no difference in expression. Genes are listed in the order in which they appear in the heatmap.

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